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

AUTHORS:

K. Okurowska[1], E. Karunakaran*[1,2]

AFFILIATIONS:

1 Sheffield Collaboratorium for Antimicrobial Resistance and Biofilms (SCARAB), University of Sheffield, Mappin Street, Sheffield, United Kingdom.
2 Department of Chemical and Biological Engineering, University of Sheffield.

*Corresponding author: Esther Karunakaran (e.karunakaran@sheffield.ac.uk)

ABSTRACT

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

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

INTRODUCTION

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

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

Currently, treatments aim to achieve minimum inhibitory concentration (MIC) of the drug at the site of infection (Gokhale, 2008). If MIC value indicates that a bacterium is susceptible to 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. at 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 $3x10^5$ 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 µg/µL 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 OD_{600nm} was measured using
164 spectrophotometer Jenway (VWR, UK). The inoculum was pipetted in a 96-well plate with a
165 final concentration $8x10^6$ 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 37^0C 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 µl
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 µl 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 µl of the recovery medium with the
179 bacteria was diluted in series up to 10^4 in 180 µl 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 1µg μL^{-1} to 512 µg μL^{-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 µg 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^{-}
209 ^{1} 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 $8x10^{6}$ 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 µg mL^{-}**
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 µg 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.

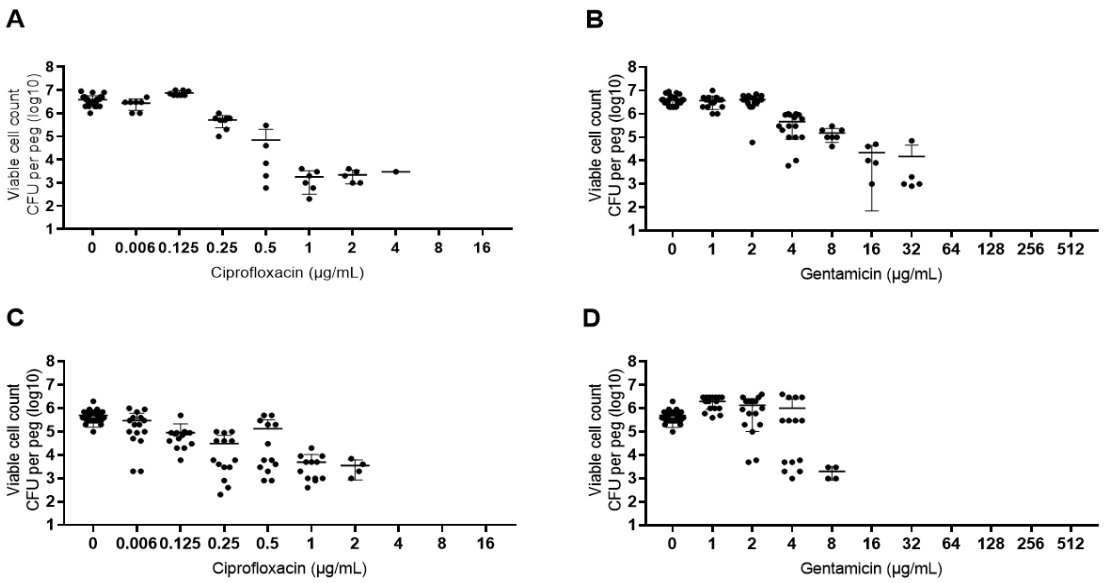


Fig.1. MBEC assay results representing colony forming units of *P. aeruginosa* PAO1 (A, B) and PA14 (C,D) treated with ciprofloxacin and gentamicin.

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

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

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

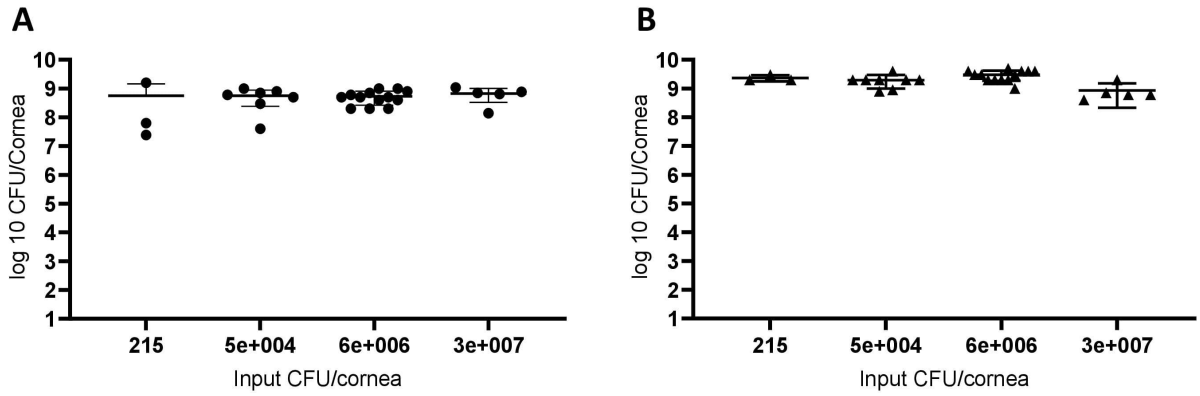


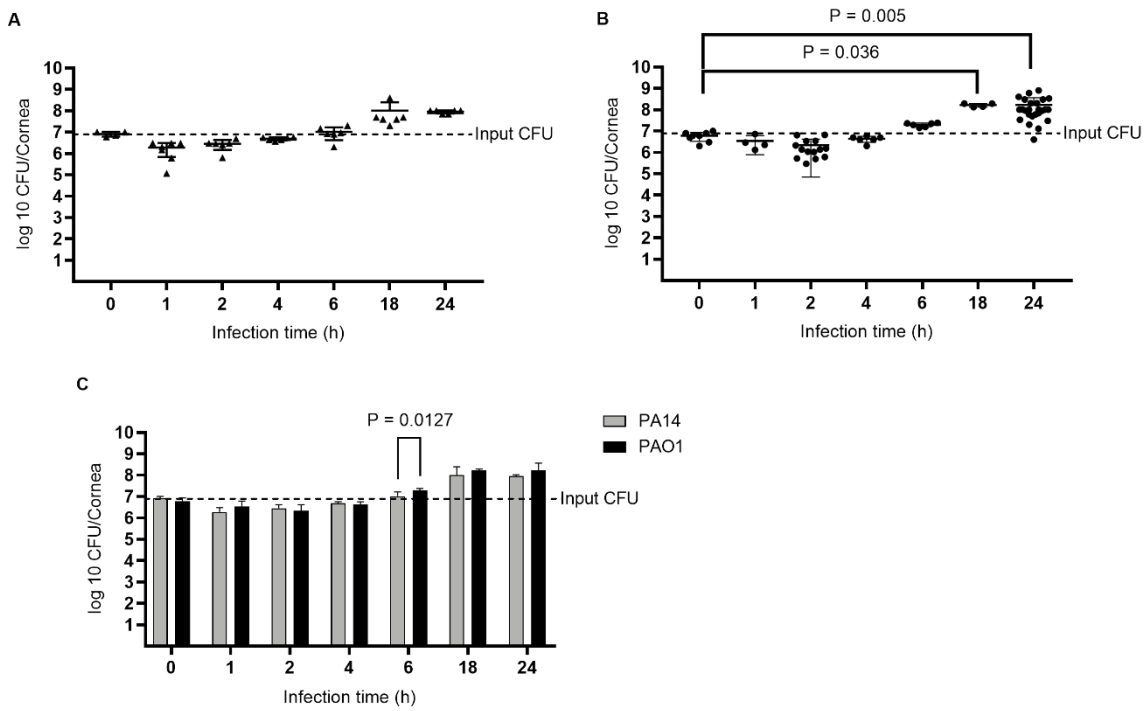
Fig 2. Number of viable *P. aeruginosa* PA14 retrieved from porcine cornea after infection with 215, $5x10^4$, $6x10^6$ and $3x10^7$ CFU per cornea. Corneas were infected for 24 hours (A) and 48 hours (B). Each dot represents results from one cornea. Error bars represent standard deviation. Statistical significance was calculated with Kruskall-Wallis test followed by Dunn's multiple comparisons test *p-value <0.05. Raw data is available in Supplementary Materials, Data Availability Section.

