



Deposited via The University of Sheffield.

White Rose Research Online URL for this paper:

<https://eprints.whiterose.ac.uk/id/eprint/160753/>

Version: Accepted Version

Article:

Okurowska, K., Roy, S., Thokala, P. et al. (2020) Establishing a porcine ex vivo cornea model for studying drug treatments against bacterial keratitis. *Journal of Visualized Experiments*.

<https://doi.org/10.3791/61156>

© 2020 Journal of Visualized Experiments (JoVE). This is an author-produced version of a paper subsequently published in *Journal of Visualized Experiments*. Available under the terms of the Creative Commons Attribution-NonCommercial Licence (<http://creativecommons.org/licenses/by-nc/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. You may not use the material for commercial purposes.

Reuse

This article is distributed under the terms of the Creative Commons Attribution-NonCommercial (CC BY-NC) licence. This licence allows you to remix, tweak, and build upon this work non-commercially, and any new works must also acknowledge the authors and be non-commercial. You don't have to license any derivative works on the same terms. More information and the full terms of the licence here: <https://creativecommons.org/licenses/>

Takedown

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.

Journal of Visualized Experiments

Establishing a porcine ex vivo cornea model for studying drug treatments against bacterial keratitis. --Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61156R1
Full Title:	Establishing a porcine ex vivo cornea model for studying drug treatments against bacterial keratitis.
Section/Category:	JoVE Immunology and Infection
Keywords:	Cornea, ex vivo, porcine, keratitis, Pseudomonas aeruginosa, infection, Antibiotics, organotypic
Corresponding Author:	Esther Karunakaran, PhD The University of Sheffield Sheffield, UNITED KINGDOM
Corresponding Author's Institution:	The University of Sheffield
Corresponding Author E-Mail:	e.karunakaran@sheffield.ac.uk
Order of Authors:	Katarzyna Okurowska Sanhita Roy Praveen Thokala Lynda Partridge Prashant Garg Sheila MacNeil Peter Nigel Monk Esther Karunakaran, PhD
Additional Information:	
Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US\$2,400)
Please indicate the city, state/province, and country where this article will be filmed . Please do not use abbreviations.	Sheffield, South Yorkshire, United Kingdom

1 TITLE:

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

5 AUTHORS:

6 Katarzyna Okurowska^{1,2}, Sanhita Roy³, Praveen Thokala⁴, Lynda Partridge^{1,5}, Prashant Garg³,
7 Sheila MacNeil^{1,6}, Peter N. Monk^{1,7}, Esther Karunakaran^{1,2}

9 AFFILIATIONS:

- 10 1. Sheffield Collaboratorium for Antimicrobial Resistance and Biofilms (SCARAB),
11 University of Sheffield, Sheffield, United Kingdom
- 12 2. Department of Chemical and Biological Engineering, University of Sheffield, Sheffield,
13 United Kingdom
- 14 3. Hyderabad Eye Research Foundation, L V Prasad Eye Institute, Hyderabad, India
- 15 4. Health Economics and Decision Science, School of Health and Related Research,
16 University of Sheffield, Sheffield, United Kingdom
- 17 5. Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield,
18 United Kingdom
- 19 6. Department of Materials Science and Engineering, University of Sheffield, Sheffield,
20 United Kingdom
- 21 7. Department of Infection, Immunity and Cardiovascular Disease, University of
22 Sheffield, Sheffield, United Kingdom

24 EMAILS:

25 K. Okurowska: k.emery@sheffield.ac.uk
26 S.Roy: sanhita@lvpei.org
27 P. Thokala: p.thokala@sheffield.ac.uk
28 L. Partridge: l.partridge@sheffield.ac.uk
29 P. Garg: prashant@lvpei.org
30 S. MacNeil: s.macneil@sheffield.ac.uk
31 P. N. Monk: p.monk@sheffield.ac.uk
32 E. Karunakaran: e.karunakaran@sheffield.ac.uk

