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TITLE:

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

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KEYWORDS:

Pseudomonas aeruginosa, keratitis, cornea, ex vivo, eye, porcine

SUMMARY:

This article describes a step-by-step protocol to set up an ex vivo porcine model of bacterial keratitis. *Pseudomonas aeruginosa* is used as a prototypic organism. This innovative model mimics in vivo infection as bacterial proliferation is dependent on the ability of the bacterium to damage corneal tissue.

ABSTRACT:

When developing novel antimicrobials, the success of animal trials is dependent on accurate extrapolation of antimicrobial efficacy from in vitro tests to animal infections in vivo. The existing in vitro tests typically overestimate antimicrobial efficacy as the presence of host tissue as a diffusion barrier is not accounted for. To overcome this bottleneck, we have developed an ex vivo porcine corneal model of bacterial keratitis using *Pseudomonas aeruginosa* as a prototypic organism. This article describes the preparation of the porcine cornea and protocol for establishment of the infection. Bespoke glass molds enable straightforward setup of the cornea for infection studies. The model mimics in vivo infection as bacterial proliferation is dependent on the ability of the bacterium to damage corneal tissue. Establishment of infection is verified as an increase in the number of colony forming units assessed via viable plate counts. The results demonstrate that infection can be established in a highly reproducible fashion in the ex vivo corneas using the method described here. The model can be extended in the future to mimic keratitis caused by microorganisms other than *P. aeruginosa*. The ultimate aim of the model is to investigate the effect of antimicrobial chemotherapy on the progress of bacterial infection in a scenario more representative of in vivo infections. In so doing, the model described here will reduce the use of animals for testing, improve success rates in clinical trials and ultimately enable rapid translation of novel antimicrobials to the clinic.

INTRODUCTION

Corneal infections are important causes of blindness and occur in epidemic proportions in low- and mid-income countries. The etiology of the disease varies from region to region but bacteria account for a large majority of these cases. *Pseudomonas aeruginosa* is an important pathogen that causes a rapidly progressive disease. In many cases, patients are left with stromal scarring, irregular astigmatism, require transplant or in the worst case scenario, lose an eye[1,2].

Bacterial keratitis caused by *P. aeruginosa* is a difficult eye infection to treat particularly due to the increasing emergence of antimicrobial resistant strains of *P. aeruginosa*. Within the last decade, it has become apparent that testing and developing new treatments for corneal infections, in general, and those caused by *Pseudomonas* sp., in particular, are essential to combat the current trend in antibiotic resistance[3].

For testing the efficacy of new treatments for corneal infections, conventional in vitro microbiological methods are a poor surrogate due to the difference in bacterial physiology during laboratory culture and during infections in vivo as well as due to the lack of the host interface[4,5]. In vivo animal models, however, are expensive, time-consuming, can only deliver a small number of replicates and raise concerns about animal welfare.

In this article, we demonstrate a simple and reproducible organotypic ex vivo porcine model of keratitis that can be used to test various treatments for acute and chronic infections. We have used *P. aeruginosa* for this experiment but the model also works well with other bacteria, and organisms such as fungi and yeast which cause keratitis.

PROTOCOL:

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.

1. Sterilization

1.1. CRITICAL STEP: Disinfect all forceps and scissors by soaking for 1 h in 5% (v/v) solution of Distel in distilled water, clean with a brush, rinse with tap water and sterilize in an oven at 185 °C for a minimum of 2 h.

1.2. Sterilize all other glassware and reagents by autoclaving at 121 °C for 15 minutes or prepare reagents according to the manufacturer's instructions. Carry out the following procedures in a class II microbiology safety cabinet.

2. Sample collection

2.1. Collection of porcine eyes

2.1.1. Use large white landrace sows, a cross with a Hampshire boar. The age of the pigs was between 21 to 23 weeks when slaughtered in a local abattoir for food consumption. Stun the animals with an electric current and enucleate the eyes 2 h later in the abattoir.

2.1.2. CRITICAL STEP: Once enucleated, transfer the eyes to the lab in a sterile phosphate buffered saline (PBS) solution to prevent them from drying out and process them immediately upon arrival.

2.2. Collection of rabbit eyes

2.2.1. Excise the corneas and send to the lab in sterile PBS.

3. Preparation of the corneoscleral button

3.1. Use sterile forceps to hold the tissue surrounding the eyeball and transfer it to a Petri dish. Remove the conjunctiva and muscle tissue around the eyeball on a Petri dish using scalpel blade no. 15 and forceps.

3.2. Gently lift the eyeball while holding the optic nerve with forceps and transfer to a 0.5 L jar filled with sterile PBS.

3.3. Once all eyes are cleared of surrounding tissue, move them using sterile forceps to another 0.5 L jar filled with 3% (v/v) povidone iodine in PBS and leave for 1 min.