Effect of incubation time on progress of infection

To investigate the progress of infection over time, porcine corneas were infected with *P. aeruginosa* PA14 and *P. aeruginosa* PAO1 and a viable count was carried out on bacteria retrieved from the infected cornea after 1, 2, 4, 6, 18 and 24 hours post infection (hpi) (Fig. 3). With *P. aeruginosa* PA14, an average of 1.9 x 10^6 CFU per cornea were retrieved after 1 hpi (n =7), 2.9 x 10^6 CFU per cornea were retrieved after 2 hpi (n = 6), and 4.9 x 10^6 CFU per cornea were retrieved after 4 hpi (n = 6) (Fig. 3A). At all these time points, the number of CFU retrieved per cornea were lower than the inoculum size (7.7 x 10^6 CFU per cornea) reflecting the impact of post-incubation rinsing steps included in the protocol during which the bacterial population not securely adhered to the corneal tissue are removed. After 6 hpi, the number of bacteria retrieved from the infected cornea were approximately equal to the inoculum size despite rinsing (n = 6). Incubation beyond 6 hpi reproducibly resulted in a clear increase of CFU retrieved per cornea despite rinsing, resulting in 1.0 x 10^8 CFU per cornea at 18 hpi (n = 6) and 9.0 x 10^7 at 24 hpi (n = 6) (Fig. 3A). Difference in CFU values for PA14 retrieved at 1 hpi and 2 hpi in comparison to 18 hpi and 24 hpi was significant ($p<0.05$).

A similar trend was seen in the progress of infection in the *ex vivo* porcine cornea infected with *P. aeruginosa* PAO1 strain (Fig. 3B). An average of 3.4 x10^6 CFU per cornea were retrieved after 1 hpi (n = 4), 2.2 x 10^6 CFU per cornea were retrieved after 2 hpi (n = 14) and 4.1 x 10^6 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 x 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 x 10^7
317 CFU per cornea at 6 hpi (n = 6), 1.6 x 10^8 CFU per cornea at 18 hpi (n = 4) and 1.7 x 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.

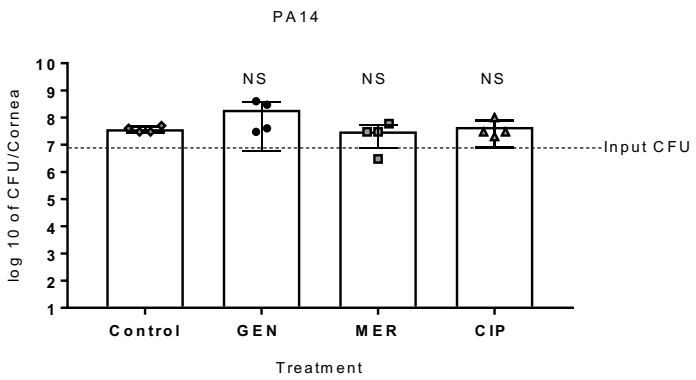


328
329 Fig. 3. Number of viable CFU of *P. aeruginosa* on porcine corneas infected for 1, 2, 4, 6, 18
330 and 24 hours with *P. aeruginosa* PA14 (A) and *P. aeruginosa* PA01 (B). Data from both strains
331 are compared on one graph (C). Inoculum CFU are shown both as CFU at t=0 infection time
332 (x-axis) 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 Kruskall-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.

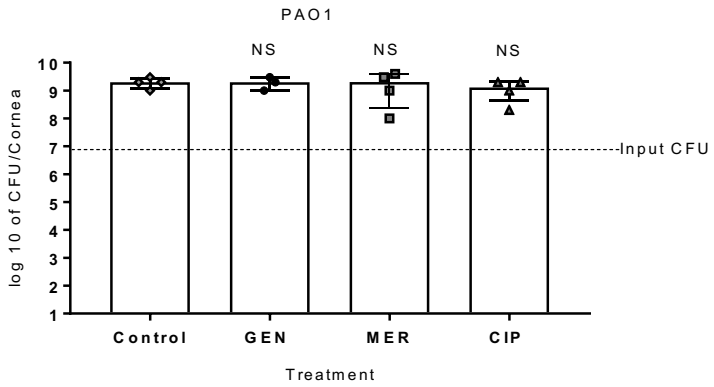
337
338 **Testing antibiotics on the *ex vivo* porcine keratitis model**

339 Next, the effect of MIC concentrations on infected tissue was investigated. *Ex vivo* porcine
340 corneas were infected on average with 1 x 10^7 CFU *P. aeruginosa* PA14 and 9 x 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.
360
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 μg 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 μg 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 $7x10^7$ CFU (1-
377 log reduction) of strain PAO1 and $2x10^7$ 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 ($5x10^6$ CFU per cornea for strain PAO1 and $9x10^5$ CFU per cornea for strain PA14)
380 despite MBEC assay suggesting that biofilms of both strains are tolerant to >512 μg mL^{-1} of
381 this antibiotic. Treatment with ciprofloxacin resulted in an average of $1x10^5$ CFU (5-log
382 reduction) for corneas infected with strain PAO1 and $5x10^4$ 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**

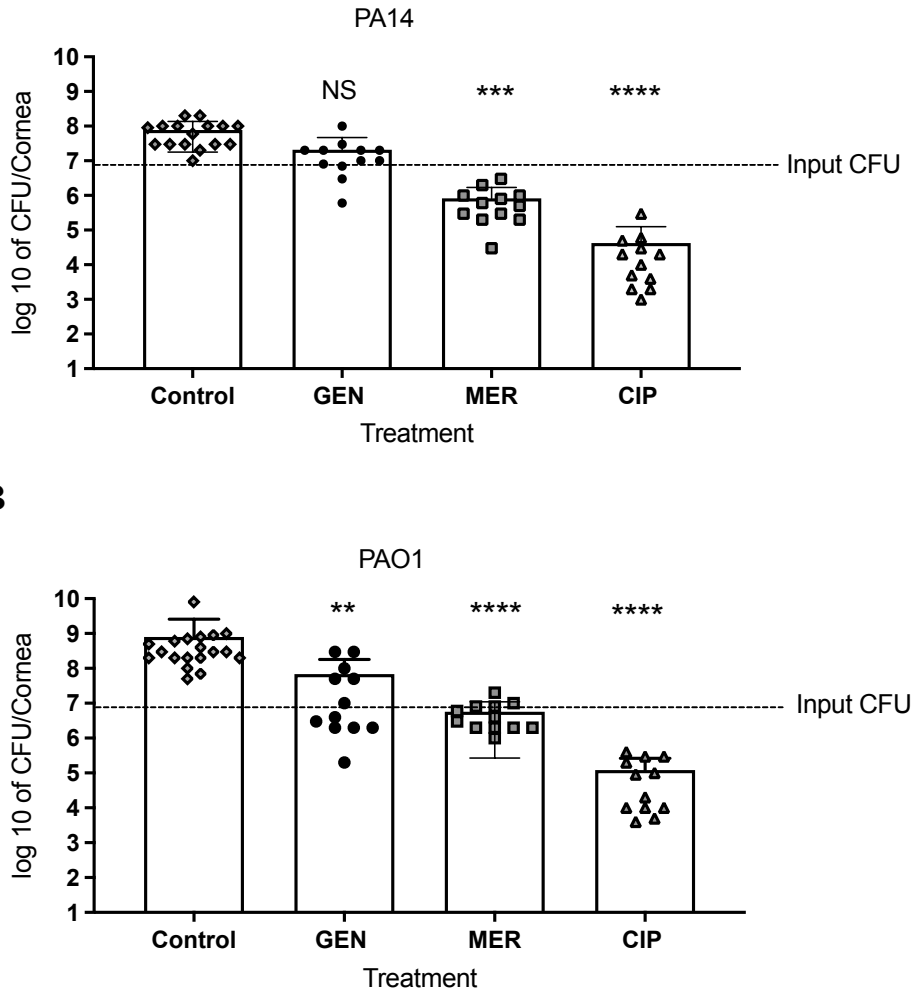
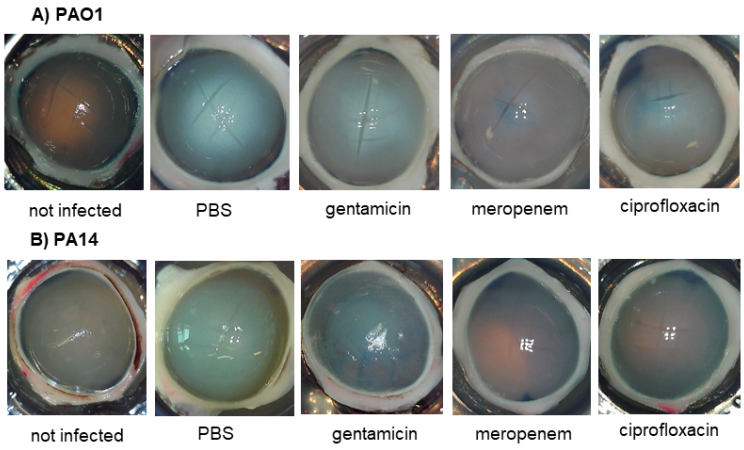


Fig. 5. Colony forming units of *P. aeruginosa* in the *ex vivo* porcine corneas infected for 6 hours with (A) PA14 or (B) PAO1. Control corneas (n = 19 & 16) were immersed in PBS while other corneas were treated with 1025 µg $mL^{-1}$of antibiotic dissolved in PBS. Following antibiotics were applied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