34 Correspondence:

35 Esther Karunakaran
36 e.karunakaran@sheffield.ac.uk

38 KEYWORDS:

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

41 SUMMARY:

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

47 ABSTRACT:

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

66 67 **INTRODUCTION**

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

74
75 Bacterial keratitis caused by *P. aeruginosa* is a difficult eye infection to treat particularly due
76 to the increasing emergence of antimicrobial resistant strains of *P. aeruginosa*. Within the last
77 decade, it has become apparent that testing and developing new treatments for corneal
78 infections, in general, and those caused by *Pseudomonas* sp., in particular, are essential to
79 combat the current trend in antibiotic resistance³.

80
81 For testing the efficacy of new treatments for corneal infections, conventional in vitro
82 microbiological methods are a poor surrogate due to the difference in bacterial physiology
83 during laboratory culture and during infections in vivo as well as due to the lack of the host
84 interface^{4,5}. In vivo animal models, however, are expensive, time-consuming, can only deliver
85 a small number of replicates and raise concerns about animal welfare.

86
87 In this article, we demonstrate a simple and reproducible organotypic ex vivo porcine model
88 of keratitis that can be used to test various treatments for acute and chronic infections. We
89 have used *P. aeruginosa* for this experiment but the model also works well with other
90 bacteria, and organisms such as fungi and yeast which cause keratitis.

91 92 **PROTOCOL:**

93 Albino laboratory rabbits were sacrificed in the laboratory for other planned experimental
94 work under home office approved protocols. The eyes were not required for experimental
95 use in those studies so they were used for this protocol.

96

97 **1. Sterilization**

98

99 1.1. CRITICAL STEP: Disinfect all forceps and scissors by soaking for 1 h in 5% (v/v) solution
100 of Distel in distilled water, clean with a brush, rinse with tap water and sterilize in an oven at
101 185 °C for a minimum of 2 h.

102

103 1.2. Sterilize all other glassware and reagents by autoclaving at 121 °C for 15 minutes or
104 prepare reagents according to the manufacturer's instructions. Carry out the following
105 procedures in a class II microbiology safety cabinet.

106

107 **2. Sample collection**

108

109 2.1. Collection of porcine eyes

110

111 2.1.1. Use large white landrace sows, a cross with a Hampshire boar. The age of the pigs was
112 between 21 to 23 weeks when slaughtered in a local abattoir for food consumption. Stun the
113 animals with an electric current and enucleate the eyes 2 h later in the abattoir.

114

115 2.1.2. CRITICAL STEP: Once enucleated, transfer the eyes to the lab in a sterile phosphate
116 buffered saline (PBS) solution to prevent them from drying out and process them immediately
117 upon arrival.

118

119 2.2. Collection of rabbit eyes

120

121 2.2.1. Excise the corneas and send to the lab in sterile PBS.

122

123 **3. Preparation of the corneoscleral button**

124

125 3.1. Use sterile forceps to hold the tissue surrounding the eyeball and transfer it to a Petri
126 dish. Remove the conjunctiva and muscle tissue around the eyeball on a Petri dish using
127 scalpel blade no. 15 and forceps.

128

129 3.2. Gently lift the eyeball while holding the optic nerve with forceps and transfer to a 0.5
130 L jar filled with sterile PBS.

131

132 3.3. Once all eyes are cleared of surrounding tissue, move them using sterile forceps to
133 another 0.5 L jar filled with 3% (v/v) povidone iodine in PBS and leave for 1 min.

134

135 3.4. Transfer eyeballs to another 0.5 L jar with sterile PBS.

136

137 3.5. Use forceps to hold the eye still on a Petri dish and make a cut near the cornea with a
138 scalpel blade no 10A.

139

140 3.6. CRITICAL STEP: Hold the edge of the cut and use scissors to excise the cornea leaving
141 about 3 mm of sclera surrounding the cornea. Ensure the sharp end of scissors does not pierce
142 the iris or the choroidal tissue and is in the supra-choroidal space.

