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Title: Establishing A Porcine Ex Vivo Cornea Model for Studying Drug Treatments against Bacterial Keratitis

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Author Questionnaire

1. **Microscopy:** Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **no**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **no**

3. **Filming location:** Will the filming need to take place in multiple locations? **no**

Introduction

1. Introductory Interview Statements

Videographer: Interviewee headshots are required. Take a headshot for each interviewee.

Authors: While filming the interview portion, our videographer will also photograph you for the [JoVE Dedicated Author Webpage](#). Please look at this [example](#). For questions about the author profile pages and pictures, please contact author.liaison@jove.com.

Authors: Please memorize the interview statements prior to your filming day.

- 1.1. **Esther Karunakaran:** The *ex vivo* porcine keratitis model presented here provides researchers developing novel antimicrobials with a representative *in vitro* model to more accurately determine antimicrobial efficacy at the preclinical stages [1].
 - 1.1.0. INTERVIEW: Named author says the above in an interview-style statement while looking slightly off-camera.
- 1.2. **Katarzyna Emery:** We have used *Pseudomonas aeruginosa* for this experiment but the model also works well with other bacteria, and organisms such as fungi and yeast [1].
 - 1.2.0. INTERVIEW: Named author says the above in an interview-style statement while looking slightly off-camera.

Answers have been edited for length and consistency with journal style guidelines.

Introduction of Demonstrator on Camera

~~1.3. Esther Karunakaran: Demonstrating the procedure will be Katarzyna Okurowska, a research technician from my laboratory [1][2].~~

~~1.3.0. INTERVIEW: Author saying the above.~~

~~1.3.1. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera.~~

Ethics Title Card

1.4. Porcine eyes were sourced from pigs sacrificed as part of the human food chain. Albino laboratory rabbits were sacrificed in the laboratory for other planned experimental work under home office approved protocols. The eyes were not required for experimental use in those studies, so they were used for this protocol.

Protocol

Please use this draft script to help you prepare for filming day.

- Filming should take no more than 10 minutes per step. If a step will take more than 10 minutes, prepare the product from that step in advance.

2. Preparation of the Corneoscleral Button

NOTE: Postshoot script from authors had messed up numbering (started with 2.1.0 instead of 2.1.1) which propagated throughout the script. Videographer should have had the correct script, so I fixed it here.

- 2.1. Use sterile forceps to transfer the eyeball to a Petri dish [1]. Remove the conjunctiva and muscle tissue around the eyeball, using a scalpel blade number 15 and forceps [2]. Then, while holding the optic nerve with the forceps, gently lift the eyeball and transfer it to a half-liter jar filled with sterile PBS [3].
 - 2.1.1. Talent transfers eyeball to Petri dish.
 - 2.1.2. Talent uses scalpel to remove tissue around eyeball.
 - 2.1.3. Talent transfers eyeball to a half-liter jar.
- 2.2. Once all eyeballs are cleared of surrounding tissue, use sterile forceps to move them to another half-liter jar, filled with 3 percent povidone iodine in PBS [1]. After the eyeballs have been in the jar for one minute, transfer them to a third jar, containing sterile PBS [2].
 - 2.2.1. Talent moves the eyeballs to another jar.
 - 2.2.2. Talent moves the eyeballs to a third jar.
- 2.3. Place an eyeball on a clean Petri dish, and use the forceps to hold it steady [0]. With a number 10A scalpel blade, make a cut near the cornea [1].
 - 2.3.1. Talent places an eyeball on a Petri dish and holds it still.
 - 2.3.2. Talent makes an incision near the cornea. NOTE: maybe slated as 2.3.1
Videographer: This is one of the most important steps for viewers to see
- 2.4. Use scissors to excise the cornea, leaving about 3 millimeters of sclera surrounding it [1]. Ensure the sharp end of the scissors is in the supra-choroidal space, and does not pierce the iris [2].
 - 2.4.1. Talent begins to excise the cornea.
 - 2.4.2. CU/ECU: Tip of scissors in the supra-choroidal space. *Videographer: This is a critical step.*

2.5. Hold the corneoscleral button with forceps and use another pair of pointed-end forceps to gently separate the uveal tissue. Then, lift the corneoscleral button to separate it from the remainder of the eyeball [1].

