

1 **Testing gentamicin, ciprofloxacin and meropenem against**
2 ***Pseudomonas aeruginosa* on *ex vivo* porcine keratitis model**

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15

16 **ABSTRACT**

17 Global trends show increase in microbial infections caused by pathogens resistant to the most
18 common antibiotics. Antibiotics in development are usually tested on rapidly dividing cells in
19 a culture medium and do not reflect complexity of infections *in vivo*, while testing *in vivo* is
20 limited, expensive and ethically concerning. This often results in development and subsequent
21 prescription of antibiotics only targeting infections in which pathogens are undergoing rapid
22 cell division and in case of persistent infections like keratitis leads to poor clinical outcomes
23 such as impaired vision or loss of an eye. In this study, we demonstrate antibiotic tolerance of
24 *Pseudomonas aeruginosa* strains PAO1 and PA14 using the *ex vivo* porcine keratitis model in
25 which bacterial physiology more closely mimics infections *in vivo* than in a culture medium.
26 MBEC and MIC was used as a guideline to establish concentration of applied antibiotics on
27 tissue. Infected *ex vivo* porcine corneas were treated with therapeutically relevant
28 concentrations of gentamicin, ciprofloxacin, chloramphenicol, clindamycin and fusidic acid.
29 Ciprofloxacin was the most potent across all tests demonstrating a positive correlation with
30 MIC but not MBEC. Nonetheless, the results demonstrated that MIC and MBEC
31 concentrations were not sufficient to clear infection even after 18 hours of continuous exposure
32 to the tested antibiotics reflecting the need for novel antibiotics that can target the persistent
33 subpopulation of these pathogens and the ability of the *ex vivo* keratitis model to be a relevant
34 platform to identify novel antibiotics with suitable activities. There was a clear visual
35 distinction between corneas infected with cytotoxic strain PA14 and invasive strain PAO1. In
36 this study both strains PA14 and PAO1 showed a high level of antibiotic tolerance, which

37 suggests that in clinical settings the treatment approach could be similar regardless of the
38 causative strain.

39

40 INTRODUCTION

41 Bacterial keratitis usually occurs because of infection following the trauma to corneal
42 epithelium caused by injury. Globally blindness caused by bacterial keratitis affects 1.5 to 2
43 million people each year (Whitcher et al., 2001, Humphries et al., 2019) however it is widely
44 acknowledged that keratitis cases globally are underreported (Ung et al., 2019). Amongst the
45 many pathogens that can cause bacterial keratitis, *Pseudomonas aeruginosa* is particularly
46 difficult to treat and is a leading cause of sight loss in the developing world. Widespread use
47 of antibiotics in livestock, availability of antimicrobial treatments without prescription and
48 inappropriate prophylactic use contributes to higher antimicrobial resistance amongst these
49 pathogens (Ting et al., 2021b, Hilliam et al., 2020, Willcox, 2011). Additionally, it is well
50 known that *P. aeruginosa* forms biofilms. Extreme multi-drug resistance and poor clinical
51 outcomes are hallmarks of biofilm infections (Maurice et al., 2018, Thi et al., 2020). Clinical
52 isolates of *P. aeruginosa* resistant to the most used antibiotics are frequently found around the
53 world (Lopez-Dupla et al., 2009, Garg et al., 1999, Willcox, 2011) and reinforce the global
54 urgency to develop new antibiotics.

55

56 Administration of antibiotics in the early stages of infection is recognised clinically as essential
57 for therapeutic success (O'Brien, 2003). Therefore, keratitis is considered as an ocular
58 emergency and treated empirically with broad-spectrum antibiotics. Patients are usually
59 prescribed fluoroquinolone monotherapy (e.g. ciprofloxacin) or a combination therapy with
60 fortified antibiotics (Gokhale, 2008, O'Brien, 2003). In few cases, prior to empirical antibiotic
61 treatment, the corneal scrape is cultured to isolate causative organism and then antibiotic
62 sensitivity testing is performed to select subsequent more targeted (evidence-based) treatment
63 options. However, identifying causative organisms is time consuming and growth and
64 identification of microorganisms occurs in only 40-60% of cases, therefore, evidence-based
65 prescription of appropriate antibiotics is not routinely undertaken in clinics (Dalmon et al.,
66 2012, Ibrahim et al., 2009, Norina et al., 2008, Varaprasathan et al., 2004).

67

68 Currently, treatments aim to achieve minimum inhibitory concentration (MIC) of the drug at
69 the site of infection (Gokhale, 2008). If MIC value indicates that a bacterium is susceptible to
70 an antibiotic, it means that there is a high probability of a positive treatment outcome however

71 bacteria require higher doses of the antibiotic to achieve a therapeutic success *in vivo*
72 (Kowalska-Krochmal and Dudek-Wicher, 2021). Effectiveness of the therapy depends on
73 multiple factors for example: the type and concentration of used antibiotics, exposure time to
74 antibiotics, drug penetration to the site of infection and the duration of the infection before the
75 drug treatment was delivered. As MIC is established on bacteria cultures *in vitro*, it does not
76 consider these tissue specific factors that affect outcome of antibiotic treatment. Additionally,
77 MIC assays indicate sensitivity of an antibiotic against planktonic (free-living) bacteria.
78 Consequently, these concentrations of antimicrobials are often found ineffective against
79 persistent infections such as bacterial keratitis which involve biofilm formation (Costerton et
80 al., 1987, Lebeaux et al., 2014, Davies, 2003). Treating biofilms often requires much higher
81 than normal concentration of antibiotics which can pose a risk of cytotoxicity. While some
82 antibiotics are toxic to corneal epithelium (e.g. gentamicin), others can delay epithelial healing
83 (ciprofloxacin) which can lead to corneal haze or keratolysis. Preservatives (e.g. benzalkonium
84 chloride) in topical ophthalmic medications are directly cytotoxic to both host and pathogen
85 cells, but can improve antimicrobial efficacy by increasing drug penetration through
86 devitalized epithelium (Eun et al., 1994, Noecker, 2001, Goldstein et al., Goldstein et al.,
87 2022). Therefore, a high throughput, *in vitro* model that is able to report on both the potency
88 of the tested antibiotics and any tissue-specific response is needed to identify novel
89 antimicrobials with suitable activities.

90

91 Currently, there is no ideal *in vitro* model for testing efficacy of existing and new antimicrobial
92 treatments. Overall, it takes more than 13 years from discovery to regulatory approval of any
93 new drug and 95% of the drugs that enter human trials fail (Scannell et al., 2012, DiMasi et al.,
94 2020, Seyhan, 2019, Gower et al., 2016). Keratitis models *in vivo* are not suitable for high
95 throughput screening, are expensive, lead to animal suffering and therefore raise ethical
96 concerns (Urwin et al., 2020). Cornea infection models *ex vivo* could be a good alternative to
97 current *in vitro* techniques and have the potential to reduce and refine the use of animals for *in*
98 *vivo* testing. However, *ex vivo* models are a relatively new concept and therefore our goal is to
99 standardise and validate *ex vivo* keratitis model for testing novel treatments.

100

101 In this study, we used the previously established *ex vivo* porcine keratitis model (Okurowska
102 et al., 2020) to test the activity of commonly used antibiotics. Corneas were infected with *P.*
103 *aeruginosa* isolates PA14 and PAO1 that were selected because the biofilm formation
104 (Wozniak et al., 2003, Colvin et al., 2011) and genetic similarities between these two strains

105 (Lee et al., 2006) are well described in the literature. Each of these clinical isolates belongs to
106 one of two major phylogenetic group: group 1, which includes strain PAO1, and group 2, which
107 includes strain PA14 (Freschi et al., 2019). Each phylogenetic group is suspected to have a
108 different effect on the host cells (Hilliam et al., 2020) and the clinical outcomes (Vallas et al.,
109 1996, Borkar et al., 2013, Fleiszig et al., 1996, Lee et al., 2003a). Strain PAO1 is considered to
110 be moderately virulent and forms more structured biofilms on solid surfaces (Goodman et al.,
111 2004) while PA14 is highly virulent, more cytotoxic and forms a weaker biofilm (Wiehlmann
112 et al., 2007, Kasetty et al., 2021, Mikkelsen et al., 2011) called a pellicle that are associated
113 with a stagnant liquid surfaces (Friedman and Kolter, 2004). Kasetty, S. et al. (2021) described
114 differences in biofilm invasion strategies between these two strains in more detail. Genes
115 encoding virulence factors in these strains is regulated by quorum-sensing (QS) systems which
116 are also well described in literature (Girard and Bloemberg, 2008, de Kievit, 2009). In this
117 study we wanted to see if differences between these two strains will be obvious during different
118 stages of infection and after treatments with antibiotics on *ex vivo* porcine keratitis model.

119

120 We tested a range of common antibiotics with various activity against *Pseudomonas* keratitis.
121 We demonstrate that our *ex vivo* porcine keratitis model can be used as a tool to test
122 effectiveness and optimal concentrations of new drugs or preservatives for ocular infections
123 quickly, at lower expense before these treatments are further validated *in vivo*. Our *ex vivo*
124 model could help to select therapeutics that have a greater chance of success in investigations
125 *in vivo*.

126

127 **MATERIALS AND METHODS**

128 **Bacterial strain used**

129 Two wild type strains of *Pseudomonas aeruginosa* (PAO1 and PA14) were a kind gift from
130 Prof. Urs Jenal, University of Basel, Switzerland. Both strains were used to infect *ex vivo*
131 porcine corneas and for establishing MIC and MBEC values.

132

133 **MIC assay**

134 The MIC value for *P. aeruginosa* PAO1 and PA14 was determined according to the EUCAST
135 guidelines (Hasselmann, 2000). The bacterial strains were inoculated in Mueller-Hinton cation
136 adjusted broth (MHB) for 24 hours at 37 °C with agitation at 110 rpm. Before each experiment
137 10 µl of 6-fold dilutions of the inoculum was spot plated on blood agar plates, and the plates
138 were incubated (Infors HT Multitron, UK) overnight at 37 °C in order to enumerate colony

139 forming units in the inoculum. Two hundred microliters of MHB containing an inoculum with
140 3×10^5 CFU per well and different concentrations of the test antibiotics was added to each well
141 in a 96-well plate. A concentration of antibiotics ranging from 0.006 to 32 $\mu\text{g}/\mu\text{L}$ was tested.
142 The MIC value was determined as the lowest concentration of an antibiotic which completely
143 inhibits visible bacterial growth after 24 hours at 37 °C in static conditions. In total six
144 antibiotics were tested: gentamicin, meropenem, ciprofloxacin, clindamycin, fusidic acid and
145 chloramphenicol. Clindamycin, fusidic acid and chloramphenicol are normally not used to treat
146 ocular infections caused by *P. aeruginosa* and were used here as a negative control. The optical
147 density at 600 nm was measured using the TECAN Spark plate reader (TECAN, Switzerland)
148 to confirm the growth inhibition. One column of each 96-well plate was designated for growth
149 control and one for sterility control. The procedure was repeated three times across different
150 days for each antibiotic.