143

144 3.7. Hold the corneoscleral button with forceps and use another pair of pointed end
145 forceps to gently separate the uveal tissue.

146

147 3.8. Lift the corneoscleral button from remaining globe and briefly rinse it in 1.5% (v/v)
148 povidone iodine solution in PBS in a 12 well plate.

149

150 3.9. Place the corneoscleral button into another 12 well plate filled with sterile PBS.

151

152 3.10. After processing all eyes (do no more than 40 eyes in one batch), place each
153 corneoscleral button to an individual Petri dish (34 mm diameter) epithelial side up and pour
154 in 3 mL of culture medium pre-warmed to 37 °C.

155

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

162

163 3.11. Incubate at 37 °C in a humidified tissue culture incubator.

164

165 4. Maintenance of the corneoscleral buttons

166

167 4.1. After 24 hours, use aseptic technique to remove media and replace with 3 mL of fresh
168 pre-warmed culture media containing antibiotics. Keep the corneoscleral buttons in media
169 with antibiotics for 48 h to disinfect the corneas. Incubate at 37 °C in a humidified tissue
170 culture incubator.

171

172 4.2. CRITICAL STEP: After 48 hours, remove the media and rinse corneas with 2 mL of PBS.
173 Then keep the corneoscleral buttons in antibiotic-free media for a minimum of two or ideally
174 three days before experimental infection, to remove residual antibiotics from the tissue.

175

176 4.3. Incubate at 37 °C in a humidified tissue culture incubator. Change media at least one
177 more time within these three days. Discard corneas if any turbidity develops in the antibiotic-
178 free medium.

179

180 5. Preparation of an inoculum

181

182 5.1. Pour 10 mL of LB broth into a 50 mL conical flask with a foam stopper.

183

184 5.2. Transfer a colony of *P. aeruginosa* strain PAO1 or strain PA14 from a fresh agar plate
185 and incubate at 37 °C for 3-4 h until the bacteria are in mid-log phase.

186

187 5.3. Transfer the culture of bacteria to a 50 mL tube and centrifuge at 3,000 x g for 5 min.
188 Remove the supernatant and re-suspend the cell pellet in PBS.

189
190 5.4. Repeat step 5.3 two more times to wash the cells. Re-suspend the cell pellet in PBS
191 and adjust the optical density at 600 nm to approximately 0.6 using sterile PBS as a blank.

192
193 **6. Infecting the corneoscleral button**

194
195 6.1. Remove media from the Petri dish and rinse corneas twice with 1 mL of sterile PBS.

196
197 6.2. Gently squeeze forceps while holding the cornea in-between. Use a 10A scalpel to
198 make four cuts – two vertical, two horizontal - in the central section of the corneoscleral
199 button through the epithelial layer to the underlying stroma.

200
201 6.3. Place a sterile glass mold in a 6-well plate with the wide part up and place the cornea
202 in the middle of the glass mold, epithelium side facing down. Make the cut right in the center
203 of the bottom part of the glass mold.

204
205 6.4. CRITICAL STEP: Pour 1 mL of 1% (w/v) low melting point agar dissolved in DMEM to fill
206 the glass mold with cornea completely.

207
208 6.5. Allow the agar to set and then invert the glass mold so that the corneal epithelium is
209 facing upwards.

210
211 6.6. Pipette 15 μ L of the bacterial culture with $OD_{600nm} = 0.6$ (for *P. aeruginosa* this equates
212 to approximately 1×10^7 colony forming units (CFU) in 15 μ L) directly into a cut area and then
213 add 85 μ L of PBS to the top to keep the corneal epithelium moist.

214
215 6.7. Add 1 mL of DMEM without antibiotics to the bottom of each well with the glass mold.
216 Incubate the 6-well plate with the infected corneoscleral buttons in a humidified incubator at
217 37 °C with 5% CO₂ for up to 24 h.