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

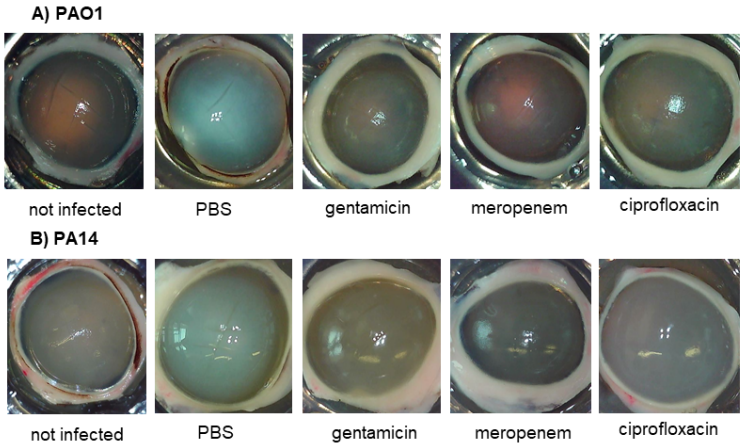
409 with PA14 (Fig. 6B). Corneas infected with both *P. aeruginosa* strains and treated with MIC
410 concentration of gentamicin looked slightly hazier in comparison to meropenem and
411 ciprofloxacin in spite of no reduction in viable colony count across all antibiotics (Fig. 4).



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 $6x10^6$ 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

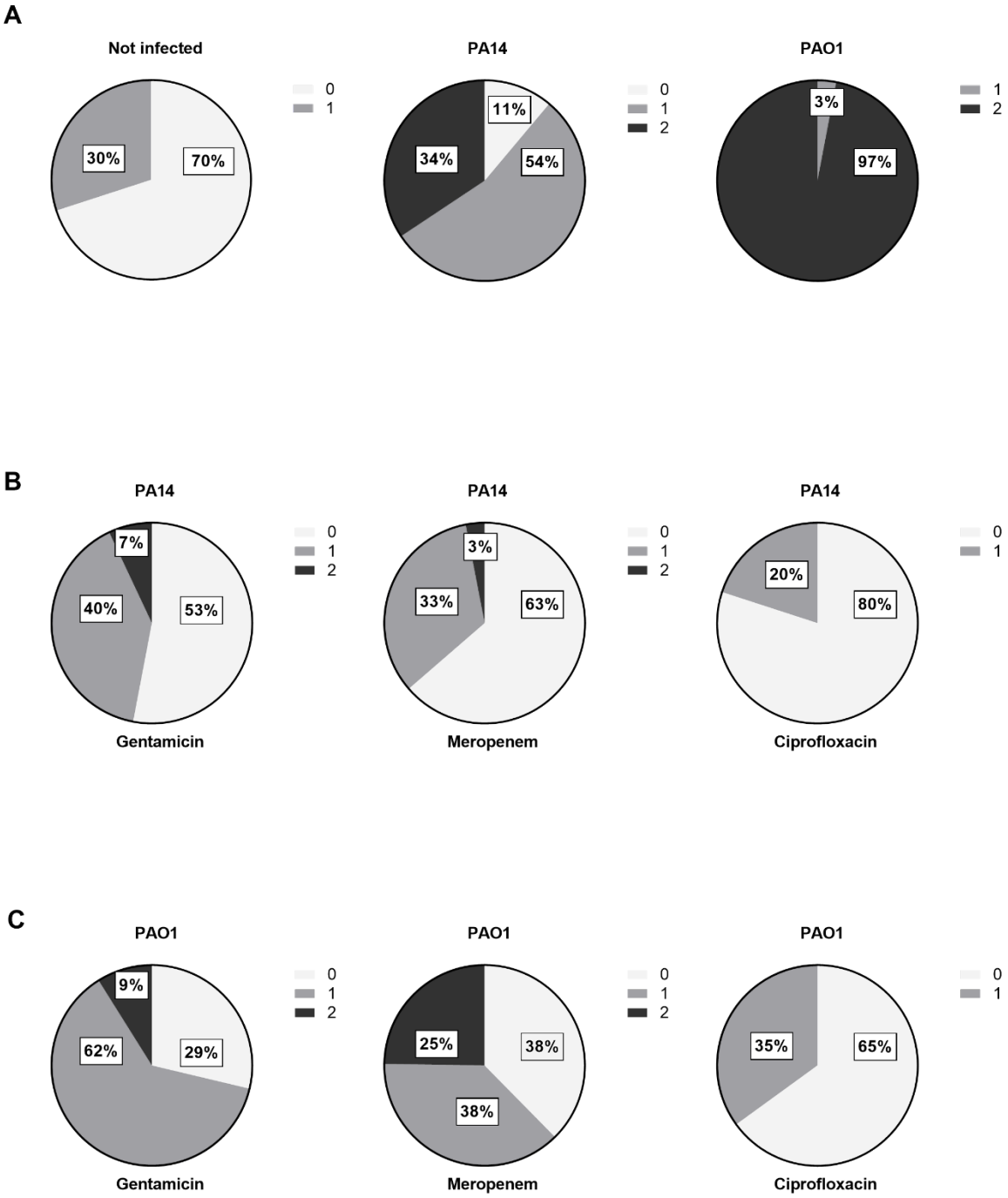
419 Treatment with higher antibiotic concentration (1025 µg mL^{-1}) decreased corneal opacity of
420 infected cornea by preventing development of milky colour (Fig. 7). This effect was especially
421 evident in cornea infected with PA14. Corneas treated with gentamicin, meropenem and
422 ciprofloxacin 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 µg mL^{-1} of gentamicin, ciprofloxacin, fusidic acid,

428 clindamycin, and chloramphenicol. Corneas shown here were infected with $6x10^6$ 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 PAO1results 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 $\mu g\ mL^{-1}$ 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 µg 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 $\mu g\ mL^{-1}$ and 2 $\mu g\ mL^{-1}$)(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

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

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

Finally, we investigated ciprofloxacin in our studies because it is considered as one of the most effective antibiotics against *P. aeruginosa* and therefore used as a first-line treatment in the UK (Hilliam et al., 2020). Our study demonstrated that ciprofloxacin was certainly the most potent antibiotic against planktonic and biofilm of *P. aeruginosa* not only *in vitro* but also on *ex vivo* cornea model. Our MIC values for ciprofloxacin indicate that both strains were susceptible according to EUCAST, 2022 with PA14 marginally more resistant than PAO1. Overall, the results for MIC were similar to those in literature. Research groups reported MIC values in range from 0.125 μg mL^{-1} (Riera et al., 2010) to 0.25 μg mL^{-1} (Shafiei et al., 2014, Bowler et al., 2012) and 1 μg mL^{-1} (Fernandez-Olmos et al., 2012) for PAO1. Some studies reported MIC 0.125 μg mL^{-1} for PA14 (Soares et al., 2019, Bruchmann et al., 2013) while ours was marginally higher (0.25-0.5 μg mL^{-1}). Our MBEC results also match trends in other studies *in vitro* which demonstrated that there was small but not significant difference in response to ciprofloxacin between biofilm formed by PA14 and PAO1 (Billings et al., 2013, Benthall et 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 form 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 x 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 x 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. aeruginos*a keratitis
579 with mixed therapeutic outcome (Punitan et al., 2019, Rootman and Krajden, 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 Krajden, 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 ofloxacins (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

685 **ACKNOWLEDGMENTS**

686 We would like to thank R. B. Elliot and Son abattoir for providing porcine corneas for research.
687 The authors would like to also thank Mr. Jonathan Emery, Dr. Mahendra Raut, Prof. Annette
688 Taylor and Ms. Hannah Regan for scoring cornea opacity.
689