3.4. Transfer eyeballs to another 0.5 L jar with sterile PBS.

3.5. Use forceps to hold the eye still on a Petri dish and make a cut near the cornea with a scalpel blade no 10A.

3.6. CRITICAL STEP: Hold the edge of the cut and use scissors to excise the cornea leaving about 3 mm of sclera surrounding the cornea. Ensure the sharp end of scissors does not pierce the iris or the choroidal tissue and is in the supra-choroidal space.

3.7. Hold the corneoscleral button with forceps and use another pair of pointed end forceps to gently separate the uveal tissue.

3.8. Lift the corneoscleral button from remaining globe and briefly rinse it in 1.5% (v/v) povidone iodine solution in PBS in a 12 well plate.

3.9. Place the corneoscleral button into another 12 well plate filled with sterile PBS.

3.10. After processing all eyes (do no more than 40 eyes in one batch), place each corneoscleral button to an individual Petri dish (34 mm diameter) epithelial side up and pour in 3 mL of culture medium pre-warmed to 37 °C.

NOTE: The composition of the culture medium is as follows: Dulbecco's modified Eagle's medium (DMEM): Ham's [1:1] supplemented with 5 $\mu g \cdot mL^{-1}$ insulin and 10 $ng \cdot mL^{-1}$ epidermal growth factor (EGF), 10% (v/v) foetal calf serum (FCS), 100 $U \cdot mL^{-1}$ penicillin, 100 $U \cdot mL^{-1}$ streptomycin and 2.5 $\mu g \cdot mL^{-1}$ amphotericin B. As an optional step, the medium can be supplemented with 50 $g \cdot L^{-1}$ dextran to prevent swelling of the excised cornea during the further incubation steps.

3.11. Incubate at 37 °C in a humidified tissue culture incubator.

4. Maintenance of the corneoscleral buttons

4.1. After 24 hours, use aseptic technique to remove media and replace with 3 mL of fresh pre-warmed culture media containing antibiotics. Keep the corneoscleral buttons in media with antibiotics for 48 h to disinfect the corneas. Incubate at 37 °C in a humidified tissue culture incubator.

4.2. CRITICAL STEP: After 48 hours, remove the media and rinse corneas with 2 mL of PBS. Then keep the corneoscleral buttons in antibiotic-free media for a minimum of two or ideally three days before experimental infection, to remove residual antibiotics from the tissue.

4.3. Incubate at 37 °C in a humidified tissue culture incubator. Change media at least one more time within these three days. Discard corneas if any turbidity develops in the antibiotic-free medium.

5. Preparation of an inoculum

5.1. Pour 10 mL of LB broth into a 50 mL conical flask with a foam stopper.

5.2. Transfer a colony of *P. aeruginosa* strain PAO1 or strain PA14 from a fresh agar plate and incubate at 37 °C for 3-4 h until the bacteria are in mid-log phase.

5.3. Transfer the culture of bacteria to a 50 mL tube and centrifuge at 3,000 x *g* for 5 min. Remove the supernatant and re-suspend the cell pellet in PBS.

5.4. Repeat step 5.3 two more times to wash the cells. Re-suspend the cell pellet in PBS and adjust the optical density at 600 nm to approximately 0.6 using sterile PBS as a blank.

6. Infecting the corneoscleral button

6.1. Remove media from the Petri dish and rinse corneas twice with 1 mL of sterile PBS.

6.2. Gently squeeze forceps while holding the cornea in-between. Use a 10A scalpel to make four cuts – two vertical, two horizontal - in the central section of the corneoscleral button through the epithelial layer to the underlying stroma.

6.3. Place a sterile glass mold in a 6-well plate with the wide part up and place the cornea in the middle of the glass mold, epithelium side facing down. Make the cut right in the center of the bottom part of the glass mold.

6.4. CRITICAL STEP: Pour 1 mL of 1% (w/v) low melting point agar dissolved in DMEM to fill the glass mold with cornea completely.

6.5. Allow the agar to set and then invert the glass mold so that the corneal epithelium is facing upwards.

6.6. Pipette 15 µL of the bacterial culture with $OD_{600nm} = 0.6$ (for *P. aeruginosa* this equates to approximately 1×10^7 colony forming units (CFU) in 15 µL) directly into a cut area and then add 85 µL of PBS to the top to keep the corneal epithelium moist.

6.7. Add 1 mL of DMEM without antibiotics to the bottom of each well with the glass mold. Incubate the 6-well plate with the infected corneoscleral buttons in a humidified incubator at 37 °C with 5% CO_2 for up to 24 h.

6.8. Set up uninfected control cornea alongside every experiment. To set up uninfected control, replace the 15 µL of bacterial culture in step 6.6 with sterile PBS.

7. Homogenization of the cornea to harvest the bacteria

7.1. Discard the DMEM medium from the bottom of the 6 well plate and add 1 mL of sterile PBS to rinse the bottom of the well.