151

152 **MBEC assay**

153 Biofilm susceptibility testing assay was performed using a Calgary device (Innovotech,
154 Canada) where the biofilm was grown on a peg (Harrison et al., 2010). First, growth conditions
155 were verified by an equivalence test for biofilm formation (Figure 1 in Supplementary
156 Materials) as described by Harrison et al. (2010). The bacterial strains were streaked out on LB
157 agar plate from cryogenic stock and incubated overnight at 37 °C. A single colony from the
158 agar subculture was used to inoculate 5 mL MHB and the suspension was incubated in a 50
159 mL Falcon tube while shaking at 110 rpm for 24 hours at 37 °C (Infors HT Multitron, UK).
160 The bacterial suspension was centrifuged at 4000 g in Eppendorf 5710R (Thermo Fisher, UK)
161 for 5 minutes. After discarding the supernatant, the pellet was re-suspended in 5 mL of sterile
162 MHB. The inoculum was prepared in a fresh centrifuge tube by diluting the suspension of
163 bacteria to optical density (OD) of 0.05 at 600 nm. The $\text{OD}_{600\text{nm}}$ was measured using
164 spectrophotometer Jenway (VWR, UK). The inoculum was pipetted in a 96-well plate with a
165 final concentration 8×10^6 CFU of *P. aeruginosa* PAO1 or PA14 per well (150 μl inoculum in
166 each well). One column in a 96-well plate was used as a control and contained media without
167 bacteria added. Pegs from Calgary Device were immersed in the inoculum. The 96-well plate
168 was double sealed with parafilm, placed inside a plastic box to reduce evaporation and
169 incubated (statically) overnight at 37°C with 70% humidity in the incubator (Infors HT
170 Multitron, UK) to allow biofilm formation on pegs. Before each experiment 10 μl of 6-fold
171 dilutions of the inoculum was spot plated on blood agar plates, and the plates were incubated
172 overnight at 37 °C in order to enumerate colony forming units (CFU) in the inoculum. After

173 overnight incubation the pegs were rinsed twice for 1 minute in two 96-well plates with 200 μ l
174 of sterile water per well to remove bacteria that did not attach to pegs (planktonic cells).

175

176 For equivalence assay, the pegs were then transferred to a 96-well plate with 200 μ l of LB with
177 1% Tween 20 per well, sonicated for 10 minutes at 60 Hz to disrupt bacteria from the biofilm
178 on pegs into a recovery medium. After sonication, 20 μ l of the recovery medium with the
179 bacteria was diluted in series up to 10^4 in 180 μ l of sterile water. All dilutions were plated out
180 on LB agar plates for CFU count and incubated at 37 °C overnight.

181

182 For MBEC assay the pegs were transferred after rinsing steps to a 96-well plate with antibiotics
183 in MHB. The plate was incubated overnight and then rinsed and sonicated in the same way as
184 equivalence assay plates. Ciprofloxacin, meropenem and gentamicin were tested with
185 concentrations starting from $1\mu\text{g } \mu\text{L}^{-1}$ to $512\mu\text{g } \mu\text{L}^{-1}$. Minimum biofilm eradication
186 concentration (MBEC) value represents the wells with the lowest concentration of an antibiotic
187 where the biofilm was completely eradicated i.e. there was no growth from biofilms across all
188 replicates. One column of each 96-well plate was designated for untreated control and one for
189 sterility control. The procedure was repeated four times across different days for each antibiotic
190 with four technical replicates each time.

191

192 **Testing antibiotics on *ex vivo* porcine cornea model**

193 In this study, porcine eyes were extracted within four hours from slaughter and transported
194 from the abattoir (R.B. Elliott and Son Abattoir, Calow, England) in a Nalgene container filled
195 with sterile phosphate buffer saline (PBS, Sigma, Germany). The age of pigs varied between
196 26 to 28 weeks. The corneas were excised in the laboratory within two hours from delivery and
197 used for experiments within a week from excision. The pigs were sacrificed for human
198 consumption and not for the purpose of this study.

199

200 Porcine eyes were prepared for infection as described previously (Okurowska et al., 2020).
201 Briefly, the porcine eyes from the abattoir were washed with sterile PBS before and after
202 disinfection with 3% povidone iodine diluted with PBS for 60 seconds. Corneas with scleral
203 rings were dissected, rinsed with 1.5% povidone iodine and then soaked in PBS before
204 transferring to warm Dulbecco's modified Eagle's medium (DMEM, Fisher Scientific, UK)
205 supplemented with growth factors and antibiotics. The composition of the culture medium was
206 as follows: DMEM: Ham's (Sigma, Germany) [1:1] supplemented with $5\mu\text{g mL}^{-1}$ insulin (SLS,

207 UK) and 10 ng mL⁻¹ epidermal growth factor (EGF) (SLS, UK), 10 % fetal calf serum (FCS)
208 (Pan-Biotech, UK), 100 U mL⁻¹ penicillin, 100 U mL⁻¹ streptomycin (SLS, UK) and 2.5 µg mL⁻¹
209 amphotericin B (Sigma, UK). The corneas were incubated in medium with antimicrobials for
210 24 hours at 37 °C and then washed once with 2 mL PBS and incubated in antimicrobial-free
211 medium for 48 hours to remove residual antibiotics. The medium was replaced every day
212 during this time. On the infection day porcine corneas were infected with 8x10⁶ CFU in 200 µl
213 of PBS and incubated for 6 hours. After the incubation, the PBS along with suspended bacteria
214 were removed with a sterile 1 mL pipette tip and replaced either with 200 µL of PBS (control
215 corneas) or with a PBS with added antibiotic (treated corneas). The corneas were treated with
216 either 1024 µg mL⁻¹ or with MIC concentration of ciprofloxacin, meropenem, gentamicin for
217 18 hours at 37 °C. All corneas were photographed with Dino-lite Xcope camera (AnMo
218 Electronics Corporation, Taiwan). Ninety cornea images were independently scored for opacity
219 by five people using following grading system: 0 – no haze, cornea clear; 1 – faint opacity or
220 cloudiness visible; 2 – cornea looks swollen, white or hazy patch clearly visible. All graphs
221 were plotted using GraphPad Prism version 8.4.1.

222

223 **Statistics**

224 Statistical analysis of viable cell counts for experiment comparing two strains of *Pseudomonas*
225 was carried out by unpaired t-tests with Holm-Sidak correction while effect of treatment versus
226 placebo was calculated using Kruskal-Wallis multiple comparisons test, using GraphPad Prism
227 version 8.4.1. *P*-values lower than 0.05 were considered significant.

228

229 **Data availability**

230 All supporting data are provided in the Supplementary Materials file.

231

232 **RESULTS**

233

234 **MIC assay**

235 MIC assays revealed that both strains of *P. aeruginosa* used in this study were sensitive to
236 gentamicin, meropenem and had intermediate resistance to ciprofloxacin (Table 1) while they
237 were resistant to clindamycin, fusidic acid and chloramphenicol (MIC > 32 µg mL⁻¹). The MIC
238 values for gentamicin were identical for both strains (2 - 4 µg mL⁻¹). However, some small
239 differences were observed between strains treated with meropenem and ciprofloxacin. Strain
240 PA14 was marginally more susceptible to meropenem (0.25 µg mL⁻¹) while strain PAO1 was
241 marginally more susceptible to ciprofloxacin (0.125-0.25 µg mL⁻¹).

242

243 **Table 1. Determination of MIC and MBC of *P. aeruginosa* for PAO1 and PA14 isolates**
 244 **against gentamicin, meropenem and ciprofloxacin . Values in the table represent $\mu\text{g mL}^{-1}$.**
 245 **1.**
 246

| Generic name (class) Break points (EUCAST, 2022) | PAO1 | | PA14 | | Mechanism of action |
|---|----------------|------------------------------|--------------|---------------------------|---|
| | MIC | MBEC | MIC | MBEC | |
| Gentamicin (aminoglycoside) ≤ 4 S; ≥ 16 (R) | 2-4 | 64 (16X - 32X MIC) | 2-4 | 16 (4X - 8X MIC) | Broad spectrum, inhibits synthesis of bacterial proteins by binding to 30S ribosomes |
| Meropenem (carbapenem) ≤ 2 (S); ≥ 8 (R) | 0.5-1 | >512 | 0.25 | >512 | Broad spectrum, inhibition of bacterial cell wall synthesis |
| Ciprofloxacin (fluoroquinolone) ≤ 0.001 (S); ≥ 0.5 (R) | 0.125- 0.25 | 4-8 (16X - 64X MIC) | 0.25- 0.5 | 4 (8X - 16X MIC) | Inhibits DNA replication by inhibiting bacterial DNA topoisomerase and DNA-gyrase |

247

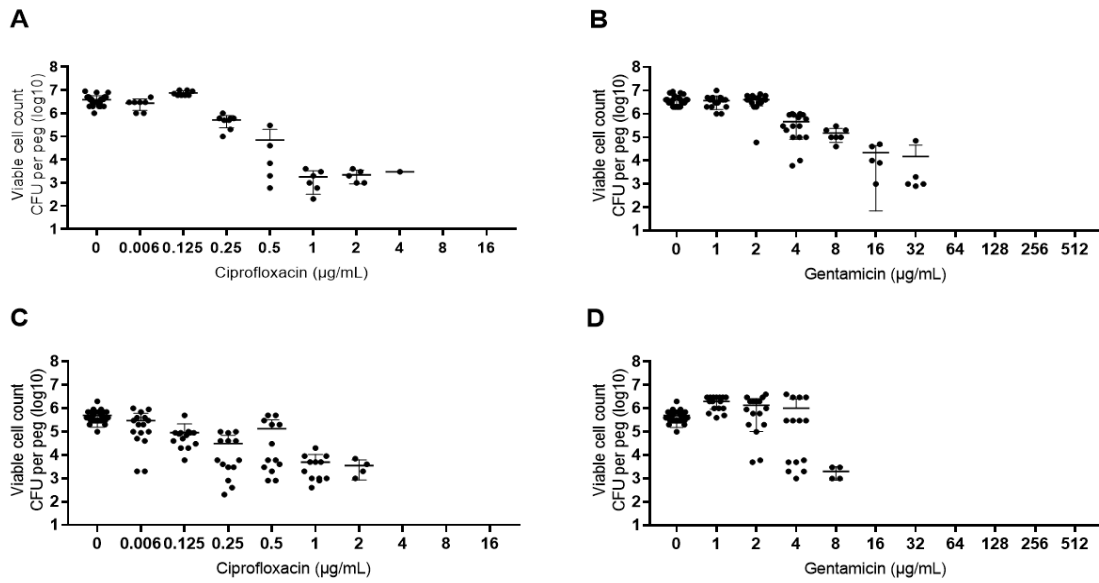
248

249 **MBEC assay**

250 Following MIC results, determination of MBECs for biofilms of both strains using Calgary
 251 device was carried only on gentamicin, meropenem and ciprofloxacin. MBEC value represents
 252 the wells with the lowest concentration of an antibiotic where the biofilm was completely
 253 eradicated i.e. there was no growth from biofilms across all replicates (Figure 1).