218
219 6.8. Set up uninfected control cornea alongside every experiment. To set up uninfected
220 control, replace the 15 μ L of bacterial culture in step 6.6 with sterile PBS.

221
222 **7. Homogenization of the cornea to harvest the bacteria**

223
224 7.1. Discard the DMEM medium from the bottom of the 6 well plate and add 1 mL of sterile
225 PBS to rinse the bottom of the well.

226
227 7.2. Remove PBS gently by pipetting without touching the central part of the corneoscleral
228 button. Remove the glass ring using sterile forceps and place it in the 5% Distel.

229
230 7.3. Gently rinse the top of the corneoscleral button with 1 mL of PBS twice [optional].

231
232 7.4. Hold the edge of the corneoscleral button with fine tip forceps and detach it from the
233 agar underneath.

234

235 7.5. Remove the corneoscleral button and place on a sterile Petri dish. Remove the
236 remaining sclera using a scalpel and then transfer the cornea to a 50 mL tube filled with ice
237 cold 1-2 mL of PBS.

238

239 7.6. Use a fine tip homogenizer to shear the top of the infected cornea. The tissue does
240 not have to be completely liquidized. The homogenizer helps to detach bacteria from the
241 corneal epithelium and the cut area.

242

243 7.7. Vortex the cornea in PBS for a few seconds to mix the contents.

244

245 7.8. Add 20 μL of the homogenate to 180 μL of PBS and perform serial dilutions in a 96 well
246 plate.

247

248 7.9. Serially dilute the suspension to 10^{-4} and 10^{-5} dilution and pipette 10 μL of the diluted
249 homogenate with bacteria onto a blood agar plate. Incubate the plate for 8 hours and count
250 the number of CFU. When testing the effect of antimicrobials, the appropriate dilution factor
251 must be arrived at experimentally.

252

253 7.10. In every experiment, homogenize one cornea immediately after infection and perform
254 viable plate count to ensure that the infective dose is approximately 1×10^7 CFU per cornea.

255

256 REPRESENTATIVE RESULTS

257 The design of the glass molds are an innovative and original idea, the use of which allowed us
258 to set up the model in a consistent fashion with minimal/no issues with contamination. The
259 molds were prepared by a glass blower at the University of Sheffield based on a design (**Figure**
260 **1A**). The experimental setup maintains the convex shape of the cornea and holds bacteria on
261 the top of the epithelium where infection takes place (**Figure 1B**).

262

263 Porcine corneas usually swell after few days in medium. This is normal and we found that
264 there was no significant difference between corneas with and without addition of dextran,
265 which is usually added to prevent swelling of the cornea (**Figure 1H**). The corneas are typically
266 wounded to help the bacteria penetrate the epithelium. Although there was no significant
267 difference in the progress of infection between wounded (cut) and unwounded (uncut)
268 corneas, we noticed more variations between replicates in uncut corneas (**Figure 1C**).
269 Washing the corneas twice with PBS removes excess bacteria that did not attach to the
270 epithelium. There was a significant difference in CFU between washed and unwashed porcine
271 corneas infected with *P. aeruginosa* PAO1 for 24 hours (**Figure 1D**). There was no significant
272 difference in CFU counts between porcine and rabbit corneas infected with PA14 and PAO1
273 (**Figure 1E,1F**). The results for both models were reproducible. After 24 hours, the cornea
274 infected with either *Pseudomonas* strain always develop opacity and the cut area becomes
275 more visible and open in comparison to the uninfected cornea (**Figure 1G**).