2.5.1. Talent holds the corneoscleral button with forceps and uses another pair of forceps to separate the uveal tissue. *Videographer: This is one of the most important steps for viewers to see.*

~~2.5.2. Talent lifts the corneoscleral button from the rest of the eyeball.~~ **NOTE:**
Cornea is lifter from eyeball in previous shot

2.6. Briefly rinse the button in a 1.5 percent solution of povidone iodine in PBS. Then, place the button in sterile PBS in a 12-well plate [1].

2.6.1. Talent rinses corneoscleral button in iodine solution and then places button in a 12-well plate of PBS.

2.7. After processing no more than 40 eyeballs, place each corneoscleral button in its own 34-millimeter Petri dish, with the epithelial side up [1]. To each dish, add 3 milliliters of culture medium, pre-warmed to 37 degrees Celsius [2]. Incubate the Petri dishes for 24 hours at 37 degrees Celsius in a humidified tissue culture incubator [3].

2.7.1. Talent moves corneoscleral buttons from 12-well plate to individual Petri dishes.

2.7.2. Talent adds culture medium to Petri dishes.

2.7.3. Talent moves Petri dishes to incubator.

3. Maintenance of the Corneoscleral Buttons

3.1. Use aseptic technique to remove the media from the Petri dishes, and replace it with 3 milliliters of fresh, pre-warmed culture media containing antibiotics [1-TXT].

3.1.1. Talent replaces media in Petri dishes. **TEXT: Note: Media with antibiotics**

3.2. After incubating the corneas for 48 hours, remove the media again, and rinse the corneas with 2 milliliters of PBS [1]. Then, add antibiotic-free media, and incubate for two to three days to remove residual antibiotics from the tissue. Change the media at least one more time within these three days [2-TXT].

3.2.1. Talent removes media and rinses corneas with PBS.

3.2.2. Talent adds antibiotic-free media. **TEXT: Note: Media without antibiotics**

3.3. Discard corneas if any turbidity develops in the antibiotic-free medium [1].

3.3.1. Petri dish showing turbidity in medium. *Videographer: This is one of the most important steps for viewers to see.*

4. Preparation of an Inoculum

- 4.1. Add 10 milliliters of LB to a 50-milliliter conical flask with a foam stopper. Inoculate the broth with a colony of *P. aeruginosa* from a fresh agar plate [2] and incubate at 37 degrees Celsius for 3 to 4 hours, until the bacteria are in mid-log phase [3].
 - ~~4.1.1. Talent adds broth to flask.~~
 - 4.1.2. Talent inoculates flask with bacteria from an agar plate. NOTE: May have been slated as 4.1.1.
 - 4.1.3. Talent places flask in incubator.
- 4.2. Transfer the bacterial culture to a 50-milliliter tube [1] and centrifuge it at 3,000 times *g* for 5 minutes [2]. Remove the supernatant [3] and resuspend the cell pellet in PBS [4].
 - 4.2.1. Talent transfers culture to a tube.
 - 4.2.2. Talent places tube in centrifuge.
 - 4.2.3. Talent removes the supernatant.
 - 4.2.4. Added shot: Talent resuspends the pellet.
- 4.3. After washing the cells two more times and resuspending them in PBS [0], adjust the optical density of the suspension at 600 nanometers to approximately 0.6, using sterile PBS as a blank [1].