254

255 Despite MIC results showing sensitivity to meropenem (Table 1), biofilms of both *P.*
 256 *aeruginosa* strains demonstrated tolerance to meropenem exceeding the concentrations tested
 257 ($>512 \mu\text{g mL}^{-1}$). For PAO1 strain, MBEC values were 16-64 times higher than MIC for
 258 ciprofloxacin and 16-32 times higher than MIC for gentamicin (Fig. 1A and 1B). MBEC values
 259 for PA14 strain for ciprofloxacin were 8-16 times higher than MIC and 4-8 times higher than
 260 MIC for gentamicin (Fig. 1C and 1D). These results suggest that the biofilm on pegs formed
 261 by strain PA14 was more sensitive to gentamicin and ciprofloxacin compared to strain PAO1
 262 (Table 1). MBEC testing made the difference between two strains more noticeable. With
 263 reference to the break point system (Table 1) and subsequent clinical relevance, the MBEC
 264 results demonstrate that biofilms formed by *P. aeruginosa* could be classified as resistant to
 265 gentamicin, meropenem and ciprofloxacin.



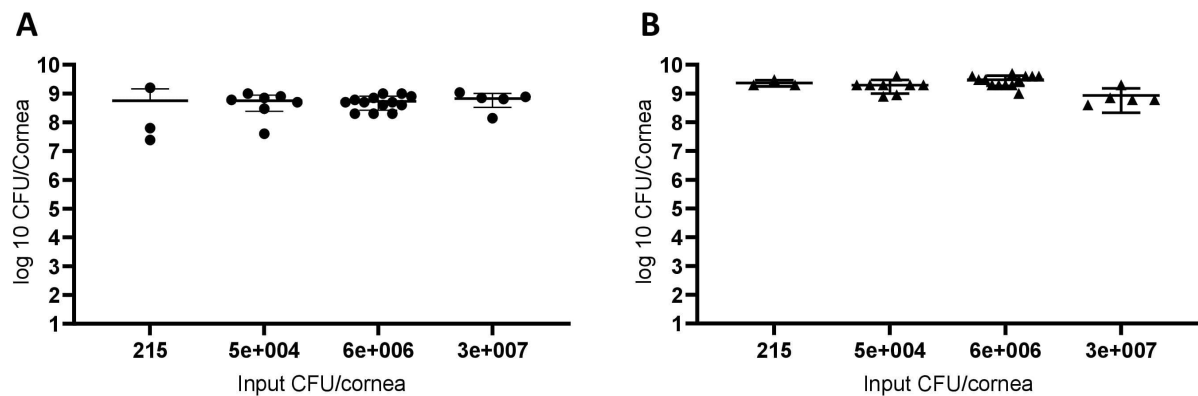
266
267 Fig.1. MBEC assay results representing colony forming units of *P. aeruginosa* PAO1 (A, B)
268 and PA14 (C,D) treated with ciprofloxacin and gentamicin.
269

270 Investigation of antimicrobial efficacy on the *ex vivo* porcine keratitis model

271 Effect of inoculum size on final Colony Forming Unit (CFU)

272 To establish the inoculum size needed to initiate an infection on porcine cornea, various CFUs
273 of *P. aeruginosa* PA14 were added to wounded corneas. A viable count of bacteria retrieved
274 from the infected cornea after 24 hours of infection (Fig. 2A) and 48 hours of infection (Fig.
275 2B) was carried out. Despite the starting inoculum size, an average of 6×10^8 CFUs per cornea
276 were retrieved after 24 hours and 2×10^9 CFUs per cornea after 48 hours. There was no
277 significant difference in CFU between groups and two incubation times. These results indicate
278 that the ultimate bacterial load in the porcine *ex vivo* cornea infection model is independent of
279 the initial bacterial load. Due to the good reproducibility in the number of CFU retrieved after
280 infection with a higher starting inoculum size, in further experiments, an inoculum size of
281 greater than 1×10^6 CFU per cornea was aimed for. We established that the maximum
282 incubation time for all following experiments was 24 hours because 48 hours of incubation
283 resulted in a complete lysis of the cornea by the bacteria.

284



285

286 Fig 2. Number of viable *P. aeruginosa* PA14 retrieved from porcine cornea after infection with
287 215, 5×10^4 , 6×10^6 and 3×10^7 CFU per cornea. Corneas were infected for 24 hours (A) and 48
288 hours (B). Each dot represents results from one cornea. Error bars represent standard deviation.
289 Statistical significance was calculated with Kruskal-Wallis test followed by Dunn's multiple
290 comparisons test * p -value <0.05. Raw data is available in Supplementary Materials, Data
291 Availability Section.

292

293

294 **Effect of incubation time on progress of infection**

295 To investigate the progress of infection over time, porcine corneas were infected with *P.*
296 *aeruginosa* PA14 and *P. aeruginosa* PAO1 and a viable count was carried out on bacteria
297 retrieved from the infected cornea after 1, 2, 4, 6, 18 and 24 hours post infection (hpi) (Fig. 3).

298 With *P. aeruginosa* PA14, an average of 1.9×10^6 CFU per cornea were retrieved after 1 hpi
299 ($n = 7$), 2.9×10^6 CFU per cornea were retrieved after 2 hpi ($n = 6$), and 4.9×10^6 CFU per
300 cornea were retrieved after 4 hpi ($n = 6$) (Fig. 3A). At all these time points, the number of CFU
301 retrieved per cornea were lower than the inoculum size (7.7×10^6 CFU per cornea) reflecting
302 the impact of post-incubation rinsing steps included in the protocol during which the bacterial
303 population not securely adhered to the corneal tissue are removed. After 6 hpi, the number of
304 bacteria retrieved from the infected cornea were approximately equal to the inoculum size
305 despite rinsing ($n = 6$). Incubation beyond 6 hpi reproducibly resulted in a clear increase of
306 CFU retrieved per cornea despite rinsing, resulting in 1.0×10^8 CFU per cornea at 18 hpi ($n =$
307 6) and 9.0×10^7 at 24 hpi ($n = 6$) (Fig. 3A). Difference in CFU values for PA14 retrieved at 1
308 hpi and 2 hpi in comparison to 18 hpi and 24 hpi was significant ($p < 0.05$).

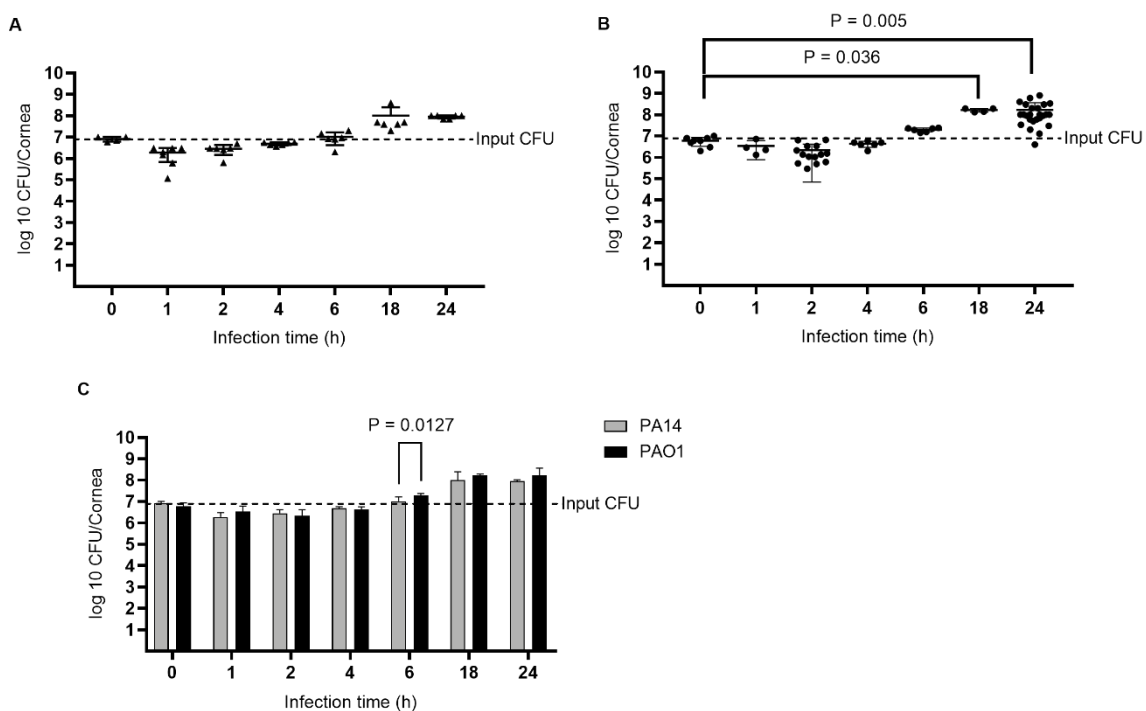
309

310 A similar trend was seen in the progress of infection in the *ex vivo* porcine cornea infected with
311 *P. aeruginosa* PAO1 strain (Fig. 3B). An average of 3.4×10^6 CFU per cornea were retrieved
312 after 1 hpi ($n = 4$), 2.2×10^6 CFU per cornea were retrieved after 2 hpi ($n = 14$) and 4.1×10^6
313 CFU per cornea were retrieved at 4 hpi ($n = 6$). Like the infection with *P. aeruginosa* PA14, at

314 all these time points, the number of CFU retrieved per cornea were lower than the inoculum
315 size (7.9×10^6 CFU per cornea). Subsequently the increase in bacteria load in the infected
316 cornea was higher compared to the inoculum size for *P. aeruginosa* PAO1 (Fig. 3C): 2.0×10^7
317 CFU per cornea at 6 hpi (n = 6), 1.6×10^8 CFU per cornea at 18 hpi (n = 4) and 1.7×10^8 CFU
318 per cornea at 24 hpi (n = 25) (Fig. 3B). Difference in CFU values for PAO1 retrieved at 1hpi,
319 2 hpi and 4hpi in comparison to 18 hpi and 24 hpi was significant ($p < 0.05$).