276

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

281 experimental set up. (C) Testing the effect of wounding on the final CFU count after
282 homogenization. Uncut (n = 16) and cut (n = 28) corneas were infected with *P. aeruginosa*
283 PAO1 and *P. aeruginosa* PA14 for 24 hours. The corneas were washed with 1 mL of PBS before
284 homogenization. Error bars indicate standard deviation. (D) Testing the effect of washing
285 corneas with 2 x 1 mL of PBS (n = 6) and not washing (n = 6) on the final CFU count after
286 infection with *P. aeruginosa* PAO1 for 24 hours. Error bars indicate standard deviation. (E)
287 Final CFU count in porcine corneas infected with *P. aeruginosa* PAO1 and *P. aeruginosa* PA14
288 for 24 hours (n = 10). Corneas were washed and cut. Error bars indicate standard deviation.
289 (F) Final CFU count in rabbit corneas infected with *P. aeruginosa* PAO1 and *P. aeruginosa* PA14
290 for 24 hours (n = 6). Corneas were washed and cut. Error bars indicate standard deviation. (G)
291 Pictures of ex vivo porcine corneas infected with *P. aeruginosa* PAO1 for 24 hours. The control
292 was wounded but no bacteria were added. The infected corneas were wounded and 10⁷ CFU
293 were added to the cut side. No CFU were recovered from the control cornea. (H) Final CFU
294 recovered after 24 hours of infection with *P. aeruginosa* PAO1 from corneas treated with
295 dextran (n = 2) and those without dextran (n = 9). Corneas were washed and cut. Error bars
296 indicate standard deviation.

297

298 **DISCUSSION:**

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

305

306 A number of studies have investigated the effect of infections on ex vivo corneas from various
307 animals such as: rabbit⁶, dog⁷, goat⁸ and pigs⁹⁻¹¹. Most of these studies focus on ways of
308 establishing⁶ and visualizing an infection⁹ but so far there have only been a few publications
309 focusing on drug testing and accurate quantification of bacteria^{6-8,12}.

310

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

325

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

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

347
348 As is the case with any model, there are limitations associated with the ex vivo porcine cornea
349 model described. The model described herein does not mimic the composition, flow and
350 replenishment of the tear film across the cornea. The mechanical action provided by blinking
351 is also not incorporated into the model. There is agreement in the literature that tear film
352 composition and dynamics, and blinking are important defense mechanisms that remove
353 foreign particles and microorganisms from the eye¹³. Indeed, the model also lacks an immune
354 response that is triggered during infection in vivo. It is likely that the progression of infection
355 in vivo in the presence of these defense mechanisms is different to that observed in the ex
356 vivo model described here. Despite these limitations, the ex vivo porcine corneal model is
357 relevant for testing the effectiveness of existing and emerging antimicrobials for two main
358 reasons: 1) the physiology of the bacteria in the ex vivo model mimics the in vivo conditions
359 as bacterial proliferation is dependent on their ability to damage the corneal tissue, and 2)
360 the model incorporates the three dimensional tissue as a diffusion barrier for therapeutics
361 much like in the in vivo situation. Therefore, the ex vivo model is advantageous over
362 conventional techniques for antimicrobial susceptibility testing.

363
364 The ex vivo porcine cornea model described here can be also used for studying different
365 strains of bacteria, fungi and yeast that cause keratitis. This ex vivo cornea model is
366 reproducible and allows one to generate replicates within a short time unlike in vivo models.
367 Instead of PBS, artificial tears or host immune defense cells can theoretically be added to
368 mimic the live scenario. Corneas are obtained from the same breed of pigs and about 21-23
369 weeks old when slaughtered. Therefore, there is less variability between replicates compared
370 to those obtained from human cadavers. The concept of using a porcine ex vivo cornea model
371 for biomedical applications has gained more popularity within the last few years because of
372 its biological similarity to the human eye which makes this model easier to compare¹⁴. There
373 is increased interest in using porcine corneas for transplantation^{15,16} or as a model for dry
374 eye¹⁷ or wound healing¹⁸.

375

376 **ACKNOWLEDGEMENTS:**

377 The authors would like to thank Elliot Abattoir in Chesterfield for providing porcine eyes. The
378 glass rings were made based on our design by the glass blower Dan Jackson from the
379 Department of Chemistry at the University of Sheffield. The authors would like to thank the
380 Medical Research Council (MR/S004688/1) for funding. The authors would like to also thank
381 Mrs Shanali Dikwella for technical help with cornea preparation. The authors would like to
382 thank Mr Jonathan Emery for help with formatting pictures.