Added shot 4.3.0: Talent filling cuvette

 - 4.3.1. Talent measures optical density of cell suspension.
 - ~~4.3.2. Talent adds PBS to cell suspension to adjust optical density.~~

5. Infecting the Corneoscleral Button

- 5.1. Remove the media from the Petri dish [1] and rinse the corneas twice with 1 milliliter of sterile PBS [2].
 - 5.1.1. Talent removes media from the Petri dish.
 - 5.1.2. Talent rinses the corneas with PBS.
- 5.2. Hold the cornea with forceps and squeeze gently. Use a 10A scalpel to make four cuts – two vertical, two horizontal - in the central section of the corneoscleral button [1], through the epithelial layer to the underlying stroma [2].
 - 5.2.1. Talent holds cornea with forceps and uses scalpel to make four cuts.

Videographer: This is one of the most important steps for viewers to see.
 - 5.2.2. ECU: Cornea, showing four cuts.
- 5.3. Place a sterile glass mold in a 6-well plate with the wide part up [1]. Then, place the cornea in the middle of the glass mold, with the epithelium side facing down and the wounded part of the cornea centered in the glass mold [2].

- 5.3.1. Glass mold being placed in 6-well plate.
- 5.3.2. Talent places cornea in the middle of the glass mold.
- 5.4. Fill the glass mold completely by adding 1 milliliter of agar solution [1-TXT]. After allowing the agar to set [2], invert the glass mold so that the corneal epithelium is facing upwards [3].
 - 5.4.1. Talent pours agar solution into glass mold. **TEXT: 1% (w/v) low melting point agar dissolved in DMEM** *Videographer, this is a critical step.*
 - 5.4.2. Glass mold, showing how it looks when completely full of set agar.
Videographer: This is one of the most important steps for viewers to see.
 - 5.4.3. Talent inverts glass mold.
- 5.5. **Katarzyna Okurowska:** The glass mold has to be sealed with agar up to the brim to prevent leaking of an inoculum or drug solution [1].
 - 5.5.1. INTERVIEW: Named author says the above in an interview-style shot while looking slightly off-camera.
- 5.6. Pipette 200 microliters of the bacterial culture directly into a cut area. Also, for each experiment, set up a control by adding 200 microliters of sterile PBS to one cornea instead of adding the bacterial culture [1].
 - 5.6.1. Talent pipettes bacterial culture onto cut areas, and pipettes sterile PBS onto one cornea.
- 5.7. Next, add 185 microliters of PBS to the top of each cornea to keep the epithelium moist [1]. Then, add 1 milliliter of DMEM without antibiotics to the bottom of each well [2-TXT].
 - 5.7.1. Talent adds PBS to all corneas. **Vid NOTE: Use 5.6.0B here**
 - 5.7.2. 5.6.0 Talent adds DMEM without antibiotics to the bottom of each well.
TEXT: Note: DMEM without antibiotics **NOTE: May be slated as 5.6.0**
- 5.8. Incubate the 6-well plate, at 37 degrees Celsius with humidity and 5 percent carbon dioxide, for up to 24 hours [1].
 - 5.8.1. Talent places 6-well plate in incubator.