690 **REFERENCES**
691

692 AHMAD, A., SMOLIN, G., OKUMOTO, M. & OHNO, S. 1977. Ticarcillin in the treatment
693 of experimental *Pseudomonas* keratitis. *British Journal of Opthalmology,* 61.
694 AKKAN, A. G., MUTLU, I., OZYAZGAN, S., GOK, A., YIGIT, U., OZUNER, Z.,
695 SENSES, V. & PEKEL, H. 1997. Penetration of topically applied ciprofloxacin,
696 norfloxacin and ofloxacin into the aqueous humor of the uninflamed human eye.
697 *Journal of Chemotherapy,* 9**,** 257-262.
698 ALIPRANDIS, E., CIRALSKY, J., LAI, H., HERLING, I. & KATZ, H. R. 2005.
699 Comparative efficacy of topical moxifloxacin versus ciprofloxacin and vancomycin in
700 the treatment of P-aeruginoso and ciprofloxacin-resistant MRSA keratitis in rabbits.
701 *Cornea,* 24**,** 201-205.
702 ANDREWS, J. M. 2001. Determination of minimum inhibitory concentrations. *Journal of*
703 *Antimicrobial Chemotherapy,* 48**,** 5-16.
704 AUGUSTIN, D. K., HEIMER, S. R., TAM, C., LI, W. Y., LE DUE, J. M., EVANS, D. J. &
705 FLEISZIG, S. M. J. 2011. Role of Defensins in Corneal Epithelial Barrier Function
706 against Pseudomonas aeruginosa Traversal. *Infection and Immunity,* 79**,** 595-605.
707 BAGGE, N., SCHUSTER, M., HENTZER, M., CIOFU, O., GIVSKOV, M., GREENBERG,
708 E. P. & HOIBY, N. 2004. Pseudomonas aeruginosa biofilms exposed to imipenem
709 exhibit changes in global gene expression and beta-lactamase and alginate production.
710 *Antimicrobial Agents and Chemotherapy,* 48**,** 1175-1187.
711 BAHARI, S., ZEIGHAMI, H., MIRSHAHABI, H., ROUDASHTI, S. & HAGHI, F. 2017.
712 Inhibition of Pseudomonas aeruginosa quorum sensing by subinhibitory
713 concentrations of curcumin with gentamicin and azithromycin. *Journal of Global*
714 *Antimicrobial Resistance,* 10**,** 21-28.
715 BENTHALL, G., TOUZEL, R. E., HIND, C. K., TITBALL, R. W., SUTTON, J. M.,
716 THOMAS, R. J. & WAND, M. E. 2015. Evaluation of antibiotic efficacy against
717 infections caused by planktonic or biofilm cultures of Pseudomonas aeruginosa and
718 Klebsiella pneumoniae in Galleria mellonella. *International Journal of Antimicrobial*
719 *Agents,* 46**,** 538-545.

720 BILLINGS, N., MILLAN, M. R., CALDARA, M., RUSCONI, R., TARASOVA, Y.,
721 STOCKER, R. & RIBBECK, K. 2013. The Extracellular Matrix Component Psl
722 Provides Fast-Acting Antibiotic Defense in Pseudomonas aeruginosa Biofilms. *Plos*
723 *Pathogens,* 9.
724 BORKAR, D. S., FLEISZIG, S. M. J., LEONG, C., LALITHA, P., SRINIVASAN, M.,
725 GHANEKAR, A. A., TAM, C., LI, W. Y., ZEGANS, M. E., MCLEOD, S. D.,
726 LIETMAN, T. M. & ACHARYA, N. R. 2013. Association Between Cytotoxic and
727 Invasive Pseudomonas aeruginosa and Clinical Outcomes in Bacterial Keratitis. *Jama*
728 *Ophthalmology,* 131**,** 147-153.
729 BOWLER, L. L., ZHANEL, G. G., BALL, T. B. & SAWARD, L. L. 2012. Mature
730 Pseudomonas aeruginosa Biofilms Prevail Compared to Young Biofilms in the
731 Presence of Ceftazidime. *Antimicrobial Agents and Chemotherapy,* 56**,** 4976-4979.
732 BOZKURT, E., MUHAFIZ, E., KEPENEK, H. S., BOZLAK, C. E. B., SALTAN, S. K. &
733 BINGOL, S. A. 2021. A New Treatment Experience in Pseudomonas Keratitis:
734 Topical Meropenem and Cefepime. *Eye & Contact Lens-Science and Clinical*
735 *Practice,* 47**,** 174-179.
736 BRADY, A. J., LAVERTY, G., GILPIN, D. F., KEARNEY, P. & TUNNEY, M. 2017.
737 Antibiotic susceptibility of planktonic-and biofilm-grown staphylococci isolated from
738 implant-associated infections: should MBEC and nature of biofilm formation replace
739 MIC? *Journal of Medical Microbiology,* 66**,** 461-469.
740 BRUCHMANN, S., DOTSCH, A., NOURI, B., CHABERNY, I. F. & HAUSSLER, S. 2013.
741 Quantitative Contributions of Target Alteration and Decreased Drug Accumulation to
742 Pseudomonas aeruginosa Fluoroquinolone Resistance. *Antimicrobial Agents and*
743 *Chemotherapy,* 57**,** 1361-1368.
744 BU, P., RISKE, P. S., ZAYA, N. E., CAREY, R. & BOUCHARD, C. S. 2007. A comparison
745 of topical chlorhexidine, ciprofloxacin, and fortified Tobramycin/Cefazolin in rabbit
746 models of Staphylococcus and Pseudomonas keratitis. *Journal of Ocular*
747 *Pharmacology and Therapeutics,* 23**,** 213-220.
748 CENDRA, M. D., CHRISTODOULIDES, M. & HOSSAIN, P. 2017. Effect of Different
749 Antibiotic Chemotherapies on Pseudomonas aeruginosa Infection In Vitro of Primary
750 Human Corneal Fibroblast Cells. *Frontiers in Microbiology,* 8.
751 CERI, H., OLSON, M. E., STREMICK, C., READ, R. R., MORCK, D. & BURET, A. 1999.
752 The Calgary Biofilm Device: New technology for rapid determination of antibiotic
753 susceptibilities of bacterial biofilms. *Journal of Clinical Microbiology,* 37**,** 1771-
754 1776.
755 CHAN, A. O. H. H., STANLEY, P. & CHANG, B. 2021. Recalcitrant *Pseudomonas*
756 *aeruginosa* keratitis with hyphaema. *Case Rep Ophthalmol,* 12**,** 214-218.
757 CIOFU, O. & TOLKER-NIELSEN, T. 2019. Tolerance and Resistance of Pseudomonas
758 aeruginosa Biofilms to Antimicrobial Agents-How P. aeruginosa Can Escape
759 Antibiotics. *Frontiers in Microbiology,* 10.
760 CLSI. 2020. Performance standards for antimicrobial susceptibility testing.
761 COLE, N., WILLCOX, M. D. P., FLEISZIG, S. M. J., STAPLETON, F., BAO, B., TOUT, S.
762 & HUSBAND, A. 1998. Different strains of Pseudomonas aeruginosa isolated from
763 ocular infections or inflammation display distinct corneal pathologies in an animal
764 model. *Current Eye Research,* 17**,** 730-735.
765 COLVIN, K. M., GORDON, V. D., MURAKAMI, K., BORLEE, B. R., WOZNIAK, D. J.,
766 WONG, G. C. L. & PARSEK, M. R. 2011. The Pel Polysaccharide Can Serve a
767 Structural and Protective Role in the Biofilm Matrix of Pseudomonas aeruginosa. *Plos*
768 *Pathogens,* 7.