383

384 **DISCLOSURES:**

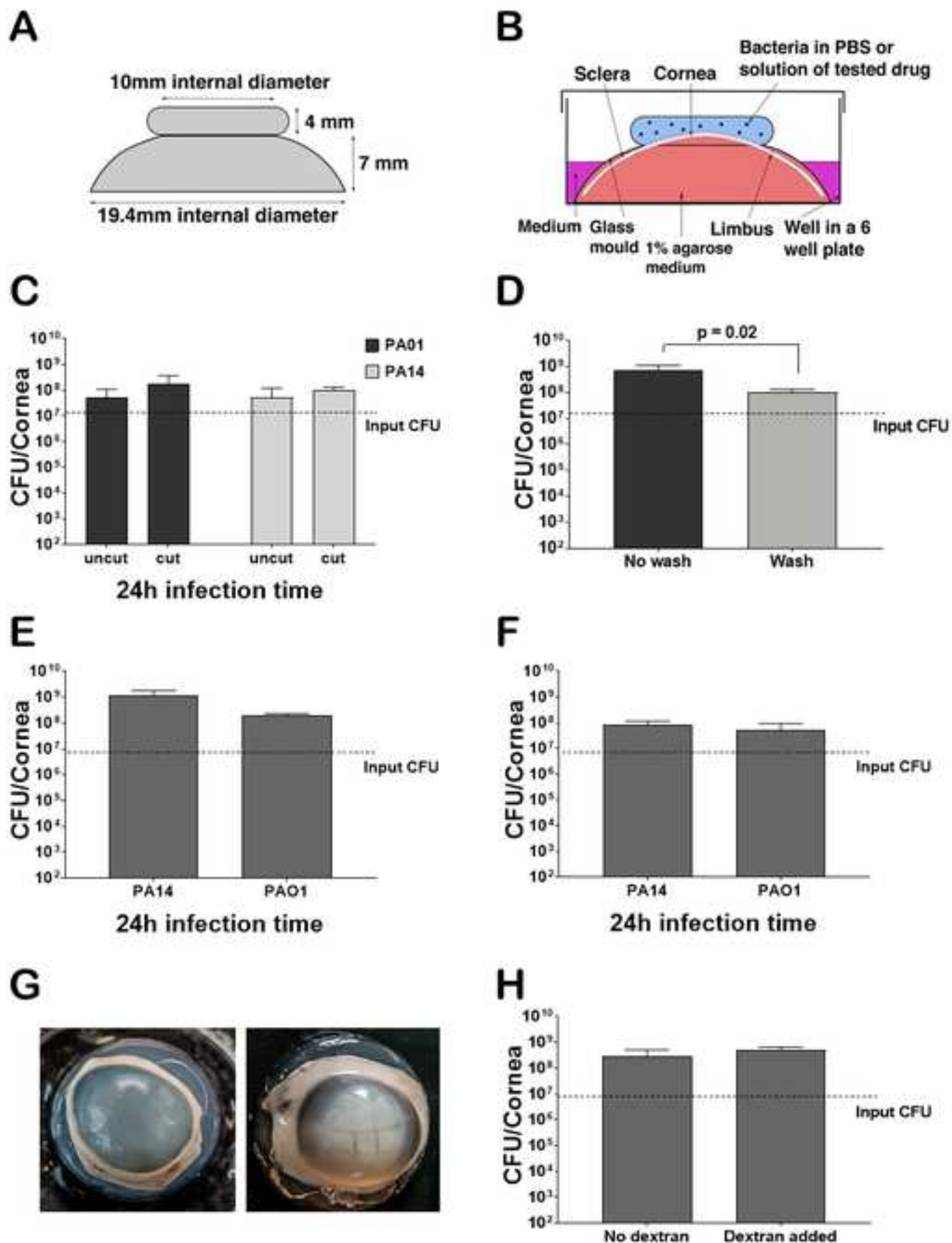
385 The authors have nothing to disclose.