7.2. Remove PBS gently by pipetting without touching the central part of the corneoscleral button. Remove the glass ring using sterile forceps and place it in the 5% Distel.

7.3. Gently rinse the top of the corneoscleral button with 1 mL of PBS twice [optional].

7.4. Hold the edge of the corneoscleral button with fine tip forceps and detach it from the agar underneath.

7.5. Remove the corneoscleral button and place on a sterile Petri dish. Remove the remaining sclera using a scalpel and then transfer the cornea to a 50 mL tube filled with ice cold 1-2 mL of PBS.

7.6. Use a fine tip homogenizer to sheer the top of the infected cornea. The tissue does not have to be completely liquidized. The homogenizer helps to detach bacteria from the corneal epithelium and the cut area.

7.7. Vortex the cornea in PBS for a few seconds to mix the contents.

7.8. Add 20 µL of the homogenate to 180 µL of PBS and perform serial dilutions in a 96 well plate.

7.9. Serially dilute the suspension to 10^{-4} and 10^{-5} dilution and pipette 10 µL of the diluted homogenate with bacteria onto a blood agar plate. Incubate the plate for 8 hours and count the number of CFU. When testing the effect of antimicrobials, the appropriate dilution factor must be arrived at experimentally.

7.10. In every experiment, homogenize one cornea immediately after infection and perform viable plate count to ensure that the infective dose is approximately 1 x 10^7 CFU per cornea.

REPRESENTATIVE RESULTS

The design of the glass molds are an innovative and original idea, the use of which allowed us to set up the model in a consistent fashion with minimal/no issues with contamination. The molds were prepared by a glass blower at the University of Sheffield based on a design (**Figure 1A**). The experimental setup maintains the convex shape of the cornea and holds bacteria on the top of the epithelium where infection takes place (**Figure 1B**).

Porcine corneas usually swell after few days in medium. This is normal and we found that there was no significant difference between corneas with and without addition of dextran, which is usually added to prevent swelling of the cornea (**Figure 1H**). The corneas are typically wounded to help the bacteria penetrate the epithelium. Although there was no significant difference in the progress of infection between wounded (cut) and unwounded (uncut) corneas, we noticed more variations between replicates in uncut corneas (**Figure 1C**). 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 (**Figure 1D**). There was no significant difference in CFU counts between porcine and rabbit corneas infected with PA14 and PAO1 (**Figure 1E,1F**). The results for both models were reproducible. After 24 hours, the cornea infected with either *Pseudomonas* strain always develop opacity and the cut area becomes more visible and open in comparison to the uninfected cornea (**Figure 1G**).

Figure 1: Ex vivo cornea infected with *Pseudomonas aeruginosa*. (**A**) Schematic picture of a glass mold used for maintaining the shape of the cornea and facilitating the introduction of bacteria and treatments. The thickness of the glass molds is 1.5 mm and is the same as the thickness of test tubes made from borosilicate glass. (**B**) Schematic picture of the

experimental set up. (**C**) Testing the effect of wounding on the final CFU count after homogenization. Uncut (n = 16) and cut (n = 28) corneas were infected with *P. aeruginosa* PAO1 and *P. aeruginosa* PA14 for 24 hours. The corneas were washed with 1 mL of PBS before homogenization. Error bars indicate standard deviation. (**D**) Testing the effect of washing corneas with 2 x 1 mL of PBS (n = 6) and not washing (n = 6) on the final CFU count after infection with *P. aeruginosa* PAO1 for 24 hours. Error bars indicate standard deviation. (**E**) Final CFU count in porcine corneas infected with *P. aeruginosa* PAO1 and *P. aeruginosa* PA14 for 24 hours (n = 10). Corneas were washed and cut. Error bars indicate standard deviation. (**F**) Final CFU count in rabbit corneas infected with *P. aeruginosa* PAO1 and *P. aeruginosa* PA14 for 24 hours (n = 6). Corneas were washed and cut. Error bars indicate standard deviation. (**G**) Pictures of ex vivo porcine corneas infected with *P. aeruginosa* PAO1 for 24 hours. The control was wounded but no bacteria were added. The infected corneas were wounded and 10^7 CFU were added to the cut side. No CFU were recovered from the control cornea. (H) Final CFU recovered after 24 hours of infection with *P. aeruginosa* PAO1 from corneas treated with dextran (n = 2) and those without dextran (n = 9). Corneas were washed and cut. Error bars indicate standard deviation.

DISCUSSION:

The main driver behind the development of this keratitis model using ex vivo porcine cornea is to provide researchers developing novel antimicrobials with a representative in vitro model to more accurately determine antimicrobial efficacy at the preclinical stages. This will provide researchers involved in developing new antimicrobials greater control over drug design and formulation at the pre-clinical stages, increase success at clinical trials, reduce use of animals by enabling targeted studies and result in faster translation of new antimicrobials to clinic.