769 COSTERTON, J. W., CHENG, K. J., GEESEY, G. G., LADD, T. I., NICKEL, J. C.,
770 DASGUPTA, M. & MARRIE, T. J. 1987. BACTERIAL BIOFILMS IN NATURE
771 AND DISEASE. *Annual Review of Microbiology,* 41**,** 435-464.
772 DALMON, C., PORCO, T. C., LIETMAN, T. M., PRAJNA, N. V., PRAJNA, L., DAS, M.
773 R., KUMAR, J. A., MASCARENHAS, J., MARGOLIS, T. P., WHITCHER, J. P.,
774 JENG, B. H., KEENAN, J. D., CHAN, M. F., MCLEOD, S. D. & ACHARYA, N. R.
775 2012. The Clinical Differentiation of Bacterial and Fungal Keratitis: A Photographic
776 Survey. *Investigative Ophthalmology & Visual Science,* 53**,** 1787-1791.
777 DART, J. K. G. & SEAL, D. V. 1988. PATHOGENESIS AND THERAPY OF
778 PSEUDOMONAS-AERUGINOSA KERATITIS. *Eye,* 2**,** S46-S55.
779 DAVIES, D. 2003. Understanding biofilm resistance to antibacterial agents. *Nature Reviews*
780 *Drug Discovery,* 2**,** 114-122.
781 DE KIEVIT, T. R. 2009. Quorum sensing in Pseudomonas aeruginosa biofilms.
782 *Environmental Microbiology,* 11**,** 279-288.
783 DIMASI, J. A., FLOREZ, M. I., STERGIOPOULOS, S., PENA, Y., SMITH, Z.,
784 WILKINSON, M. & GETZ, K. A. 2020. Development Times and Approval Success
785 Rates for Drugs to Treat Infectious Diseases. *Clinical Pharmacology & Therapeutics,*
786 107**,** 324-332.
787 ERDEM, I., KAYNAR-TASCIOGLU, J., KAYA, B. & GOKTAS, P. 2002. The comparison
788 of in the vitro effect of imipenem or meropenem combined with ciprofloxacin or
789 levofloxacin against multidrug-resistant Pseudomonas aeruginosa strains.
790 *International Journal of Antimicrobial Agents,* 20**,** 384-386.
791 ERDEM, I., KUCUKERCAN, M. & CERAN, N. 2003. In vitro activity of combination
792 therapy with cefepime, piperacillin-tazobactam, or meropenem with ciprofloxacin
793 against multidrug-resistant Pseudomonas aeruginosa strains. *Chemotherapy,* 49**,** 294-
794 297.
795 EUCAST 2022. Breakpoint tables for interpretation of MICs and zone diameters. 12.0 ed.
796 http://www.eucast.org.
797 EUN, H. C., CHUNG, J. H., JUNG, S. Y., CHO, K. H. & KIM, K. H. 1994. A
798 COMPARATIVE-STUDY OF THE CYTOTOXICITY OF SKIN IRRITANTS ON
799 CULTURED HUMAN ORAL AND SKIN KERATINOCYTES. *British Journal of*
800 *Dermatology,* 130**,** 24-28.
801 FERNANDEZ-OLMOS, A., GARCIA-CASTILLO, M., MAIZ, L., LAMAS, A.,
802 BAQUERO, F. & CANTON, R. 2012. In vitro prevention of Pseudomonas
803 aeruginosa early biofilm formation with antibiotics used in cystic fibrosis patients.
804 *International Journal of Antimicrobial Agents,* 40**,** 173-176.
805 FLEISZIG, S. M. J., WIENERKRONISH, J. P., MIYAZAKI, H., VALLAS, V., MOSTOV,
806 K. E., KANADA, D., SAWA, T., YEN, T. S. B. & FRANK, D. W. 1997.
807 Pseudomonas aeruginosa-mediated cytotoxicity and invasion correlate with distinct
808 genotypes at the loci encoding exoenzyme S. *Infection and Immunity,* 65**,** 579-586.
809 FLEISZIG, S. M. J., ZAIDI, T. S., PRESTON, M. J., GROUT, M., EVANS, D. J. & PIER,
810 G. B. 1996. Relationship between cytotoxicity and corneal epithelial cell invasion by
811 clinical isolates of Pseudomonas aeruginosa. *Infection and Immunity,* 64**,** 2288-2294.
812 FRESCHI, L., VINCENT, A. T., JEUKENS, J., EMOND-RHEAULT, J. G., KUKAVICA-
813 IBRULJ, I., DUPONT, M. J., CHARETTE, S. J., BOYLE, B. & LEVESQUE, R. C.
814 2019. The Pseudomonas aeruginosa Pan-Genome Provides New Insights on Its
815 Population Structure, Horizontal Gene Transfer, and Pathogenicity. *Genome Biology*
816 *and Evolution,* 11**,** 109-120.
817 FRIEDMAN, L. & KOLTER, R. 2004. Genes involved in matrix formation in Pseudomonas
818 aeruginosa PA14 biofilms. *Molecular Microbiology,* 51**,** 675-690.

FRUCHTPERY, J., GOLAN, G., HEMO, I., ZAUBERMAN, H. & SHAPIRO, M. 1995. EFFICACY OF TOPICAL GENTAMICIN TREATMENT AFTER 193-NM PHOTOREFRACTIVE KERATECTOMY IN AN EXPERIMENTAL PSEUDOMONAS KERATITIS MODEL. *Graefes Archive for Clinical and Experimental Ophthalmology,* 233**,** 532-534.

GARCIARODRIGUEZ, J. A., BLAZQUEZ, A. M., FRESNADILLO, M. J., SANCHEZ, E. G., SANCHEZ, J. E. G. & MARTIN, I. T. 1996. In vitro activity of meropenem against ciprofloxacin-resistant enterobacteriaceae and Pseudomonas aeruginosa. *Journal of Chemotherapy,* 8**,** 358-364.

GARG, P., SHARMA, S. & RAO, G. N. 1999. Ciprofloxacin resistant Pseudomonas keratitis. *Ophthalmology,* 106**,** 1319-1323.

GEFEN, O., FRIDMAN, O., RONIN, I. & BALABAN, N. Q. 2014. Direct observation of single stationary-phase bacteria reveals a surprisingly long period of constant protein production activity. *Proceedings of the National Academy of Sciences of the United States of America,* 111**,** 556-561.

GIRARD, G. & BLOEMBERG, G. V. 2008. Central role of quorum sensing in regulating the production of pathogenicity factors in Pseudomonas aeruginosa. *Future Microbiology,* 3**,** 97-106.

GOKHALE, N. S. 2008. Medical management approach to infectious keratitis. *Indian Journal of Ophthalmology,* 56**,** 215-220.

GOLDSTEIN, M. H., SILVA, F. Q., BLENDER, N., TRAN, T. & VANTIPALLI, S. Ocular benzalkonium chloride exposure: problems and solutions. *Eye*.

GOLDSTEIN, M. H., SILVA, F. Q., BLENDER, N., TRAN, T. & VANTIPALLI, S. 2022. Ocular benzalkonium chloride exposure: problems and solutions. *Eye,* 36**,** 361-368.

GOODMAN, A. L., KULASEKARA, B., RIETSCH, A., BOYD, D., SMITH, R. S. & LORY, S. 2004. A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in Pseudomonas aeruginosa. *Developmental Cell,* 7**,** 745-754.

GOWER, N. J. D., BARRY, R. J., EDMUNDS, M. R., TITCOMB, L. C. & DENNISTON, A. K. 2016. Drug discovery in ophthalmology: past success, present challenges, and future opportunities. *Bmc Ophthalmology,* 16.

GUPTA, S. K., VAJPAYEE, R., VARMA, B., SHARMA, N., SATPATHY, G. & VAJPAYEE, M. 1995. A COMPARATIVE-EVALUATION OF TOPICAL PEFLOXACIN AND GENTAMICIN IN EXPERIMENTALLY-INDUCED PSEUDOMONAS CORNEAL ULCERS. *Annals of Ophthalmology-Glaucoma,* 27**,** 197-202.

GUZEK, J. P., CHACKO, D., KETTERING, J. D., WESSELS, I. F. & APRECIO, R. M. 1994. COMPARISON OF TOPICAL CIPROFLOXACIN TO CONVENTIONAL ANTIBIOTIC-THERAPY IN THE TREATMENT OF EXPERIMENTAL PSEUDOMONAS-AERUGINOSA KERATITIS. *Cornea,* 13**,** 500-504.

HAAGENSEN, J., VEROTTA, D., HUANG, L. S., ENGEL, J., SPORMANN, A. M. & YANG, K. 2017. Spatiotemporal pharmacodynamics of meropenem- and tobramycin-treated Pseudomonas aeruginosa biofilms. *Journal of Antimicrobial Chemotherapy,* 72**,** 3357-3365.

HARRISON, J. J., STREMICK, C. A., TURNER, R. J., ALLAN, N. D., OLSON, M. E. & CERI, H. 2010. Microtiter susceptibility testing of microbes growing on peg lids: a miniaturized biofilm model for high-throughput screening. *Nature Protocols,* 5**,** 1236-1254.