320

321 These data demonstrate that both strains of *P. aeruginosa* were able to initiate and maintain
322 infection on porcine corneas within first few hours of incubation. In both strains, despite
323 inclusion of a washing step, there was a net increase in the number of CFU retrieved after
324 incubation compared to the inoculum which suggests that infection was well established in the
325 model. In the subsequent experiments, antibiotic treatments were added to corneas at 6 hpi
326 because there was a clearly visible increase in CFU counts at this time point in comparison to
327 input of bacteria which indicated that the infection was well-established.



328

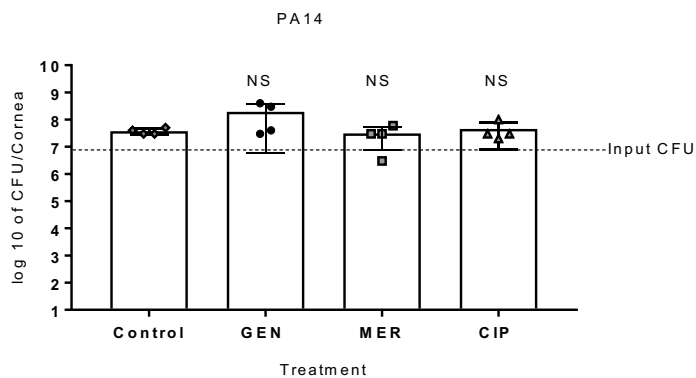
329 Fig. 3. Number of viable CFU of *P. aeruginosa* on porcine corneas infected for 1, 2, 4, 6, 18
330 and 24 hours with *P. aeruginosa* PA14 (A) and *P. aeruginosa* PAO1 (B). Data from both strains
331 are compared on one graph (C). Inoculum CFU are shown both as CFU at t=0 infection time
332 (x-axis) as well as a dotted line labelled as Input CFU. Each dot on charts A and B represents
333 one cornea. Error bars represent standard deviation. Statistical significance for graph A and B
334 is presented according to Kruskal-Wallis test while for graph C is presented according to the
335 unpaired *t*-test with Holm-Sidak correction **p*-value < 0.05 . Raw data is available in
336 Supplementary Materials, Data Availability Section.

337

338 Testing antibiotics on the *ex vivo* porcine keratitis model

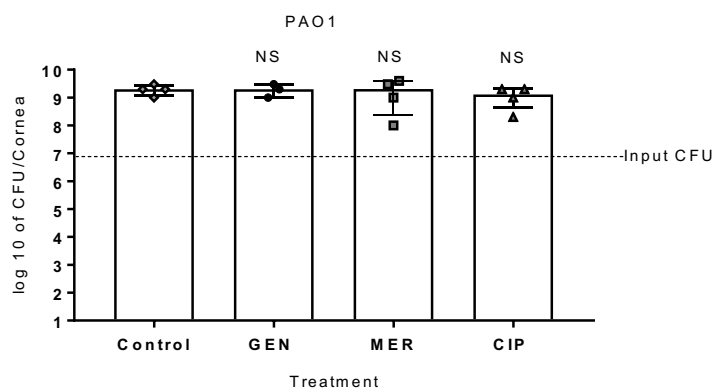
339 Next, the effect of MIC concentrations on infected tissue was investigated. *Ex vivo* porcine
340 corneas were infected on average with 1×10^7 CFU *P. aeruginosa* PA14 and 9×10^6 *P.*
341 *aeruginosa* PAO1 for 6 hours and then MIC concentrations of gentamicin, meropenem and
342 ciprofloxacin were applied for 18 hours. While MIC concentrations of antibiotics successfully
343 inhibited growth of bacteria *in vitro*, these concentrations were ineffective ($p > 0.05$) for both
344 tested strains of *P. aeruginosa* PA14 and PAO1 in *ex vivo* porcine cornea model (Fig. 4). Raw
345 data are presented in Supplementary Materials Table 2. This demonstrates that application of
346 MIC concentrations on *ex vivo* cornea is insufficient to treat ocular infections with *P.*
347 *aeruginosa* despite the fact that the infected tissue was continually exposed to the antibiotic for
348 18 hours.

349 **A**



350

351 **B**



352

353 Fig. 4. Colony forming units of *P. aeruginosa* in the *ex vivo* porcine corneas infected for 6
354 hours with (A) PA14 or (B) PAO1. Control corneas were immersed in PBS while other corneas
355 were treated with MIC concentrations of antibiotic dissolved in PBS. Following antibiotics
356 were applied on infected corneas: gentamicin (GEN) (n = 4), meropenem (MER) (n = 4) and
357 ciprofloxacin (CIP) (n = 4). Error bars are means ± SD. Kruskal-Wallis multiple comparison

358 test was performed for the pairwise statistical analysis of treated against control colony forming
359 units for each strain.

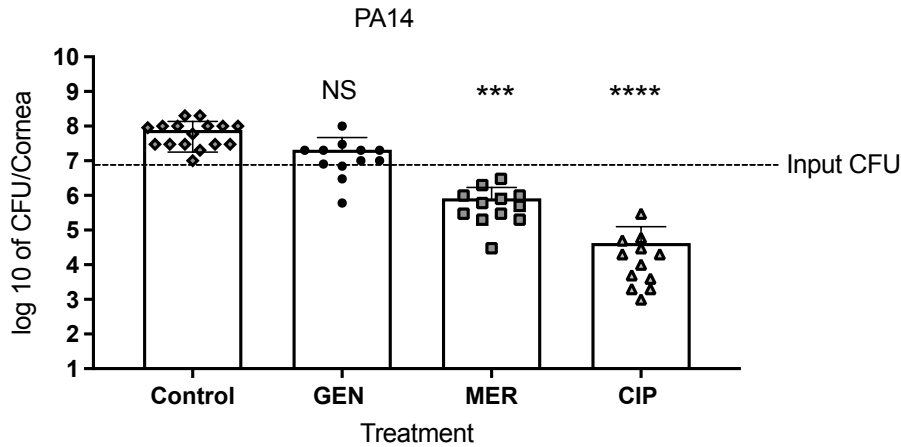
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361 Following previous results, the concentration of antibiotics (gentamicin, meropenem and
362 ciprofloxacin) that were applied on *ex vivo* porcine corneas was increased to 1025 $\mu\text{g mL}^{-1}$.
363 This concentration is 256 times MIC for gentamicin for strains PAO1 and PA14, respectively.
364 For meropenem, this concentration is 1025 times MIC for strain PAO1 and 4100 times MIC
365 meropenem for PA14. For ciprofloxacin, this concentration is 4100 times MIC for strain PAO1
366 and 2050 times MIC for strain PA14. As this concentration is higher than MIC and MBEC
367 some growth inhibition on *ex vivo* infected tissue was expected. A significant reduction in
368 bacteria load for strain PAO1 in corneas treated with gentamicin ($p = 0.0051$), meropenem (p
369 < 0.0001) and ciprofloxacin ($p < 0.0001$) was observed when compared to controls (Figure 5).
370 There was no significant reduction for corneas infected with strain PA14 and treated with
371 gentamicin ($p = 0.15$). However, treatment with meropenem ($p = 0.0001$) and ciprofloxacin (p
372 < 0.0001) had a noticeable reduction in bacteria load.

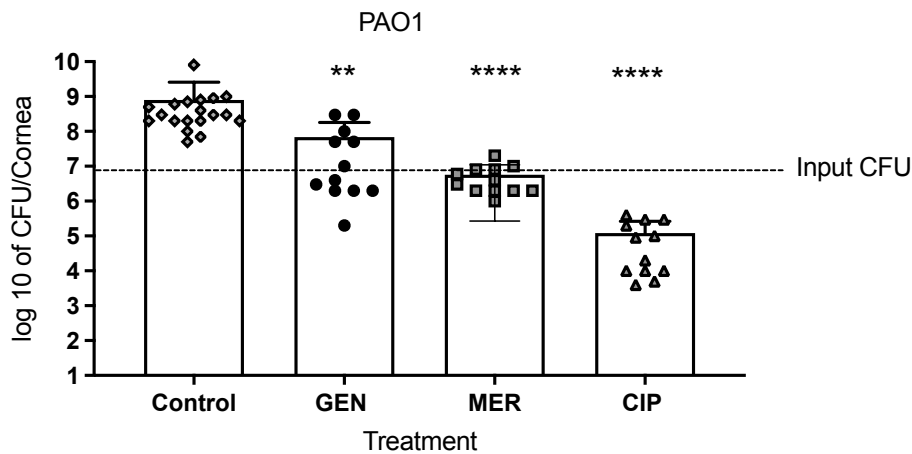
373

374 Out of three tested antibiotics at concentration 1025 $\mu\text{g mL}^{-1}$, gentamicin was the least potent,
375 possibly because the concentration of this antibiotic added to corneas in the relation to MIC
376 was much smaller in comparison to meropenem and ciprofloxacin. On average 7×10^7 CFU (1-
377 log reduction) of strain PAO1 and 2×10^7 CFU (< 1 -log reduction) of strain PA14 were recovered
378 from porcine corneas. Meropenem reduced bacteria load approximately by 2-log for both
379 strains (5×10^6 CFU per cornea for strain PAO1 and 9×10^5 CFU per cornea for strain PA14)
380 despite MBEC assay suggesting that biofilms of both strains are tolerant to $> 512 \mu\text{g mL}^{-1}$ of
381 this antibiotic. Treatment with ciprofloxacin resulted in an average of 1×10^5 CFU (5-log
382 reduction) for corneas infected with strain PAO1 and 5×10^4 CFU (4-log reduction) for corneas
383 infected with strain PA14. Treatment with clindamycin, fusidic acid and chloramphenicol had
384 no effect on bacteria count in comparison to control corneas with added PBS only, and this was
385 expected since MIC results showed that these antibiotics are ineffective on investigated *P.*
386 *aeruginosa* strains (supplementary materials Figure 2).