386

387 **REFERENCES:**

- 388 1. Vazirani, J., Wurity, S., Ali, M.H. Multidrug-Resistant *Pseudomonas aeruginosa*
389 Keratitis Risk Factors, Clinical Characteristics, and Outcomes. *Ophthalmology*. **122** (10), 2110
390 - 2114 (2015).
- 391 2. Sharma, S. Keratitis. *Bioscience Reports*. **21** (4), 419 - 444 (2001).
- 392 3. Sharma, G. et al. *Pseudomonas aeruginosa* biofilm: Potential therapeutic targets.
393 *Biologicals*. **42** (1), 1 - 7 (2014).
- 394 4. Ersoy, S. C. et al. Correcting a Fundamental Flaw in the Paradigm for Antimicrobial
395 Susceptibility Testing. *EBioMedicine*. **20**, 173 - 181 (2017).
- 396 5. Kubicek-Sutherland, J. Z. et al. Host-dependent Induction of Transient Antibiotic
397 Resistance: A Prelude to Treatment Failure. *EBioMedicine*. **2** (9), 1169 - 1178 (2015)
- 398 6. Pinnock, A. et al. Ex vivo rabbit and human corneas as models for bacterial and fungal
399 keratitis. *Graefe's Archive for Clinical and Experimental Ophthalmology*. **255** (2), 333 - 342
400 2017.
- 401 7. Harman, R. M., Bussche, L., Ledbetter, E. C., Van de Walle, G. R. Establishment and
402 Characterization of an Air-Liquid Canine Corneal Organ Culture Model To Study Acute Herpes
403 Keratitis. *Journal of Virology*. **88** (23), 13669 - 13677 (2014).
- 404 8. Madhu, S.N., Jha, K. K., Karthyayani, A. P., Gajjar, D. U. Ex vivo Caprine Model to Study
405 Virulence Factors in Keratitis. *Journal of Ophthalmic & Vision Research*. **13** (4), 383 - 391
406 (2018).
- 407 9. Vermeltfoort, P. B. J., van Kooten, T. G., Bruinsma, G. M., Hooymans, A. M. M., van der
408 Mei, H.C., Busscher, H. J. Bacterial transmission from contact lenses to porcine corneas: An ex
409 vivo study. *Investigative Ophthalmology & Visual Science*. **46** (6), 2042 - 2046 (2005).
- 410 10. Duggal, N. et al. Zinc oxide tetrapods inhibit herpes simplex virus infection of cultured
411 corneas. *Molecular Vision*. **23**, 26 - 38 (2017).
- 412 11. Brothers, K. et al. Bacterial Impediment of Corneal Cell Migration. *Investigative*
413 *Ophthalmology & Visual Science*. **56** (7) (2015).
- 414 12. Alekseev, O., Tran, A. H., Azizkhan-Clifford, J. Ex vivo Organotypic Corneal Model of
415 Acute Epithelial Herpes Simplex Virus Type I Infection. *Journal of Visualized Experiments*. 69
416 (2012).
- 417 13. Sack, R. A., Nunes, I., Beaton, A., Morris, C. Host-Defense Mechanism of the Ocular
418 Surfaces. *Bioscience Reports*. **21** (4), 463 - 480 (2001).
- 419 14. Kunzmann, B. C. et al. Establishment of a porcine corneal endothelial organ culture
420 model for research purposes. *Cell and Tissue Banking*. **19** (3), 269 - 276 (2018).