A number of studies have investigated the effect of infections on ex vivo corneas from various animals such as: rabbit[6], dog[7], goat[8] and pigs[9-11]. Most of these studies focus on ways of establishing[6] and visualizing an infection[9] but so far there have only been a few publications focusing on drug testing and accurate quantification of bacteria[6-8,12].

The primary advantage of our model is the availability of the porcine corneas as part of the food chain. The use of ex vivo porcine corneas therefore aligns with the principle of 3Rs, which is to replace, refine and reduce the use of animals in research, whilst providing a representative model of the host interface. We have observed no issues with contamination of the corneal explants if the protocol is strictly followed. The glass molds are very easy, quick and straightforward to use without any requirement for specialized equipment. The narrow ring at the top makes the addition of a small quantity of a tested drug (100 µL) or bacteria convenient. The ring of the glass mold allows PBS with bacteria or a drug solution to be retained in the central part of the cornea and prevents the bacteria from getting underneath the cornea. The ring is easy to clean and sterilize, and allows the observation of the changes that occur on the top of the cornea during infection. Strains of fluorescently-tagged bacteria can be used to visualize infection or quantify the spread of infection in the tissue using fluorescent confocal microscopy. The whole corneas can be further processed for histology or electron microscopy imaging.

The critical steps are marked in the protocol. Extra attention must be paid to these steps when carrying out the protocol to ensure successful infection. The most critical steps within the

protocol are ensuring that the corneas are treated with sufficient antibiotics to prevent infection during preparation and then that the antibiotics are sufficiently eliminated before the introduction of the infective organism, in this case *P. aeruginosa*. When setting up the experiments using this protocol, in some instances, turbidity developed during incubation in the antibiotic-free medium. This turbidity was indicative of growth of microorganisms in the antibiotic-free medium. This might be due to incomplete treatment of the cornea using the antibiotics or due to contamination during handling. These corneas were not taken forward for further experiments and were discarded. Development of turbidity when incubating corneas in antibiotic-free medium was avoided by employing frequent sterilization runs in the incubator, using disposable pipette tips with a filter and taking adequate care when sterilizing the tools used for excising the cornea from the porcine eyes. Another critical step is when the corneas are placed in the glass mold prior to infection. The glass mold enables one to maintain the convex shape of the cornea. The convexity of the cornea is a challenge for retention of either the infective dose or the therapeutic agent on the surface of the cornea. Therefore, it is essential to ensure the presence of adequate seal between the cornea and the glass mold. When there is adequate seal between the cornea and the glass mold, the ring structure above the mold creates a reservoir to retain either the infective dose or the therapeutic agent. An adequate seal is ensured by completely filling the wide section of the glass mold with DMEM agar up to the brim.

As is the case with any model, there are limitations associated with the ex vivo porcine cornea model described. The model described herein does not mimic the composition, flow and replenishment of the tear film across the cornea. The mechanical action provided by blinking is also not incorporated into the model. There is agreement in the literature that tear film composition and dynamics, and blinking are important defense mechanisms that remove foreign particles and microorganisms from the eye[13]. Indeed, the model also lacks an immune response that is triggered during infection in vivo. It is likely that the progression of infection in vivo in the presence of these defense mechanisms is different to that observed in the ex vivo model described here. Despite these limitations, the ex vivo porcine corneal model is relevant for testing the effectiveness of existing and emerging antimicrobials for two main reasons: 1) the physiology of the bacteria in the ex vivo model mimics the in vivo conditions as bacterial proliferation is dependent on their ability to damage the corneal tissue, and 2) the model incorporates the three dimensional tissue as a diffusion barrier for therapeutics much like in the in vivo situation. Therefore, the ex vivo model is advantageous over conventional techniques for antimicrobial susceptibility testing.

The ex vivo porcine cornea model described here can be also used for studying different strains of bacteria, fungi and yeast that cause keratitis. This ex vivo cornea model is reproducible and allows one to generate replicates within a short time unlike in vivo models. Instead of PBS, artificial tears or host immune defense cells can theoretically be added to mimic the live scenario. Corneas are obtained from the same breed of pigs and about 21-23 weeks old when slaughtered. Therefore, there is less variability between replicates compared to those obtained from human cadavers. The concept of using a porcine ex vivo cornea model for biomedical applications has gained more popularity within the last few years because of its biological similarity to the human eye which makes this model easier to compare[14]. There is increased interest in using porcine corneas for transplantation[15,16] or as a model for dry eye[17] or wound healing[18].

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DISCLOSURES:

The authors have nothing to disclose.