867 HASSAN, M. M., HARRINGTON, N. E., SWEENEY, E. & HARRISON, F. 2020.
868 Predicting Antibiotic-Associated Virulence ofPseudomonas aeruginosaUsing anex
869 vivoLung Biofilm Model. *Frontiers in Microbiology,* 11.
870 HASSELMANN, C. 2000. European Society of Clinical Microbiology and Infectious
871 Diseases (ESCMID) - European Committee for Antimicrobial Susceptibility Testing
872 (EUCAST) - Determination of minimum inhibitory concentration (MIC) by agar
873 dilution. *Clinical Microbiology and Infection,* 6**,** 1-7.
874 HAZLETT, L. D., JIANG, X. Y. & MCCLELLAN, S. A. 2014. IL-10 Function, Regulation,
875 and in Bacterial Keratitis. *Journal of Ocular Pharmacology and Therapeutics,* 30**,**
876 373-380.
877 HILLIAM, Y., KAYE, S. & WINSTANLEY, C. 2020. Pseudomonas aeruginosa and
878 microbial keratitis. *Journal of Medical Microbiology,* 69**,** 3-13.
879 HUE, B., DOAT, M., RENARD, G., BRANDELY, M. L. & CHAST, F. 2009. Severe
880 Keratitis Caused by Pseudomonas aeruginosa Successfully Treated with Ceftazidime
881 Associated with Acetazolamide. *Journal of Ophthalmology,* 2009.
882 HUMPHRIES, R. M., ABBOTT, A. N. & HINDLER, J. A. 2019. Understanding and
883 Addressing CLSI Breakpoint Revisions: a Primer for Clinical Laboratories. *Journal of*
884 *Clinical Microbiology,* 57.
885 IBRAHIM, Y. W., BOASE, D. L. & CREE, I. A. 2009. Epidemiological characteristics,
886 predisposing factors and microbiological profiles of infectious corneal ulcers: the
887 Portsmouth corneal ulcer study. *British Journal of Ophthalmology,* 93**,** 1319-1324.
888 JAISHANKAR, J. & SRIVASTAVA, P. 2017. Molecular Basis of Stationary Phase Survival
889 and Applications. *Frontiers in Microbiology,* 8.
890 KARLOWSKY, J. A., ZHANEL, G. G., DAVIDSON, R. J. & HOBAN, D. J. 1994.
891 POSTANTIBIOTIC EFFECT IN PSEUDOMONAS-AERUGINOSA FOLLOWING
892 SINGLE AND MULTIPLE AMINOGLYCOSIDE EXPOSURES IN-VITRO.
893 *Journal of Antimicrobial Chemotherapy,* 33**,** 937-947.
894 KASETTY, S., KATHARIOS-LANWERMEYER, S., O'TOOLE, G. A. & NADELL, C. D.
895 2021. Differential Surface Competition and Biofilm Invasion Strategies of
896 Pseudomonas aeruginosa PA14 and PAO1. *Journal of Bacteriology,* 203.
897 KOWALSKA-KROCHMAL, B. & DUDEK-WICHER, R. 2021. The Minimum Inhibitory
898 Concentration of Antibiotics: Methods, Interpretation, Clinical Relevance. *Pathogens,*
899 10.
900 KOWALSKI, R. P., KOWALSKI, T. A., SHANKS, R. M. Q., ROMANOWSKI, E. G.,
901 KARENCHAK, L. M. & MAH, F. S. 2013. In Vitro Comparison of Combination and
902 Monotherapy for the Empiric and Optimal Coverage of Bacterial Keratitis Based on
903 Incidence of Infection. *Cornea,* 32**,** 830-834.
904 KOWALSKI, R. P., ROMANOWSKI, E. G., YATES, K. A. & GORDON, Y. J. 2001.
905 Lomefloxacin is an effective treatment of experimental bacterial keratitis. *Cornea,* 20**,**
906 306-308.
907 LABORWIT, S. E., KATZ, H. R., HIRSCHBEIN, M. J., OSWALD, M. R., SNYDER, L. S.,
908 SCHWARTZ, K. S. & HERLING, I. E. 2001. Topical 0.3% ciprofloxacin vs topical
909 0.3% ofloxacin in early treatment of Pseudomonas aeruginosa keratitis in a rabbit
910 model. *Annals of Ophthalmology,* 33**,** 48-52.
911 LAUFFENBURGER, M. D. & COHEN, K. L. 1993. TOPICAL CIPRROFLOXACIN
912 VERSUS TOPICAL FORTIFIED ANTIBIOTICS IN RABBIT MODELS OF
913 STAPHYLOCOCCUS AND PSEUDOMONAS KERATITIS. *Cornea,* 12**,** 517-521.
914 LAWINBRUSSEL, C. A., REFOJO, M. F., LEONG, F. L., HANNINEN, L. & KENYON,
915 K. R. 1993. EFFECT OF PSEUDOMONAS-AERUGINOSA CONCENTRATION

916 IN EXPERIMENTAL CONTACT LENS-RELATED MICROBIAL KERATITIS.
917 *Cornea,* 12**,** 10-18.
918 LEBEAUX, D., GHIGO, J. M. & BELOIN, C. 2014. Biofilm-Related Infections: Bridging
919 the Gap between Clinical Management and Fundamental Aspects of Recalcitrance
920 toward Antibiotics. *Microbiology and Molecular Biology Reviews,* 78**,** 510-543.
921 LEE, D. G., URBACH, J. M., WU, G., LIBERATI, N. T., FEINBAUM, R. L., MIYATA, S.,
922 DIGGINS, L. T., HE, J. X., SAUCIER, M., DEZIEL, E., FRIEDMAN, L., LI, L.,
923 GRILLS, G., MONTGOMERY, K., KUCHERLAPATI, R., RAHME, L. G. &
924 AUSUBEL, F. M. 2006. Genomic analysis reveals that Pseudomonas aeruginosa
925 virulence is combinatorial. *Genome Biology,* 7.
926 LEE, E. J., COWELL, B. A., EVANS, D. H. & FLEISZIG, S. M. J. 2003a. Contribution of
927 ExsA-regulated factors to corneal infection by cytotoxic and invasive Pseudomonas
928 aeruginosa in a murine scarification model. *Investigative Ophthalmology & Visual*
929 *Science,* 44**,** 3892-3898.
930 LEE, E. J., TRUONG, T. N., MENDOZA, M. N. & FLEISZIG, S. M. J. 2003b. A
931 comparison of invasive and cytotoxic Pseudomonas aeruginosa strain-induced corneal
932 disease responses to therapeutics. *Current Eye Research,* 27**,** 289-299.
933 LEVEY, S. B., KATZ, H. R., LEVINE, E. S., ABRAMS, D. A. & MARSH, M. J. 1998.
934 Efficacy of topical ciprofloxacin 0.3% in the treatment of ulcerative keratitis in
935 humans. *Annals of Ophthalmology & Glaucoma,* 30**,** 301-304.
936 LLORENS, J. M. N., TORMO, A. & MARTINEZ-GARCIA, E. 2010. Stationary phase in
937 gram-negative bacteria. *Fems Microbiology Reviews,* 34**,** 476-495.
938 LOPEZ-DUPLA, M., MARTINEZ, J. A., VIDAL, F., ALMELA, M., SORIANO, A.,
939 MARCO, F., LOPEZ, J., OLONA, M. & MENSA, J. 2009. Previous ciprofloxacin
940 exposure is associated with resistance to beta-lactam antibiotics in subsequent
941 Pseudomonas aeruginosa bacteremic isolates. *American Journal of Infection Control,*
942 37**,** 753-758.
943 MAURICE, N. M., BEDI, B. & SADIKOT, R. T. 2018. Pseudomonas aeruginosa Biofilms:
944 Host Response and Clinical Implications in Lung Infections. *American Journal of*
945 *Respiratory Cell and Molecular Biology,* 58**,** 428-439.
946 MCDERMOTT, A. M. 2013. Antimicrobial compounds in tears. *Experimental Eye Research,*
947 117**,** 53-61.
948 MCDERMOTT, M. L., TRAN, T. D., COWDEN, J. W. & BUGGE, C. J. L. 1993.
949 CORNEAL STROMAL PENETRATION OF TOPICAL CIPROFLOXACIN IN
950 HUMANS. *Ophthalmology,* 100**,** 197-200.
951 MCDONALD, E. M., RAM, F. S. F., PATEL, D. V. & MCGHEE, C. N. J. 2014. Topical
952 antibiotics for the management of bacterial keratitis: an evidence-based review of
953 high quality randomised controlled trials. *British Journal of Ophthalmology,* 98**,**
954 1470-1477.
955 MIKKELSEN, H., MCMULLAN, R. & FILLOUX, A. 2011. The Pseudomonas aeruginosa
956 Reference Strain PA14 Displays Increased Virulence Due to a Mutation in ladS. *Plos*
957 *One,* 6.
958 MONAHAN, L. G., TURNBULL, L., OSVATH, S. R., BIRCH, D., CHARLES, I. G. &
959 WHITCHURCH, C. B. 2014. Rapid Conversion of Pseudomonas aeruginosa to a
960 Spherical Cell Morphotype Facilitates Tolerance to Carbapenems and Penicillins but
961 Increases Susceptibility to Antimicrobial Peptides. *Antimicrobial Agents and*
962 *Chemotherapy,* 58**,** 1956-1962.
963 NOECKER, R. 2001. Effects of common ophthalmic preservatives on ocular health.
964 *Advances in Therapy,* 18**,** 205-215.