387 **A**



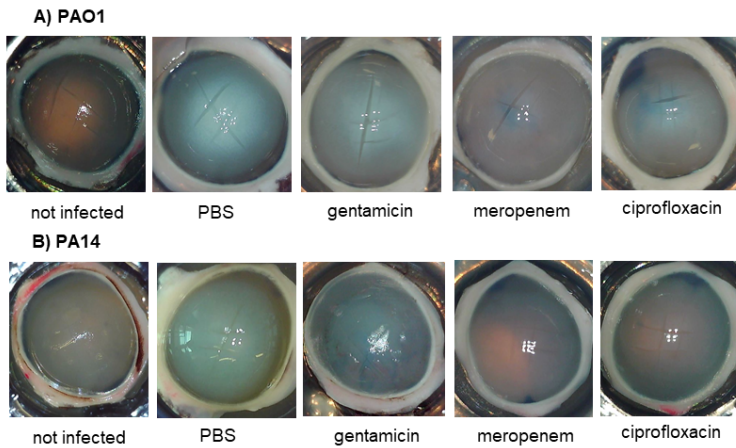
388
389 **B**



390
391 Fig. 5. Colony forming units of *P. aeruginosa* in the *ex vivo* porcine corneas infected for 6
392 hours with (A) PA14 or (B) PAO1. Control corneas (n = 19 & 16) were immersed in PBS
393 while other corneas were treated with 1025 µg mL⁻¹ of antibiotic dissolved in PBS. Following
394 antibiotics were applied on infected corneas: gentamicin (GEN) (n = 12), meropenem (MER)
395 (n = 12) and ciprofloxacin (CIP) (n = 12). Error bars are means ± SD. Kruskal-Wallis multiple
396 comparison test was performed for the pairwise statistical analysis of treated against untreated
397 colony forming units for each strain; significant difference (*p* value < 0.05) is denoted with *.

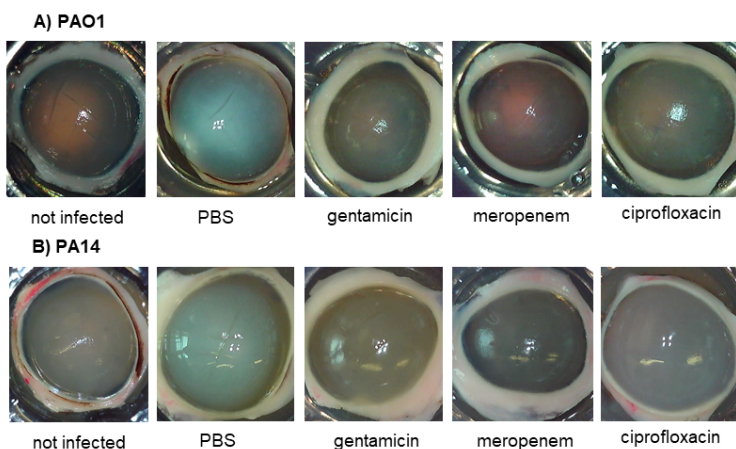
398
399
400 All infected and treated corneas were photographed before homogenisation (Fig.6&7). *Ex vivo*
401 corneas often swell a little while kept in media for a few days which may give them a slightly
402 hazy look and affect the opacity grading (see not infected corneas Fig. 6.). Clinically, *P.*
403 *aeruginosa* keratitis usually manifests with a presence of a large epithelial defect related to
404 stromal necrosis that appears as a ring-like, milky in colour stromal infiltrate. *P. aeruginosa*
405 infection on *ex vivo* porcine cornea manifested with similar features compared to clinical
406 infections *in vivo* (Fig. 6&7 PBS). Visually there was less white discoloration on all corneas
407 treated with MIC concentration of gentamicin, meropenem and ciprofloxacin in comparison to
408 the untreated infected corneas (PBS). This difference is even more obvious on corneas infected

409 with PA14 (Fig. 6B). Corneas infected with both *P. aeruginosa* strains and treated with MIC
410 concentration of gentamicin looked slightly hazier in comparison to meropenem and
411 ciprofloxacin in spite of no reduction in viable colony count across all antibiotics (Fig. 4).



412
413 Fig. 6. Representative images of not infected and infected *ex vivo* porcine corneas without
414 (PBS) treatment and treated with gentamicin, meropenem and ciprofloxacin. Corneas shown
415 here were infected with 6×10^6 CFU of strain PAO1 (A) and strain PA14 (B) and treated with
416 MIC concentrations of antibiotics after infection had progressed for 6 hours. Corneas were
417 imaged and immediately homogenised for viable counting.
418

419 Treatment with higher antibiotic concentration ($1025 \mu\text{g mL}^{-1}$) decreased corneal opacity of
420 infected cornea by preventing development of milky colour (Fig. 7). This effect was especially
421 evident in cornea infected with PA14. Corneas treated with gentamicin, meropenem and
422 ciprofloxacin looked clear and visually impossible to distinguish from uninfected showing a
423 direct effect of treatment on opacity (Fig. 7). Despite high bacteria count, gentamicin treatment
424 preserved corneal transparency.



425
426 Fig. 7. Representative images of not infected and infected *ex vivo* porcine corneas without
427 (PBS) treatment and treated with $1025 \mu\text{g mL}^{-1}$ of gentamicin, ciprofloxacin, fusidic acid,

428 clindamycin, and chloramphenicol. Corneas shown here were infected with 6×10^6 CFU of
429 strain PAO1 (A) and strain PA14 (B) 6-hour prior to antibiotic treatment. Corneas were imaged
430 and immediately homogenised for viable counting.

431
432 The presence and intensity of discolouration and opacity for ninety-nine corneas was
433 undertaken by assessing the images to verify if infection or the effect of a treatment could be
434 determined visually. The images of corneas were allocated to the following three grades: 0 –
435 the corneas looked clear and not infected; 1 - corneas looked infected, slightly hazy and cloudy;
436 2 – corneas looked infected, swollen and white/milky in colour. Images of corneas were blind
437 scored by five different people and presented as percentages (Fig. 8).

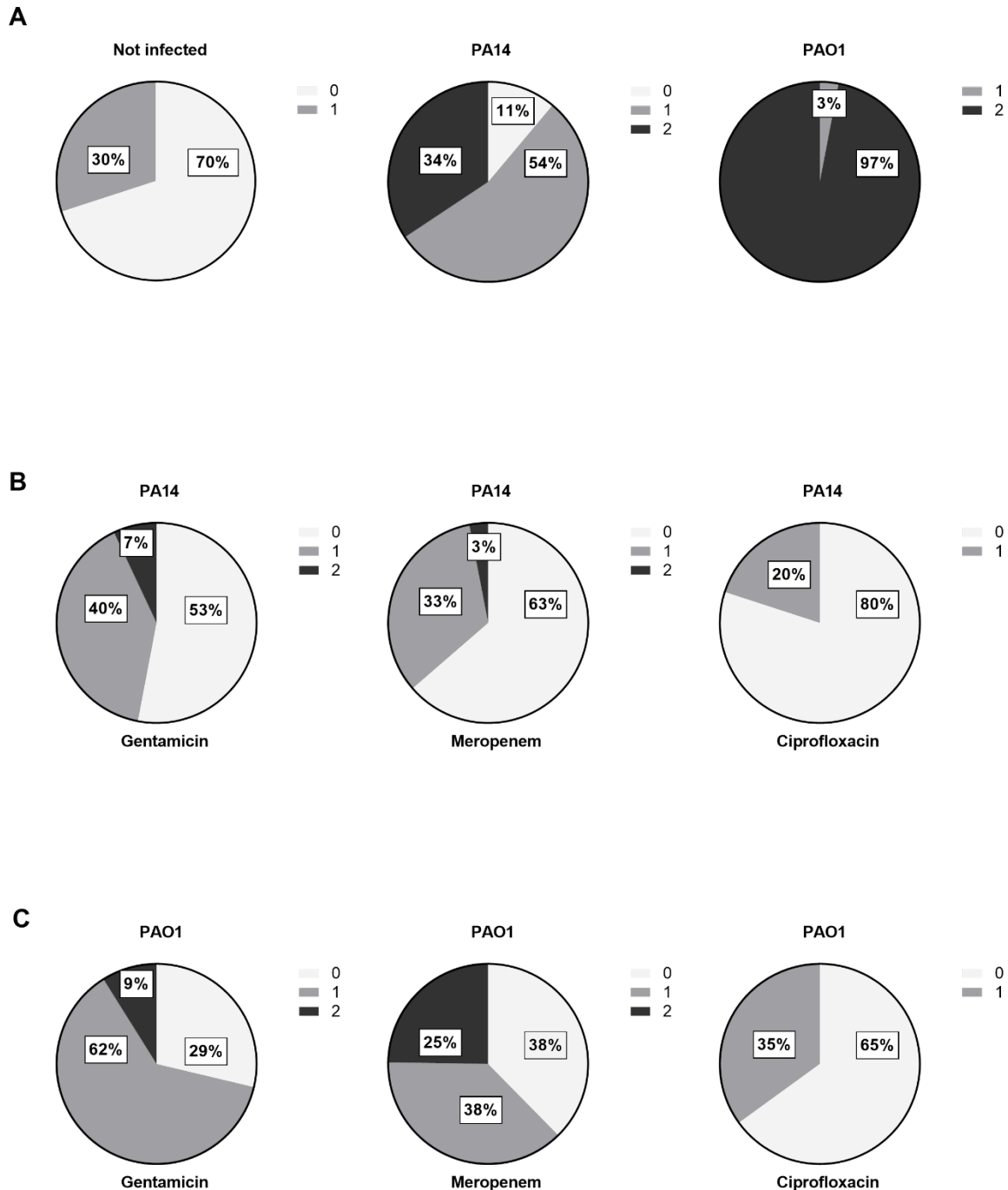
438
439 Majority of uninfected corneas (70%) were correctly identified while 30% were graded 1 (Fig.
440 8A). This is because the scoring of uninfected corneas could have been affected by swelling
441 that naturally occurs during incubation in media for few days and could make some corneas
442 appear less transparent than normal. Lack of previous experience in scoring, changes in the
443 natural light in the room and quality of some images could also have an effect on cornea
444 grading.

445
446 Some untreated corneas infected with strain PA14 looked clear (11%) whilst majority were
447 correctly identified as infected (54% scored grade 1 and 34% scored grade 2) (Fig. 8A).
448 Majority of untreated corneas (97%) were correctly identified as being infected with strain
449 PAO1 due to the development of an obvious haziness (grade 2) suggesting infection with strain
450 PAO1 results in ulceration and severe tissue damage on *ex vivo* porcine cornea (Fig. 8A).