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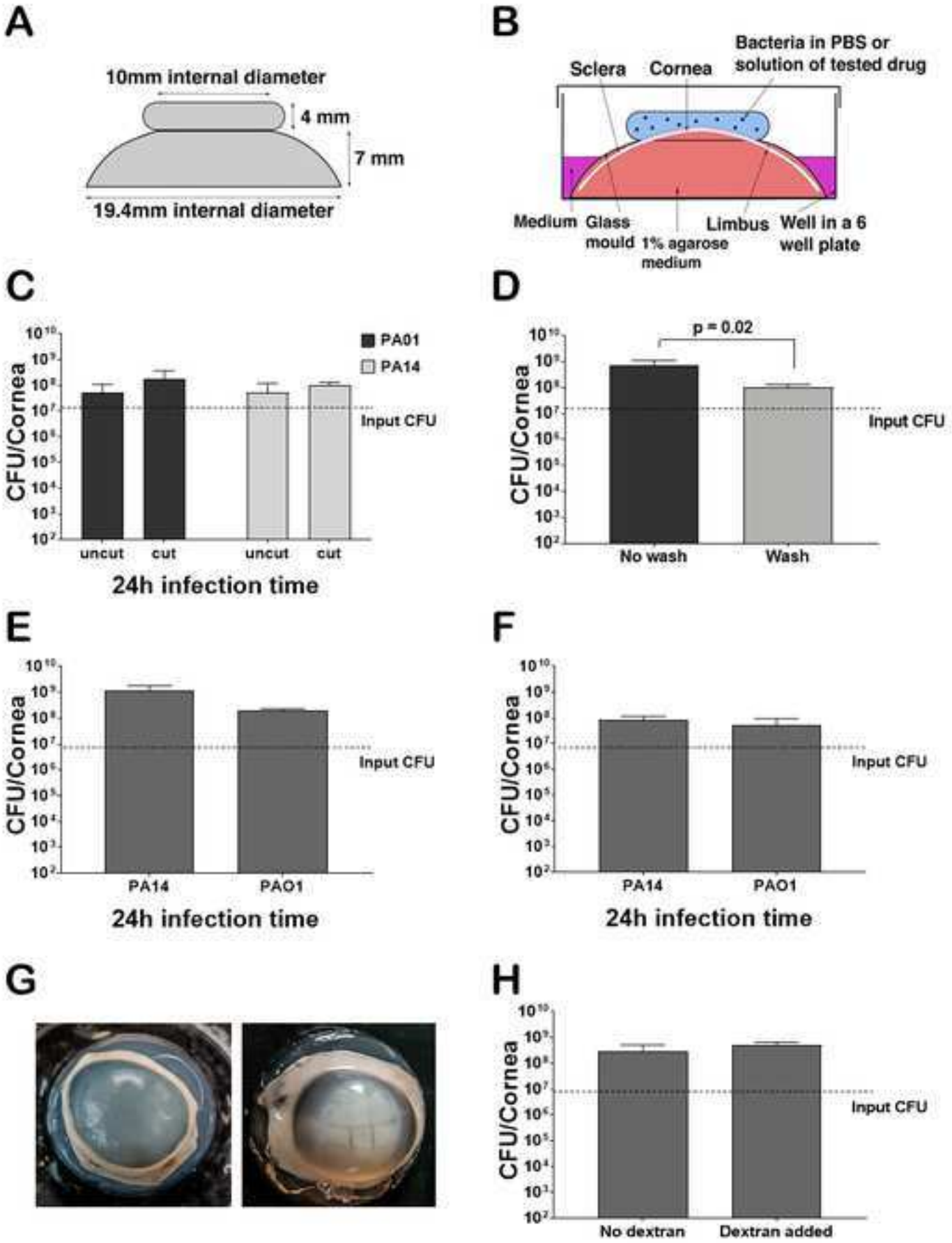


Table of Materials

Click here to access/download;Table of Materials;Revised_JoVE_Table_of_Materials.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
50 mL Falcon tube	SLS	352070	
Amphotericin B	Sigma	A2942	
Cellstar 12 well plate	Greiner Bio-One	665180	
Dextran	Sigma	31425-100mg-F	
Distel	Fisher Scientific	12899357	
DMEM + glutamax	SLS	D0819	
Dual Oven Incubator	SLS	OVe1020	Sterilising oven
Epidermal growth factor	SLS	E5036-200UG	
F12 HAM	Sigma	N4888	
Foetal calf serum	Labtech International	CA-115/500	
Forceps	Fisher Scientific	15307805	
Handheld homogeniser 220	Fisher Scientific	15575809	Homogeniser
Heracell VIOS 160i	Thermo Scientific	15373212	Tissue culture incubator
Heraeus Megafuge 16R	VWR	521-2242	Centrifuge
Insulin, recombinant Human	SLS	91077C-1G	
LB agar	Sigma	L2897	
Multitron	Infors	Not appplicable	Bacterial incubator
PBS	SLS	P4417	
Penicillin-Streptomycin	SLS	P0781	
Petri dish	Fisher Scientific	12664785	
Petri dish 35x10mm CytoOne	Starlab	CC7672-3340	
Povidone iodine	Weldricks pharmacy	2122828	
Safe 2020	Fisher Scientific	1284804	Class II microbiology safety cabinet
Scalpel blade number 15	Fisher Scientific	O305	
Scalpel Swann Morton	Fisher Scientific	11849002	

AUTHORS' RESPONSE TO EDITORIAL AND REVIEWERS' COMMENTS

Dear Editor,

The authors would like to thank the editorial board and the reviewers for taking to time to review the manuscript and comment on it. The authors found the comments valuable and have made modifications to the original manuscript to incorporate the suggested changes. The quality of the manuscript has improved as a result. Please find below the authors' responses to the specific comments raised. We trust the changes we have made are satisfactory and that the manuscript will be accepted for publication in JoVE.