965 NORINA, T. J., RAIHAN, S., BAKIAH, S., EZANEE, M., LIZA-SHARMINI, A. T. &
966 WAN, H. W. H. 2008. Microbial keratitis: aetiological diagnosis and clinical features
967 in patients admitted to Hospital Universiti Sains Malaysia. *Singapore Medical*
968 *Journal,* 49**,** 67-71.
969 O'BRIEN, T. P. 2003. Management of bacterial keratitis: beyond exorcism towards
970 consideration of organism and host factors. *Eye,* 17**,** 957-974.
971 OBRIEN, T. P., SAWUSCH, M. R., DICK, J. D. & GOTTSCH, J. D. 1988. TOPICAL
972 CIPROFLOXACIN TREATMENT OF PSEUDOMONAS KERATITIS IN
973 RABBITS. *Archives of Ophthalmology,* 106**,** 1444-1446.
974 OCAMPO-SOSA, A. A., CABOT, G., RODRIGUEZ, C., ROMAN, E., TUBAU, F.,
975 MACIA, M. D., MOYA, B., ZAMORANO, L., SUAREZ, C., PENA, C.,
976 DOMINGUEZ, M. A., MONCALIAN, G., OLIVER, A., MARTINEZ-MARTINEZ,
977 L. & SPANISH NETWORK RES INFECT DIS, R. E. I. 2012. Alterations of OprD in
978 Carbapenem-Intermediate and -Susceptible Strains of Pseudomonas aeruginosa
979 Isolated from Patients with Bacteremia in a Spanish Multicenter Study. *Antimicrobial*
980 *Agents and Chemotherapy,* 56**,** 1703-1713.
981 OGUZ, H., OZBILGE, H., OGUZ, E. & GURKAN, T. 2005. Effectiveness of topical
982 taurolidine versus ciprofloxacin, ofloxacin, and fortified cefazolin in a rabbit
983 Staphylococcus aureus keratitis model. *Current Eye Research,* 30**,** 155-161.
984 OKUROWSKA, K., ROY, S., THOKALA, P., PARTRIDGE, L., GARG, P., MACNEIL, S.,
985 MONK, P. N. & KARUNAKARAN, E. 2020. Establishing a Porcine Ex Vivo Cornea
986 Model for Studying Drug Treatments against Bacterial Keratitis. *Jove-Journal of*
987 *Visualized Experiments*.
988 OZTURK, F., KORTUNAY, S., KURT, E., ILKER, S. S., BASCI, N. E. & BOZKURT, A.
989 1999. Penetration of topical and oral ciprofloxacin into the aqueous and vitreous
990 humor in inflamed eyes. *Retina-the Journal of Retinal and Vitreous Diseases,* 19**,**
991 218-222.
992 PANDE, R. A. & BHAILUME, P. V. 2014. Use of topical meropenem in management
993 of hospital acquired Pseudomonas ocular infections. *Journal of Clinical Opthalmology,* 1**,** 23-
994 25.
995 PANKUCH, G. A., LIN, G. R., SEIFERT, H. & APPELBAUM, P. C. 2008. Activity of
996 meropenem with and without ciprofloxacin and colistin against Pseudomonas
997 aeruginosa and Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy,*
998 52**,** 333-336.
999 PRESTON, M. J., FLEISZIG, S. M. J., ZAIDI, T. S., GOLDBERG, J. B., SHORTRIDGE, V.
1000 D., VASIL, M. L. & PIER, G. B. 1995. RAPID AND SENSITIVE METHOD FOR
1001 EVALUATING PSEUDOMONAS-AERUGINOSA VIRULENCE FACTORS
1002 DURING CORNEAL INFECTIONS IN MICE. *Infection and Immunity,* 63**,** 3497-
1003 3501.
1004 PUNITAN, R., SULAIMAN, S. A., HASAN, H. B. & SHATRIAH, I. 2019. Clinical and
1005 Antibacterial Effects of Tualang Honey on Pseudomonas-induced Keratitis in Rabbit
1006 Eyes. *Cureus,* 11.
1007 PUSIC, P., SONNLEITNER, E., KRENNMAYR, B., HEITZINGER, D. A., WOLFINGER,
1008 M. T., RESCH, A. & BLASI, U. 2018. Harnessing Metabolic Regulation to Increase
1009 Hfq-Dependent Antibiotic Susceptibility in Pseudomonas aeruginosa. *Frontiers in*
1010 *Microbiology,* 9.
1011 RHEE, M. K., KOWALSKI, R. P., ROMANOWSKI, E. G., MAH, F. S., RITTERBAND, D.
1012 C. & GORDON, Y. J. 2004. A laboratory evaluation of antibiotic therapy for
1013 ciprofloxacin-resistant Pseudomonas aeruginosa. *American Journal of*
1014 *Ophthalmology,* 138**,** 226-230.

1015 RIERA, E., MACIA, M. D., MENA, A., MULET, X., PEREZ, J. L., GE, Y. G. & OLIVER,
1016 A. 2010. Anti-biofilm and resistance suppression activities of CXA-101 against
1017 chronic respiratory infection phenotypes of Pseudomonas aeruginosa strain PAO1.
1018 *Journal of Antimicrobial Chemotherapy,* 65**,** 1399-1404.
1019 ROLFE, M. D., RICE, C. J., LUCCHINI, S., PIN, C., THOMPSON, A., CAMERON, A. D.
1020 S., ALSTON, M., STRINGER, M. F., BETTS, R. P., BARANYI, J., PECK, M. W. &
1021 HINTON, J. C. D. 2012. Lag Phase Is a Distinct Growth Phase That Prepares Bacteria
1022 for Exponential Growth and Involves Transient Metal Accumulation. *Journal of*
1023 *Bacteriology,* 194**,** 686-701.
1024 ROOTMAN, D. S. & KRAJDEN, M. 1993. CONTINUOUS-FLOW PERFUSION OF
1025 GENTAMICIN WITH A SCLERAL SHELL REDUCES BACTERIAL COLONY
1026 COUNTS IN EXPERIMENTAL PSEUDOMONAS KERATITIS. *Journal of Ocular*
1027 *Pharmacology,* 9**,** 271-276.
1028 SCANNELL, J. W., BLANCKLEY, A., BOLDON, H. & WARRINGTON, B. 2012.
1029 Diagnosing the decline in pharmaceutical R&D efficiency. *Nature Reviews Drug*
1030 *Discovery,* 11**,** 191-200.
1031 SCHUURMANS, J. M., HAYALI, A. S. N., KOENDERS, B. B. & TER KUILE, B. H. 2009.
1032 Variations in MIC value caused by differences in experimental protocol. *Journal of*
1033 *Microbiological Methods,* 79**,** 44-47.
1034 SEYHAN, A. A. 2019. Lost in translation: the valley of death across preclinical and clinical
1035 divide – identification of problems and overcoming obstacles. *Translational Medicine*
1036 *Communications***,** 18.
1037 SHAFIEI, M., ALI, A. A., SHAHCHERAGHI, F., SABOORA, A. & NOGHABI, K. A.
1038 2014. Eradication of Pseudomonas aeruginosa Biofilms Using the Combination of n-
1039 butanolic Cyclamen coum Extract and Ciprofloxacin. *Jundishapur Journal of*
1040 *Microbiology,* 7.
1041 SILBIGER, J. & STERN, G. A. 1992. EVALUATION OF CORNEAL COLLAGEN
1042 SHIELDS AS A DRUG DELIVERY DEVICE FOR THE TREATMENT OF
1043 EXPERIMENTAL PSEUDOMONAS KERATITIS. *Ophthalmology,* 99**,** 889-892.
1044 SILVA, G. C. M., JABOR, V. A. P., BONATO, P. S., MARTINEZ, E. Z. & FARIA-E-
1045 SOUSA, S. J. 2017. Penetration of 0.3% ciprofloxacin, 0.3% ofloxacin, and 0.5%
1046 moxifloxacin into the cornea and aqueous humor of enucleated human eyes. *Brazilian*
1047 *Journal of Medical and Biological Research,* 50.
1048 SIQUEIRA, V. L. D., CARDOSO, R. F., CALEFFI-FERRACIOLI, K. R., SCODRO, R. B.
1049 D., FERNANDEZ, M. A., FIORINI, A., UEDA-NAKAMURA, T., DIAS-FILHO, B.
1050 P. & NAKAMURAA, C. V. 2014. Structural Changes and Differentially Expressed
1051 Genes in Pseudomonas aeruginosa Exposed to Meropenem-Ciprofloxacin
1052 Combination. *Antimicrobial Agents and Chemotherapy,* 58**,** 3957-3967.
1053 SOARES, A., ROUSSEL, V., PESTEL-CARON, M., BARREAU, M., CARON, F.,
1054 BOUFFARTIGUES, E., CHEVALIER, S. & ETIENNE, M. 2019. Understanding
1055 Ciprofloxacin Failure in Pseudomonas aeruginosa Biofilm: Persister Cells Survive
1056 Matrix Disruption. *Frontiers in Microbiology,* 10.
1057 STIEBEL-KALISH, H., GATON, D. D., WEINBERGER, D., LOYA, N., SCHWARTZ-
1058 VENTIK, M. & SOLOMON, A. 1998. A comparison of the effect of hyaluronic acid
1059 versus gentamicin on corneal epithelial healing. *Eye,* 12**,** 829-833.
1060 SUEKE, H., KAYE, S., WILKINSON, M. C., KENNEDY, S., KEARNS, V., ZHENG, Y.,
1061 ROBERTS, P., TUFT, S. & NEAL, T. 2015. Pharmacokinetics of Meropenem for
1062 Use in Bacterial Keratitis. *Investigative Ophthalmology & Visual Science,* 56**,** 5731-
1063 5738.