451
452 Overall, corneas infected with strain PA14 and treated with antibiotics and (Fig. 8B) developed
453 less haze in comparison to strain PAO1 (Fig. 8C). Smaller percentage of cornea infected with
454 either strain and treated with $1025 \mu\text{g mL}^{-1}$ gentamicin and meropenem were scored grade 2 in
455 comparison to untreated (Fig. 8B & 8C versus Fig. 8A). None of the corneas infected with
456 either strain and treated with ciprofloxacin were graded 2 and majority were graded 0 which
457 suggests that ciprofloxacin had a beneficial effect on preserving corneal transparency.

458
459 Opacity scoring demonstrated visual differences in opacity between infection with *P.*
460 *aeruginosa* cytotoxic strain PA14 and infection with invasive strain PAO1. The PAO1 strain
461 showed the most visually obvious reduction in opacity for both untreated as well as treated
462 corneas. However, higher transparency of corneas treated with antibiotics should not be solely

463 used to determine effectiveness of a drug because, for example, in case of gentamicin the
 464 reduction in colony forming units was very minimal even though the transparency was
 465 improved. These data demonstrate that images of *ex vivo* corneas could be used as one of
 466 preliminary indicators showing the immediate response of infection to a treatment.



467
 468 Fig. 8. Graph shows the percentage of scored *ex vivo* porcine corneas (n = 99) that were not
 469 infected (n = 4) versus infected with *P. aeruginosa* strain PA14 (n = 42) and PAO1 (n = 44)
 470 for 24 hours (A). Some corneas were treated with 1025 $\mu\text{g mL}^{-1}$ of gentamicin, meropenem or
 471 ciprofloxacin after infection with PA14 (B) and PAO1 (C). Corneas were allocated to following

472 groups: 0 – corneas clear, no haze or infection visible; 1- corneas looked infected, slightly hazy
473 and cloudy; 2 – corneas looked infected, swollen and white/milky in colour.

474

475

476 **DISCUSSION**

477

478 We previously established an *ex vivo* porcine model of *Pseudomonas aeruginosa* keratitis
479 (Okurowska et al., 2020). In this study we demonstrate that our *ex vivo* porcine cornea model
480 can be used for testing novel treatments against keratitis. We first established MIC and MBEC
481 values for gentamicin, meropenem and ciprofloxacin using cytotoxic (strain PA14) and
482 invasive (strain PAO1) strains of *P. aeruginosa*. Next, we tested the input of bacteria needed
483 to develop an infection and then monitored the development of an infection over time. Finally,
484 we investigated differences in response to antibiotic treatments between cytotoxic (strain
485 PA14) and invasive (strain PAO1) strains of *P. aeruginosa* on *ex vivo* porcine keratitis model.

486

487 Comparing MIC and MBEC results to literature was challenging because of the variance in
488 experimental protocols between research groups (Kowalska-Krochmal and Dudek-Wicher,
489 2021, Schuurmans et al., 2009) but overall, our findings followed the trend in literature.
490 Gentamicin, meropenem and ciprofloxacin were the most effective against both studied strains
491 of *P. aeruginosa*. MBEC values of all tested antibiotics were much higher in comparison to
492 MIC. Our results tie in well with previous studies showing that higher concentrations of
493 antibiotics are needed to eradicate biofilms compared to their planktonic counterparts (Bagge
494 et al., 2004, Brady et al., 2017, Bowler et al., 2012, Ceri et al., 1999).

495

496 Gentamicin was the first antibiotic that we tested for MIC against *P. aeruginosa* because it
497 often is used in early stages of keratitis (Dart and Seal, 1988). We found that MIC results in
498 our study, were in alignment with those found in literature and demonstrated that both strains
499 of *P. aeruginosa* (PA14 and PAO1) were equally sensitive to gentamicin. Our MIC values
500 were either identical (Bowler et al., 2012, Ceri et al., 1999) or similar to those reported by other
501 research groups ($1 \mu\text{g mL}^{-1}$ and $2 \mu\text{g mL}^{-1}$)(Andrews, 2001, Bahari et al., 2017, Pusic et al.,
502 2018). While MIC values did not demonstrate any obvious differences between investigated
503 two strains, MBEC results clearly had shown that PAO1 was more resistant to gentamicin in
504 comparison to PA14. Similar to other studies, MBEC values showed that biofilms were
505 resistant to gentamicin in *P. aeruginosa* (Bowler et al., 2012, Ceri et al., 1999, Billings et al.,
506 2013). These results suggest that whilst gentamicin could be used to eradicate planktonic forms

507 of *P. aeruginosa*, treating the biofilm formed by this bacterium would require much higher
508 concentrations. Resistance of *P. aeruginosa* biofilm towards gentamicin could be explained by
509 the fact that gentamicin belongs to aminoglycosides group of antibiotics, that are known to
510 bind to various components in the biofilm matrix (Ciofu and Tolker-Nielsen, 2019) such as
511 exopolysaccharides Psl (Billings et al., 2013) and Pel (Colvin et al., 2011).

512

513 We selected meropenem for our studies because of good corneal penetration, low cytotoxicity
514 (Sueke et al., 2015) and promising results in live rabbit keratitis model. Our MIC values for *P.*
515 *aeruginosa* PAO1 showed sensitivity towards meropenem and were identical ($0.5 \mu\text{g mL}^{-1}$)
516 (Riera et al., 2010, Ocampo-Sosa et al., 2012) or close ($1-2 \mu\text{g mL}^{-1}$) (Haagensen et al., 2017,
517 Bowler et al., 2012, Monahan et al., 2014) to those found in literature. MIC value for strain
518 PA14 in our study are also in accordance with findings reported by others ($0.25 \mu\text{g mL}^{-1}$)
519 (Hassan et al., 2020, Ocampo-Sosa et al., 2012). MBEC values in literature for *P. aeruginosa*
520 PAO1 biofilm treated with meropenem were consistently much higher than MICs (Haagensen
521 et al., 2017, Bowler et al., 2012). Overall, while MIC results show sensitivity, our MBEC
522 results ties well with previous studies showing resistance in *P. aeruginosa* towards this
523 antibiotic (Bowler et al., 2012, Haagensen et al., 2017). These findings support the notion that
524 meropenem is more effective against actively dividing, planktonic bacteria or early-stage
525 biofilm, while less effective against established biofilms (Bowler et al., 2012).

526

527 Finally, we investigated ciprofloxacin in our studies because it is considered as one of the most
528 effective antibiotics against *P. aeruginosa* and therefore used as a first-line treatment in the UK
529 (Hilliam et al., 2020). Our study demonstrated that ciprofloxacin was certainly the most potent
530 antibiotic against planktonic and biofilm of *P. aeruginosa* not only *in vitro* but also on *ex vivo*
531 cornea model. Our MIC values for ciprofloxacin indicate that both strains were susceptible
532 according to EUCAST, 2022 with PA14 marginally more resistant than PAO1. Overall, the
533 results for MIC were similar to those in literature. Research groups reported MIC values in
534 range from $0.125 \mu\text{g mL}^{-1}$ (Riera et al., 2010) to $0.25 \mu\text{g mL}^{-1}$ (Shafiei et al., 2014, Bowler et
535 al., 2012) and $1 \mu\text{g mL}^{-1}$ (Fernandez-Olmos et al., 2012) for PAO1. Some studies reported MIC
536 $0.125 \mu\text{g mL}^{-1}$ for PA14 (Soares et al., 2019, Bruchmann et al., 2013) while ours was
537 marginally higher ($0.25-0.5 \mu\text{g mL}^{-1}$). Our MBEC results also match trends in other studies *in*
538 *vitro* which demonstrated that there was small but not significant difference in response to
539 ciprofloxacin between biofilm formed by PA14 and PAO1 (Billings et al., 2013, Benthall et
540 al., 2015).

541

542 In initial studies to establish *ex vivo* keratitis model, we tested various bacterial loads in the
543 inoculum to initiate infection. It was found that as few as 215 CFU of *P. aeruginosa* PA14 per
544 cornea was enough to initiate infection in our *ex vivo* model and the CFU retrieved from the
545 cornea after 24 hours were the same regardless of the input. We speculate that this phenomenon
546 happens at 24 hours post infection because the nutrients become more limited at this time point
547 and similarly to batch cultures, the bacteria reached a stationary growth phase (Llorens et al.,
548 2010, Rolfe et al., 2012). Additionally, changes in pH, accumulation of toxic metabolites and
549 many other factors force bacteria to enter the stationary phase (Jaishankar and Srivastava, 2017,
550 Llorens et al., 2010) and it is common in the wild (Gefen et al., 2014). The proliferation of
551 bacteria on *ex vivo* cornea is not limited by antimicrobial compounds in tears (McDermott,
552 2013) or host response (Hazlett et al., 2014) as it would normally happen in live scenario.
553 Lower inoculum was found to decrease the prevalence of ulcerative microbial keratitis in live
554 animals (Lawinbrussel et al., 1993) therefore researchers often initiate infection using an
555 inoculum load higher than or equal to 1×10^6 CFU of *Pseudomonas* sp. per eye (Tam et al.,
556 2011, Lawinbrussel et al., 1993, Preston et al., 1995, Augustin et al., 2011). However, in
557 Lawinbrussel et al. (1993) study, the corneas were not wounded but lower inoculum was
558 introduced with contact lens and the infection time was extended to 9 days in rabbits *in vivo*
559 (Lawinbrussel et al., 1993), therefore comparing our findings to literature is challenging.
560 Additionally, as previously discussed (Urwin et al., 2020), we are unable to compare our results
561 to other studies *ex vivo* due to lack of standardised protocol. As a higher inoculum allows
562 reliable bacterial quantification and makes bacterial visualization on infected corneas easier
563 (Ting et al., 2021a) we subsequently used an inoculum containing at least 1×10^6 CFU per
564 cornea in further experiments.

565

566 To identify whether it is possible to distinguish between infections caused by cytotoxic and
567 invasive strains of *P. aeruginosa*, we compared progress of infection over time between
568 cytotoxic *P. aeruginosa* PA14 and invasive *P. aeruginosa* PAO1 strain by monitoring the
569 number of CFU retrieved from the cornea over time. Growth plateau observed after 18 hours
570 of incubation with both strains suggests stationary phase was reached for both strains at this
571 point. This suggests that the enhanced cytotoxicity of *P. aeruginosa* PA14 did not seem to
572 confer a selective advantage during infection of the wounded *ex vivo* porcine cornea. These
573 observations led us to conclude that enhanced cytotoxicity did not dramatically affect progress
574 of infection in our porcine keratitis model.