- 421 15. Oh, J. Y. et al. Processing Porcine Cornea for Biomedical Applications. *Tissue*
422 *Engineering Part C-Methods*. **15** (4), 635 - 645 (2009).
- 423 16. Shi, W. Y. et al. Protectively Decellularized Porcine Cornea versus Human Donor
424 Cornea for Lamellar Transplantation. *Advanced Functional Materials*. **29**, 1902491 - 1902503
425 (2019).
- 426 17. Menduni, F., Davies, L. N., Madrid-Costa, D., Fratini, A., Wolffsohn, J. S.
427 Characterisation of the porcine eyeball as an in-vitro model for dry eye. *Contact Lens &*
428 *Anterior Eye*. **41** (1), 13 - 17 (2018).
- 429 18. Castro, N., Gillespie, S. R., Bernstein, A. M. Ex vivo Corneal Organ Culture Model for
430 Wound Healing Studies. *Journal of Visualized Experiments*. 144 (2019).



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	
	Labtech		
Foetal calf serum	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 applicable	Bacterial incubator
PBS	SLS	P4417	
Penicillin-Streptomycin	SLS	P0781	
Petri dish	Fisher Scientific	12664785	
Petri dish 35x10mm CytoOne	Starlab	CC7672-3340	
	Weldricks		
Povidone iodine	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](https://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](https://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](https://doi.org/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:

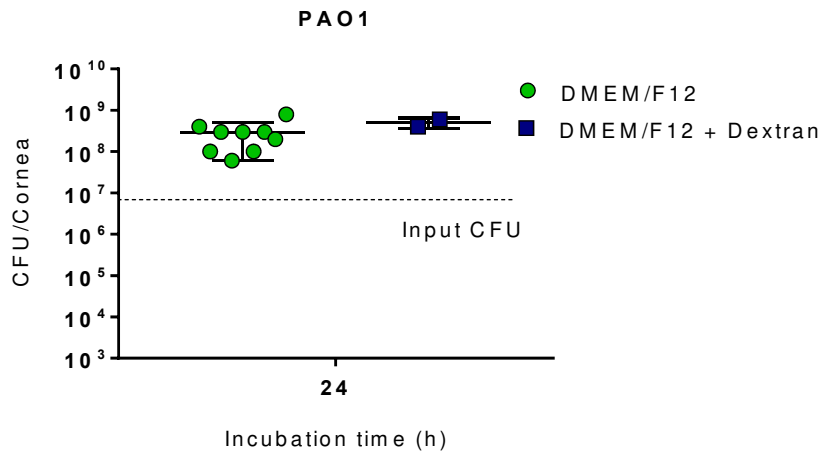
Manuscript Summary:

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:

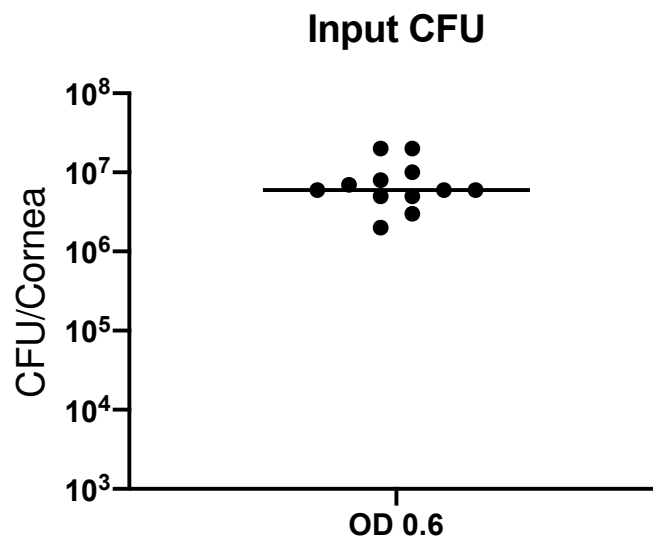
1-You mentioned that the corneas swell up over the course of the experiments. In my experience, this can potentially skew some results, especially in studies where structural integrity is important such as studying biofilm formation. The cornea can increase significantly in thickness reaching well above 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 10⁷ 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 10⁷ 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