On behalf of the authors,

Esther Karunakaran.

Editorial comments:

General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: This has been carried out.

2. Please include all authors' emails in the manuscript itself.

Response: The email addresses of all authors has been added to the manuscript under a separate subsection titled emails.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Distel, Falcon, Lonza

Response: All trademark and registered symbols have now been removed from the manuscript and from the Table of Materials and Reagents.

Protocol:
1. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Response: Care has been taken to ensure the "how" of each step is covered. Step 7 in part 3 has been split in to two separate steps (Part 3 steps 7 and 8) in the revised manuscript to aid clarity.

Specific Protocol steps:

1. 3.3: This is a bit confusing- do you mean to leave the eyeball in PBS for 1 minute?

Response: Yes, that is correct. The authors have modified the wording in step 3.3 to aid clarity.

Figures:
1. Figure 1: The text is generally hard to read, including in the original image file.

Response: The font size of the text in the figures has been increased in the revised manuscript.

2. Figure 1A: 'tiameter' is a typo.

Response: The typo has been corrected in the revised manuscript.

Discussion:
1. Please revise the Discussion to explicitly cover the following in detail in 3–6 paragraphs with citations:
a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

Response: Critical steps have been identified in the protocol and marked as "CRITICAL STEP" in the protocol section. Two additional paragraphs have been included in the discussion section of the revised manuscript discussing the critical steps, options for troubleshooting the technique and limitations of the technique.

References:
1. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

Response: The references have been re-formatted according to the suggested style.

Table of Materials:

1. Please remove trademark (™) and registered (®) symbols from the Table of Materials.

Response: The trademark and registered symbols have been removed from the revised Table of Materials.

2. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Response: The authors confirm that the Table of Materials contain information on all materials and equipment used.

Reviewers' comments:

Reviewer #1:

Well written article and described technique.

Ln52 the issues with conventional in-vitro models should be referenced

Response: Two references that describe the issues with conventional models have been added to the revised manuscript.

> *1. Ersoy, S. C. et al (2017) Correcting a Fundamental Flaw in the Paradigm for Antimicrobial Susceptibility Testing. EBioMedicine. dx.doi.org/10.1016/j.ebiom.2017.05.026*
>
> *2. Kubicek-Sutherland, J. Z. et al. (2015) Host-dependent Induction of Transient Antibiotic Resistance: A Prelude to Treatment Failure. EBioMedicine. dx.doi.org/10.1016/j.ebiom.2015.08.012*

It would be good to estimate the number of in-vivo experiments conducted yearly to emphasise the need for reliable ex-vivo models

Response: This is a great suggestion and the authors agree that the suggested comparison would emphasise the need for ex-vivo models. Unfortunately, the authors do not perform in-vivo experiments, and therefore are unable to provide an estimate of the number of in-vivo experiments one can conduct yearly. The authors estimate that the maximum number of ex-vivo corneas one researcher can process is 40 corneas per week, so one can process approximately 2000 corneas per year. This is a reasonable throughput. No change has been made in the revised manuscript concerning this suggestion.

Ln70 what effect does the 2 hour delay from enucleation have. Are the pigs blanched or disinfected?

Response: The pigs are neither blanched nor disinfected. The eyes are closed after the killing and the pigs are refrigerated. The 2 hour delay from enucleation is due to practical issues around staff availability and processing time in the abattoir and cannot be avoided. Based on the literature on enucleation of eyes from human cadaveric donors for transplantation, no adverse effect is expected during the 2 hour delay. For instance, according to Mohamed et al. 2016, human corneas removed within 6 to 10 hours from death can be used for tissue transplantation, provided the cadavers are refrigerated.

> *Mohamed, A. et al. (2016) Outcome of transplanted donor corneas with more than 6 h of death-to-preservation time. Indian Journal of Ophthalmology. DOI: 10.4103/0301-4738.194338*

Why the choice of glass for the moulds compared to a 3D printed design?

Response: Glass was the preferred material due to practical reasons. The authors had ready accessibility to the services of a glass blower rather than a 3D printer. The authors therefore found securing glass moulds less time consuming and cost effective. Using glass as the material for moulds also meant that the moulds can be sterilised by autoclaving between use which minimised issues related to microbial contamination.

Lu 118 indicate PA01 and PA14 are strains

Response: The requested change has been made in the revised manuscript (Part 5 step 2).