575

576 Finally, when the antibiotics were tested on the *ex vivo* keratitis model, we discovered that
577 gentamicin was not effective at concentration of 1.25 mg mL⁻¹. Studies on rabbits *in vivo* used
578 various concentrations of gentamicin (1.6, 3, 5 & 13 mg mL⁻¹) to treat *P. aeruginosa* keratitis
579 with mixed therapeutic outcome (Punitan et al., 2019, Rootman and Kraiden, 1993, Fruchtpery
580 et al., 1995, Silbiger and Stern, 1992, Gupta et al., 1995, Kowalski et al., 2013, Ahmad et al.,
581 1977). One case study showed that the treatment with 14 mg mL⁻¹ gentamicin failed even
582 though the *P. aeruginosa* strains isolated from human cornea were identified as sensitive to
583 this antibiotic (Chan et al., 2021). Some of the concentrations used in mentioned studies were
584 cytotoxic because it was found that as little as of 3 mg mL⁻¹ of this antibiotic is cytotoxic in
585 human corneal epithelial cells *in vitro* (Tsai et al., 2010) and impairs the wound healing in
586 rabbits *in vivo* (Stiebel-Kalish et al., 1998). This suggests that potential harm of higher
587 gentamicin concentrations used to treat infections may outweigh the benefit, especially with
588 prolonged exposure. Again, it is very difficult to compare our results from infection treatment
589 outcome to *in vivo* because of differences in wounding techniques (bacteria are often injected
590 into the stroma), number of CFU in inoculum, exposure time and concentration of an antibiotic
591 and the host immune response between studies. Although we did not measure if gentamicin
592 reached MIC in tissue, this antibiotic shows a good corneal tissue penetration; therefore, the
593 concentration of this antibiotic more likely reached MIC values (Rootman and Kraiden, 1993,
594 Yau et al., 1986). Additionally, wounding corneas in our study created a defect that is expected
595 to increase penetration of an antibiotic (McDermott et al., 1993). Also gentamicin demonstrates
596 post antibiotic affect (PAE) where bacteria growth is inhibited following exposure even after
597 the drug concentration has fallen below MIC (Karlowsky et al., 1994). According to the
598 literature, cytotoxic strains of *Pseudomonas aeruginosa* (PA14) remain mostly outside the host
599 cells, while invasive strains (PAO1) reside and replicate inside corneal cells during infection.
600 Therefore, it is believed that antibiotics that do not penetrate host cell membranes such as
601 tobramycin or gentamicin are often less effective against invasive strains of *P. aeruginosa*,
602 while ofloxacin (e.g. ciprofloxacin) that penetrate host cell membranes can be used to target
603 these strains (Lee et al., 2003b, Cendra et al., 2017). Our results for gentamicin demonstrated
604 an opposite effect because the antibiotic significantly reduced viable count only for strain
605 PAO1 in the *ex vivo* keratitis model.

606

607 Meropenem has a low toxicity, good corneal tissue penetration (Sueke et al., 2015) and it was
608 found to be very effective in *Pseudomonas* keratitis in concentrations of 50 mg mL⁻¹ in rabbits

609 *in vivo* (Bozkurt et al., 2021) and humans (Pande and Bhailume, 2014) without any side effects.
610 Some studies found that meropenem concentrations of 5 mg mL⁻¹ increased cellular activity in
611 corneal epithelial cell lines and the cell viability was still high (96%) after meropenem
612 treatment (Sueke et al., 2014, Sueke et al., 2015). Meropenem reduced the bacterial load in our
613 *ex vivo* corneas similarly to other studies on *ex vivo* rabbits or human (Bozkurt et al., 2021).
614 Low toxicity at high concentrations and reduction of bacteria load in studies *in vivo* suggests
615 that meropenem could be a good alternative drug against keratitis in the future (Pande and
616 Bhailume, 2014, Bozkurt et al., 2021). However, the resistance towards this antibiotic in
617 MBEC data is concerning (Bowler et al., 2012, Haagensen et al., 2017). Haagensen et al. (2017)
618 demonstrated that meropenem was highly effective in early stages of *P. aeruginosa* PAO1
619 biofilm formation. We also achieved good reduction of bacteria load after application of
620 meropenem after 6 hours post infection, during possibly early stages of biofilm formation.
621 More studies need to be conducted to assess the effectiveness of this drug and possibility of its
622 use in clinical practice. Some studies report that combining meropenem and ciprofloxacin can
623 have a synergistic effect against some clinical isolates of *P. aeruginosa* (Erdem et al., 2002,
624 Erdem et al., 2003, Siqueira et al., 2014, Pankuch et al., 2008, GarciaRodriguez et al., 1996)
625 which could be tested on our *ex vivo* porcine keratitis model in the future.

626
627 Ciprofloxacin has a very good tissue penetration property. Exposure to this antibiotic for as
628 short as 10 minutes has been demonstrated to result in concentrations exceeding MIC in human
629 cornea *ex vivo* (Silva et al., 2017, McDermott et al., 1993, Akkan et al., 1997, Ozturk et al.,
630 1999) therefore we suspect that the 18 hour continuous exposure to this antibiotic in our study
631 very likely resulted in MIC concentration in corneal tissue. Ciprofloxacin was very effective
632 in eradicating *P. aeruginosa* at higher concentrations in our experiments which is in line with
633 studies *in vivo*. The treatment was equally effective against cytotoxic and invasive strains of *P.*
634 *aeruginosa*. Several studies found that treating corneas with ciprofloxacin significantly
635 reduced or completely ceased infection with *P. aeruginosa* in live rabbits (Obrien et al., 1988,
636 Aliprandis et al., 2005, Guzek et al., 1994, LaBorwit et al., 2001, Bu et al., 2007, Kowalski et
637 al., 2001, Lauffenburger and Cohen, 1993, Oguz et al., 2005, Rhee et al., 2004) and humans
638 (Levey et al., 1998). Although it was found that phenotypic adaptation towards persistence to
639 this antibiotic happens very early if supra-MIC concentrations are used and as a result
640 ciprofloxacin may fail to eradicate biofilm (Soares et al., 2019). Using higher concentrations
641 of ciprofloxacin (0.3%) can cause crystalline corneal precipitation in humans (Wilhelmus and
642 Abshire, 2003, Wilhelmus et al., 1993, McDonald et al., 2014).

643

644 A study on primary human corneal fibroblasts *in vitro* showed that antimicrobial treatment
645 failed to clear bacteria located intracellularly (Cendra et al., 2017) which may explain the
646 presence of remaining bacteria that survived treatment with higher than MIC concentration of
647 ciprofloxacin, meropenem and gentamicin in our *ex vivo* model. Our *ex vivo* data clearly show
648 that topical MIC drug concentrations at the site of infection are not sufficient to rapidly kill *P.*
649 *aeruginosa*. Similarly to studies on mouse *in vivo*, we found no significant difference in
650 response towards antimicrobials between cytotoxic and invasive strain of *P. aeruginosa* (Lee
651 et al., 2003b) in our *ex vivo* keratitis model.

652

653 Visual acuity is clinically one of the parameters showing therapeutic response in patients
654 (Borkar et al., 2013, Hue et al., 2009). We observed that corneal damage caused by *P.*
655 *aeruginosa* in our *ex vivo* keratitis model looked visibly similar to images found in clinical
656 case reports (Hue et al., 2009). The response to different treatments can be observed and used
657 to foresee the outcome which makes this model even more advantageous in comparison to
658 other *in vitro* models. Genetic differences between cytotoxic and invasive *P. aeruginosa* strains
659 led to different effects on epithelial cells (Fleiszig et al., 1997) which may be observed visually
660 (Cole et al., 1998). However, other researchers found a lack of correlation between the number
661 of viable bacteria remaining after antibiotic treatment and disease severity assessed visually
662 from images (Lee et al., 2003b) in a similar way to ours. This was verified in our study where
663 the corneas looked healthy after gentamicin treatment despite of high colony count. In our
664 experiments, the invasive strain PAO1 had initially the highest observable opacity with and
665 without an antibiotic treatment in comparison to the cytotoxic strain PA14. A similar
666 conclusion was reached by Borkar et al. (2013) where the ulcer size from invasive strains of *P.*
667 *aeruginosa* in human keratitis was significantly bigger than from cytotoxic although
668 genotypically invasive strains were associated with better visual acuity at enrolment. Some
669 studies on mice showed that the damage in the centre of the cornea is not only due to bacterial
670 damage but also a result of neutrophil infiltration (Fleiszig et al., 1996, Cole et al., 1998, Borkar
671 et al., 2013) however our model *ex vivo* lacks neutrophils therefore ulceration comes from
672 bacterial action.

673

674 The limitation of present studies naturally includes absence of fully operating host-defences in
675 *ex vivo* model. Nevertheless, the response to treatment with tested antibiotics was in line with
676 trends found in literature and showed that observations our *ex vivo* keratitis model is very

677 similar to other animal models *in vivo* and to findings in clinical studies on humans. Therefore,
678 our *ex vivo* porcine cornea model is a practical tool for rapidly and cost effectively screening
679 the efficacy of ocular drugs with good sensitivity and reliability. We contend that our *ex vivo*
680 model could be used to reduce and refine use of live animals in keratitis studies. The
681 observations from our *ex vivo* keratitis model could advance discovery of new ocular drugs,
682 facilitate their rapid translation to the market and serve as a guidance for clinical application in
683 the future.