6. Homogenization of the Cornea

- 6.1. Discard the DMEM from the 6-well plate and add 1 milliliter of sterile PBS to each well to rinse it [1]. Remove the PBS gently, without touching the central part of the corneoscleral button [2]. Remove the glass mold using sterile forceps and place it in 5 percent Distel [3].
 - 6.1.1. Talent discards medium in 6-well plate and adds PBS.
 - 6.1.2. Talent removes PBS. **NOTE: Slated as 6.1.1** *Videographer, please show placement of pipette tip clearly.*
 - 6.1.3. Talent removes the glass mold.
- 6.2. Gently rinse the top of the corneoscleral button twice with 1 milliliter of PBS [1-TXT].
 - 6.2.1. Talent rinses corneoscleral button with PBS. **TEXT: Note: PBS rinse is optional.**
- 6.3. Use fine-tip forceps to lift the edge of the corneoscleral button, detach it from the agar underneath, and transfer it to a 50-milliliter tube filled with 1 to 2 milliliters of ice-cold PBS.
 - 6.3.1. Talent lifts the edge of the cornea, detaches it from the agar, and transfers it to a 50-milliliter tube.
- 6.4. To help detach bacteria from the corneal epithelium and the cut area, add PBS to the tube and use a fine-tip homogenizer to shear the top of the infected cornea. The tissue does not have to be completely liquidized [0 – 1, then added]. ~~[2]~~.
 - 6.4.1. 6.4.1. Talent uses homogenizer to shear the top of the infected cornea.
 - 6.4.0. Talent adds PBS to tube and mixes by vortexing. **NOTE: This shot should go before 6.4.1.**
Added shot: 6.4.3 CU of homogenate
- 6.5. Vortex to resuspend settled bacteria and add 20 microliters of the homogenized cornea [1] to 180 microliters of PBS, then perform serial dilutions of the homogenate in a 96-well plate [2-TXT].
 - 6.5.1. **Added shot: Talent vortex tube with the cornea for a few seconds.** **NOTE: Slating might be off in this step due to added shot**
 - 6.5.2. Talent adds homogenized cornea to PBS and begins performing serial dilutions. **TEXT: When testing antimicrobials, dilution factor must be arrived at experimentally.**
- 6.6. Pipette 10 microliters of the diluted homogenate onto a blood agar plate [1]. After incubating the plate for 16 hours, count the number of colony-forming units [2][3].
 - 6.6.1. Talent pipettes homogenate onto a blood agar plate.

- 6.6.2. Talent counting colony-forming units. *Video editor: Show 6.6.2 and 6.6.3 together as a multipanel.*
- 6.6.3. CU: Blood agar plate, showing individual colonies.

Results

7. Results: Ex Vivo Model Mimics In Vivo Infection

- 7.1. Porcine corneas usually swell after few days in medium. There was no significant difference in colony forming units obtained from corneas with and without addition of dextran, which is usually added to prevent swelling of the cornea [1].

7.1.1. LAB MEDIA: Figure 1. [Video editor, show only Figure 1H.](#)

- 7.2. Corneas are typically wounded to help the bacteria penetrate the epithelium. Although there was no significant difference in the progress of infection between wounded and unwounded corneas, there was more variation among replicates in unwounded corneas [1].

7.2.1. LAB MEDIA: Figure 1. [Video editor, show only Figure 1C.](#)

- 7.3. Washing the corneas twice with PBS removes excess bacteria that did not attach to the epithelium. There was a significant difference in CFU between washed and unwashed porcine corneas infected with *P. aeruginosa* PAO1 for 24 hours [1].

7.3.1. LAB MEDIA: Figure 1. [Video editor, show only Figure 1D.](#)

- 7.4. There was no significant difference in CFU counts between porcine and rabbit corneas infected with PA14 and PAO1. The results for both models were reproducible [1].

7.4.1. LAB MEDIA: Figure 1. [Video editor, show only Figure 1E and 1F.](#)

- 7.5. After 24 hours, the corneas infected with either Pseudomonas strain always develop opacity, and the cut area becomes more visible and open in comparison to the uninfected cornea [1].

7.5.1. LAB MEDIA: Figure 1. [Video editor, show only Figure 1G.](#)

Conclusion

8. Conclusion Interview Statements

8.1. **Katarzyna Okurowska**: Infected and treated corneas that were processed with this method can be further used for waxing, cryopreservation, sectioning, staining with hematoxylin and eosin, immunostaining, and confocal microscopy [1].

8.1.1. INTERVIEW: Named author says the above in an interview-style statement while looking slightly off-camera.

8.2. **Esther Karunakaran**: The protocol contributes to greater control over drug design and formulation at the pre-clinical stages, which would increase success at clinical trials, reduce use of animals, and result in faster translation of new antimicrobials to clinic.

8.2.1. INTERVIEW: Named author says the above in an interview-style statement while looking slightly off-camera.