What is the thickness of the glass in Figure 1A.

Response: The glass moulds were cut out of standard size laboratory tubes made of borosilicate glass. An additional sentence has been added to the figure legend to clarify this point.

Could do with a control to show minimal/no CFU and ideally the CFU over several time points

Response: Uninfected control cornea were always set up alongside each batch. Everytime, no colony forming units were recovered from uninfected controls. An additional step has been included in the protocol section (Part 6 step 9) of the revised manuscript to emphasise this point. An additional sentence has been added to the end of the figure legend to emphasise that no CFU were recovered from the uninfected controls. The authors have followed the progression of infection over several time points as suggested by the reviewer. However, this has not been included in this manuscript as it will be included in a forthcoming publication. Therefore, no change has been made regarding this suggestion in the revised manuscript.

Reviewer #2:

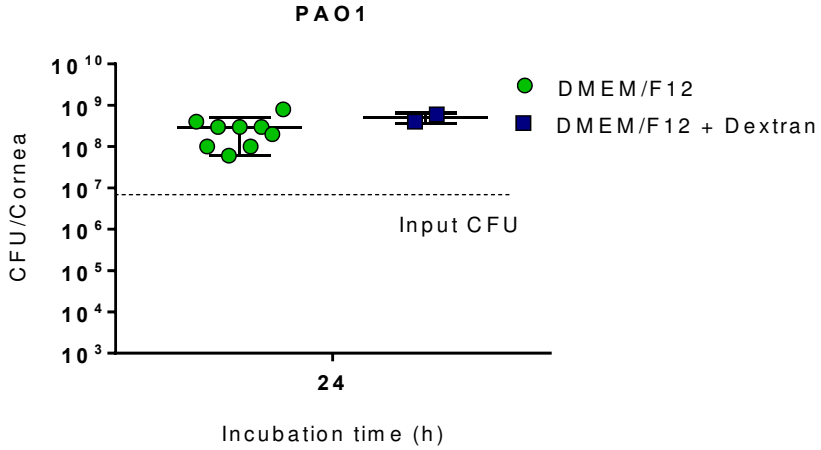
Manuscript Summary:

The glass mould is a fairly innovative and elegant idea that can help standardise infection experiments to yield reproducible results. There are, however, some details that can be optimised to fully utilise the potential of this model.

Major Concerns:

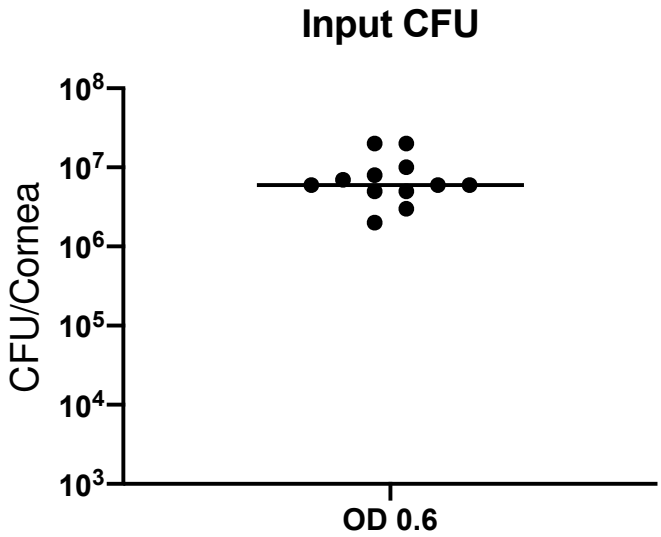
1-You mentioned that the corneas swell up over the course of the experiments. In my experience, this can potentially skew some results, especially in studies where structural integrity is important such as studying biofilm formation. The cornea can increase significantly in thickness reaching well above 1000 microns. This can also affect live confocal imaging studies if this model is to be used, as the resolution would be significantly affected by the thickened cornea with its relatively disorganised collagen fibres and inter-fibrillary spaces. Adding Dextran to culture medium may help mitigate those problems.

Response: The authors agree with the reviewer that the addition of dextran will mitigate swelling of the corneas. The authors conducted an experiment to compare the CFU recovered after 24 hours of infection from dextran treated and untreated cornea. We observed no significant difference in the number of CFU recovered suggesting that addition of dextran did not affect infection of the cornea. Please see graph below. The authors note that it was difficult to filter sterilise dextran which resulted in a lot of corneas developing contamination. This is why addition of dextran is suggested in the original manuscript as an optional step. The authors have included this data in the revised manuscript (Figure 1H).