684

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689

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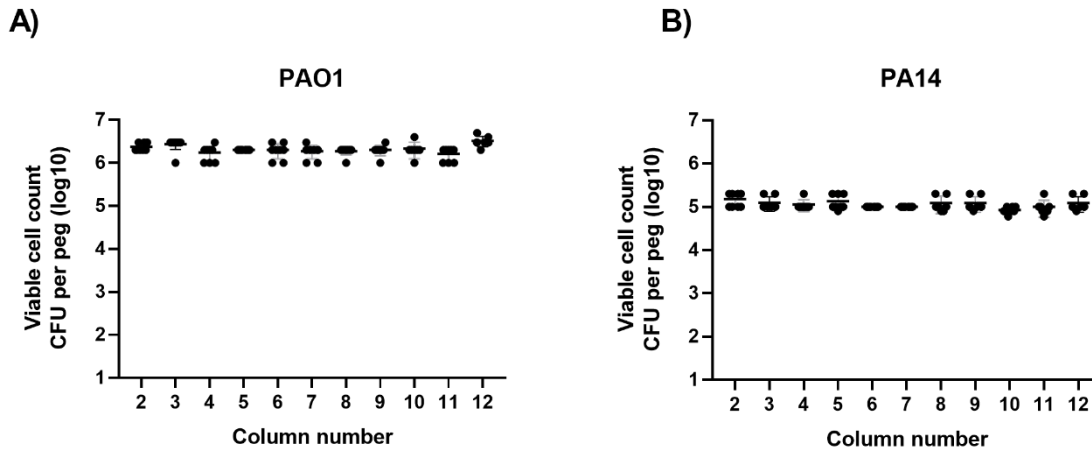
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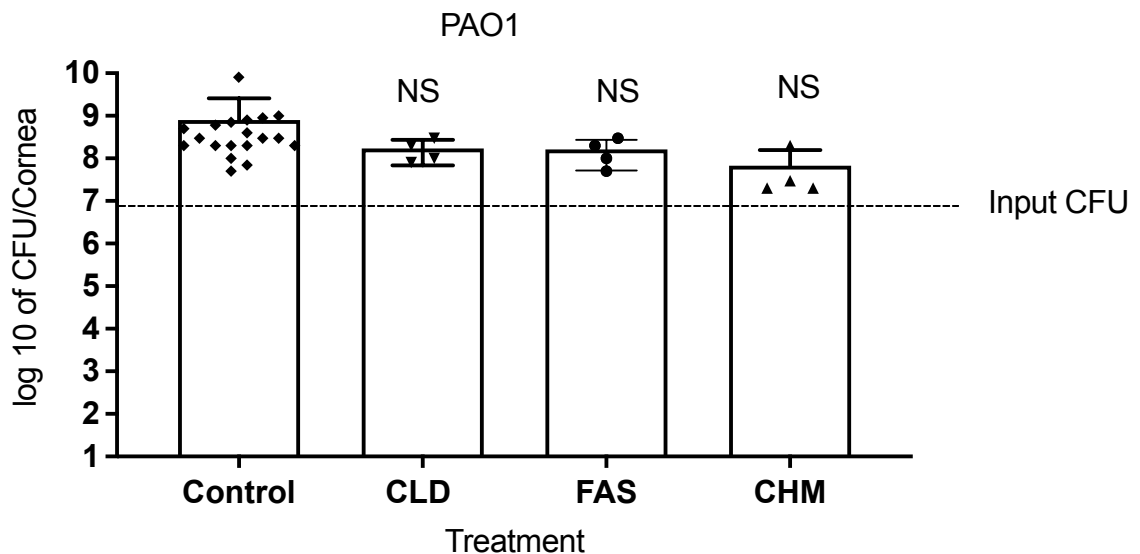
Supplementary materials

1120
1121 Figure 1. Equivalence assay results representing colony forming units of *P. aeruginosa* PAO1
1122 (A) and PA14 (C) retrieved from pegs across all columns in a 96-well plate.



1123
1124
1125 Figure 2. Colony forming units of *P. aeruginosa* in the *ex vivo* porcine corneas infected for 6
1126 hours with (A) PAO1 or (B) PA14. Control corneas were immersed in PBS while other corneas
1127 were treated with 1025µg/mL of antibiotic dissolved in PBS. Following antibiotics were
1128 applied on infected corneas: clindamycin (CLD) (n = 4), fusidic acid (FAS) (n = 4) and
1129 chloramphenicol (CHM) (n = 4). Error bars are means ± SD. Unpaired *t*-tests were performed
1130 for the pairwise statistical analysis of treated against untreated colony forming units for each
1131 strain; significant difference (*p* value < 0.05) is denoted with *.

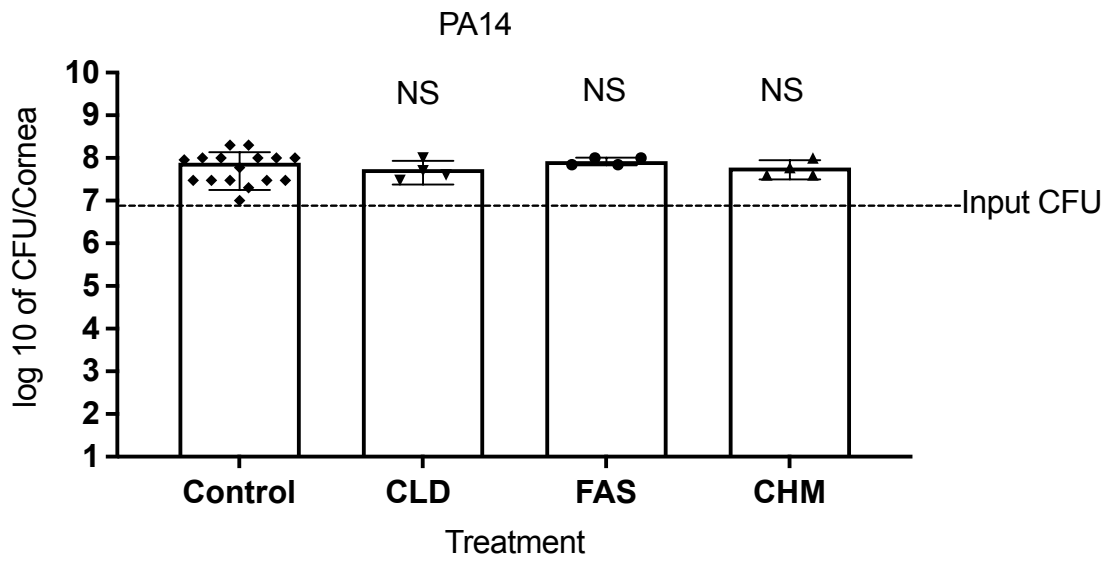
1132 A
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B



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1139

1140 Table 2. Data summary of average colony forming units of *P. aeruginosa* in the ex vivo porcine
 1141 corneas infected for 6 hours with PAO1 or PA14 and treated with MIC concentrations of
 1142 gentamicin, ciprofloxacin and meropenem.

1143

1144
 1145

PAO1

| Treatment | Concentration $\mu\text{g mL}^{-1}$ | Average | N | SD | % Reduction |
|---------------|--|---------|---|--------|-------------|
| PBS | 0 | 3.E+09 | 4 | 8.E+08 | 1146 |
| Gentamicin | 4 | 2.E+09 | 4 | 8.E+08 | 1148 |
| Ciprofloxacin | 0.25 | 2.E+09 | 4 | 1.E+09 | 26.00549 |
| Meropenem | 1 | 1.E+09 | 4 | 9.E+08 | 43.62550 |
| | | | | | 53.65151 |

PA14

| Treatment | Concentration $\mu\text{g mL}^{-1}$ | Average | N | SD |
|---------------|--|---------|---|--------|
| PBS | 0 | 4.E+07 | 4 | 1.E+07 |
| Gentamicin | 4 | 2.E+08 | 4 | 2.E+08 |
| Ciprofloxacin | 0.5 | 3.E+07 | 4 | 2.E+07 |
| Meropenem | 0.25 | 5.E+07 | 4 | 5.E+07 |

1152

1153 Table 3. Data summary of average colony forming units of *P.aeruginosa* in the ex vivo porcine
 1154 corneas infected for 6 hours with PAO1 or PA14 and treated with gentamicin, ciprofloxacin,
 1155 meropenem, fusidic acid, clindamycin and chloramphenicol.

1156

PAO1

| Treatment | Concentration mg/mL | Average CFU | SD | N | % Reduction | LOG Reduction |
|-----------------|------------------------|----------------|--------|----|-------------|---------------|
| PBS | 0 | 8.E+08 | 2.E+09 | 19 | | |
| Gentamicin | 1.025 | 7.E+07 | 1.E+08 | 12 | 91.260 | 1 log |
| Ciprofloxacin | 1.025 | 1.E+05 | 1.E+05 | 12 | 99.985 | 5 log |
| Meropenem | 1.025 | 5.E+06 | 4.E+06 | 12 | 99.340 | 2 log |
| Fusidic acid | 1.025 | 2.E+08 | 8.E+07 | 4 | 76.452 | <1 log |
| Clindamycin | 1.025 | 2.E+08 | 9.E+07 | 4 | 78.411 | <1 log |
| Chloramphenicol | 1.025 | 8.E+07 | 1.E+08 | 4 | 89.972 | 1 log |

1157

PA14

| Treatment | Concentration mg/mL | Average CFU | SD | N | % Reduction | LOG Reduction |
|-----------------|------------------------|----------------|--------|----|-------------|---------------|
| PBS | 0 | 1.E+08 | 6.E+07 | 16 | | |
| Gentamicin | 1.025 | 2.E+07 | 2.E+07 | 12 | 79.266 | < 1 log |
| Ciprofloxacin | 1.025 | 5.E+04 | 2.E+04 | 12 | 99.955 | 4 log |
| Meropenem | 1.025 | 9.E+05 | 1.E+06 | 12 | 99.125 | 2 log |
| Fusidic acid | 1.025 | 9.E+07 | 2.E+07 | 4 | 13.586 | <1 log |
| Clindamycin | 1.025 | 5.E+07 | 3.E+07 | 4 | 45.897 | <1 log |
| Chloramphenicol | 1.025 | 7.E+07 | 4.E+07 | 4 | 33.724 | <1 log |

1158

1159 Table 4. Data summary of opacity scoring for ex vivo porcine corneas. The table shows a
 1160 percent of corneas graded 0, 1 and 2, depending on degree of opacity. Images showed ninety

1161 corneas infected with either cytotoxic strain PA14, or invasive strain PAO1 of *P. aeruginosa*
 1162 and with or without a treatment with antibiotics.

| Infecting strain of <i>P. aeruginosa</i> | Treatment | Average % of corneas graded | | |
|--|-----------|-----------------------------|----|-----|
| | | 0 | 1 | 2 |
| uninfected | PBS | 70 | 30 | 0 |
| PA14 | PBS | 11 | 54 | 34 |
| | CHM | 45 | 55 | 0 |
| | CLD | 80 | 20 | 0 |
| | FAS | 16 | 56 | 28 |
| | GEN* | 10 | 55 | 35 |
| | MER* | 70 | 25 | 5 |
| | CIP* | 40 | 40 | 20 |
| | GEN | 53 | 40 | 7 |
| | MER | 63 | 33 | 3 |
| | CIP | 80 | 20 | 0 |
| PAO1 | PBS | 0 | 3 | 97 |
| | CHM | 50 | 50 | 0 |
| | CLD | 25 | 45 | 30 |
| | FAS | 0 | 0 | 100 |
| | GEN* | 0 | 13 | 87 |
| | MER* | 30 | 45 | 25 |
| | CIP* | 15 | 45 | 40 |
| | GEN | 29 | 63 | 9 |
| | MER | 38 | 38 | 25 |
| | CIP | 65 | 35 | 0 |

1163 • - corneas treated with MIC concentration of an antibiotic