2- The method of quantifying the infection dose seems to be crude, only approximating the number of CFU/15 ul used. If this model was to be used in infection experiments testing therapeutic agents for example, a more accurate quantification must be used to allow the measurement of the effect of a therapeutic agent, for example, or the growth pattern of bacteria. In step 10, you recommend diluting to 10-4 or 10-5, but it is not clear what such a recommendation is based on. If the initial infection dose is not accurately quantified, and verified by viable counting, such a dilution may yield very variable, even unexpected, results

Response: The authors apologise for lack of clarity in the manuscript regarding the quantification of the infective dose. Every time the experiment was performed, the infective dose was verified by viable plate count to ensure that the target infective dose of 1 x 10^7 CFU per cornea was delivered to the cornea in the 15 uL used. Please see graph below. An additional step has been added to the revised manuscript (Part 7 step 12) to emphasise this.



The recommended dilution in step 10, is to allow the recovery of sufficient CFU on the agar plate during viable plate count to obtain a reproducible result. Since the infective dose is 1 x 10^7 CFU per cornea, a

10-5 dilution is necessary after 24 hours incubation to recover at least 30 CFU per agar plate for viable plate count i.e. the minimum recommended CFU for reproducible viable plate count. The authors agree that when testing the effect of therapeutics the required dilution factor must be arrived at experimentally for reproducible results. An additional sentence has been added to part 7 step 11 to emphasise this point.

3- In Part 6. Infecting the corneoscleral button: Step 3 is not very clear. You mentioned a sterile glass ring - is that the same as your proposed glass mould? How do you "seal the glass ring"? Do you pour some of the agar-containing DMEM between the mould and the cornea? This part needs more elaboration.

Response: The authors have modified the wording of this step (Part 6 step 3) to improve clarity. The sealing of the glass ring is done by adding sufficient DMEM agar (1 mL) to fill the mould completely. The authors appreciate that this is difficult to get across and feel this is a crucial step, the clarity of which will be additionally aided by the video produced by the journal. The wording of part 6 step 4 has been modified to improve clarity.

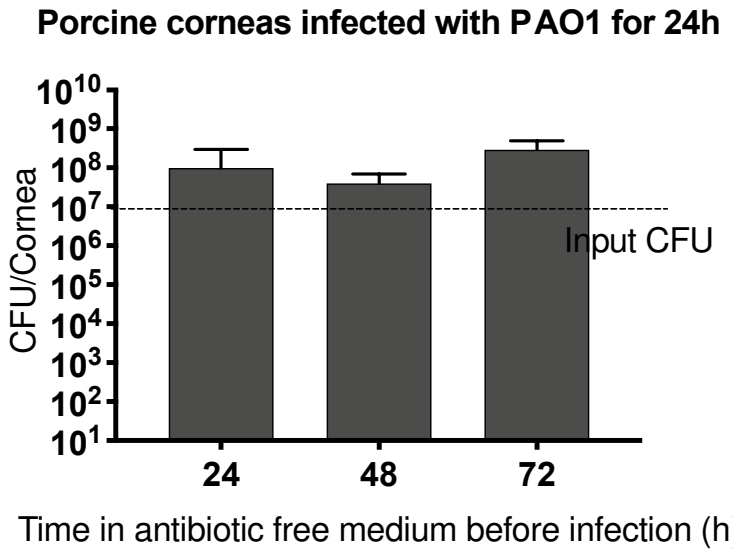
4- In step 6: You mentioned "bacterial culture", do you mean the infective solution? In my experience, 100 microlitres of PBS can evaporate fairly quickly off the surface of the cornea over 24h, leaving a dry, distorted epithelial surface. This may affect the results of infection experiments carried out using this model, especially imaging studies, like scanning electron microscopy, for instance.

Response: All incubation steps were carried out in a humidified incubator with relative humidity levels at 90%. The authors have not observed evaporation of the PBS within the timeframes of incubation reported in the manuscript.

Minor Concerns:

1- the use of antibiotics in the culture medium can affect bacterial growth, despite washing and maintaining in antibiotic-free media for 3 days. One way to ensure the lack of the undesirable effect of antibiotics is to observe the growth of bacteria (e.g. growth curve) in the supernatant from this antibiotic-free medium immediately before commencing the infection experiment.

Response: The authors thank the reviewer for the suggestion, and for particularly stating this point as a minor concern. As the reviewer notes, the corneas placed in antibiotic-containing medium are washed multiple times in PBS before transferring the corneas to the antibiotic-free medium. There is typically a 24 hour incubation in antibiotic-free medium. There is a subsequent removal and replacement of the antibiotic-free medium and a further incubation for 48 hours before infection. The authors have performed an experiment in which CFU recovered per cornea after a 24 hour infection was enumerated and compared from cornea placed in antibiotic-free media for 24, 48 and 72 hours. Please see graph below. No significant difference was observed in the number of CFU recovered suggesting that antibiotics are reduced to negligible levels (if not completely eliminated) even after 24 hours of incubation in antibiotic-free medium. Therefore, the authors believe that antibiotics used will not adversely affect the infection experiment.


Porcine corneas infected with PAO1 for 24h
CFU/Cornea
10^10
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
Input CFU
24
48
72
Time in antibiotic free medium before infection (h)