1064 SUEKE, H., NEAL, T., TUFT, S. J., WILKINSON, M., ZHENG, Y., WINSTANLEY, C. &
1065 KAYE, S. 2014. Corneal Pharmacokinetics of Meropenem. *Investigative*
1066 *Ophthalmology & Visual Science,* 55.
1067 TAM, C., LEDUE, J., MUN, J. J., HERZMARK, P., ROBEY, E. A., EVANS, D. J. &
1068 FLEISZIG, S. M. J. 2011. 3D Quantitative Imaging of Unprocessed Live Tissue
1069 Reveals Epithelial Defense against Bacterial Adhesion and Subsequent Traversal
1070 Requires MyD88. *Plos One,* 6.
1071 THI, M. T. T., WIBOWO, D. & REHM, B. H. A. 2020. Pseudomonas aeruginosa Biofilms.
1072 *International Journal of Molecular Sciences,* 21.
1073 TING, D. S. J., HO, C. S., CAIRNS, J., ELSAHN, A., AL-AQABA, M., BOSWELL, T.,
1074 SAID, D. G. & DUA, H. S. 2021a. 12-year analysis of incidence, microbiological
1075 profiles and in vitro antimicrobial susceptibility of infectious keratitis: the Nottingham
1076 Infectious Keratitis Study. *British Journal of Ophthalmology,* 105**,** 328-333.
1077 TING, D. S. J., HO, C. S., DESHMUKH, R., SAID, D. G. & DUA, H. S. 2021b. Infectious
1078 keratitis: an update on epidemiology, causative microorganisms, risk factors, and
1079 antimicrobial resistance. *Eye,* 35**,** 1084-1101.
1080 TSAI, T. H., CHEN, W. L. & HU, F. R. 2010. Comparison of fluoroquinolones: cytotoxicity
1081 on human corneal epithelial cells. *Eye,* 24**,** 909-917.
1082 UNG, L., BISPO, P. J. M., SHANBHAG, S. S., GILMORE, M. S. & CHODOSH, J. 2019.
1083 The persistent dilemma of microbial keratitis: Global burden, diagnosis, and
1084 antimicrobial resistance. *Survey of Ophthalmology,* 64**,** 255-271.
1085 URWIN, L., OKUROWSKA, K., CROWTHER, G., ROY, S., GARG, P.,
1086 KARUNAKARAN, E., MACNEIL, S., PARTRIDGE, L. J., GREEN, L. R. &
1087 MONK, P. N. 2020. Corneal Infection Models: Tools to Investigate the Role of
1088 Biofilms in Bacterial Keratitis. *Cells,* 9.
1089 VALLAS, V., WIENERKRONISH, J. P., MOSTOV, K. E. & FLEISZIG, S. M. J. 1996.
1090 Cytotoxic strains of Pseudomonas aeruginosa can damage the intact corneal surface.
1091 *Investigative Ophthalmology & Visual Science,* 37**,** 4026-4026.
1092 VARAPRASATHAN, G., MILLER, K., LIETMAN, T., WHITCHER, J. P., CEVALLOS,
1093 V., OKUMOTO, M., MARGOLIS, T. P., MIAO, Y. H. & CUNNINGHAM, E. T.
1094 2004. Trends in the etiology of infectious corneal ulcers at the F. I. Proctor
1095 Foundation. *Cornea,* 23**,** 360-364.
1096 WHITCHER, J. P., SRINIVASAN, M. & UPADHYAY, M. P. 2001. Corneal blindness: a
1097 global perspective. *Bulletin of the World Health Organization,* 79**,** 214-221.
1098 WIEHLMANN, L., WAGNER, G., CRAMER, N., SIEBERT, B., GUDOWIUS, P.,
1099 MORALES, G., KOHLER, T., VAN DELDEN, C., WEINEL, C., SLICKERS, P. &
1100 TUMMLER, B. 2007. Population structure of Pseudomonas aeruginosa. *Proceedings*
1101 *of the National Academy of Sciences of the United States of America,* 104**,** 8101-8106.
1102 WILHELMUS, K. R. & ABSHIRE, R. L. 2003. Corneal ciprofloxacin precipitation during
1103 bacterial keratitis. *American Journal of Ophthalmology,* 136**,** 1032-1037.
1104 WILHELMUS, K. R., HYNDIUK, R. A., CALDWELL, D. R., ABSHIRE, R. L.,
1105 FOLKENS, A. T. & GODIO, L. B. 1993. 0.3-PERCENT CIPROFLOXACIN
1106 OPHTHALMIC OINTMENT IN THE TREATMENT OF BACTERIAL
1107 KERATITIS. *Archives of Ophthalmology,* 111**,** 1210-1218.
1108 WILLCOX, M. D. P. 2011. Review of resistance of ocular isolates of Pseudomonas
1109 aeruginosa and staphylococci from keratitis to ciprofloxacin, gentamicin and
1110 cephalosporins. *Clinical and Experimental Optometry,* 94**,** 161-168.
1111 WOZNIAK, D. J., WYCKOFF, T. J. O., STARKEY, M., KEYSER, R., AZADI, P.,
1112 O'TOOLE, G. A. & PARSEK, M. R. 2003. Alginate is not a significant component of
1113 the extracellular polysaccharide matrix of PA14 and PAO1 Pseudomonas aeruginosa

biofilms. *Proceedings of the National Academy of Sciences of the United States of America,* 100**,** 7907-7912.
YAU, C. W., BUSIN, M. & KAUFMAN, H. E. 1986. OCULAR CONCENTRATION OF GENTAMICIN AFTER PENETRATING KERATOPLASTY. *American Journal of Ophthalmology,* 101**,** 44-48.

Supplementary materials

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

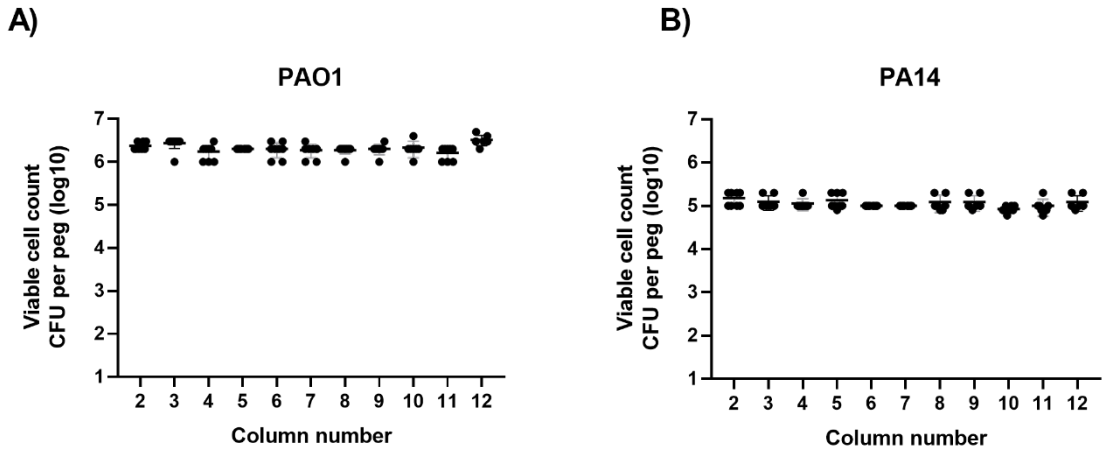
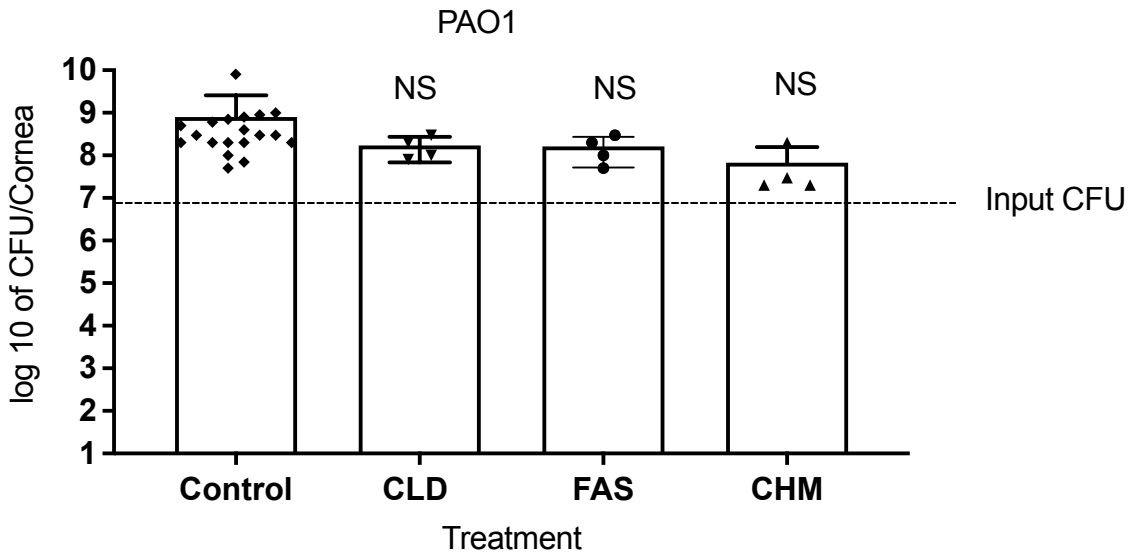
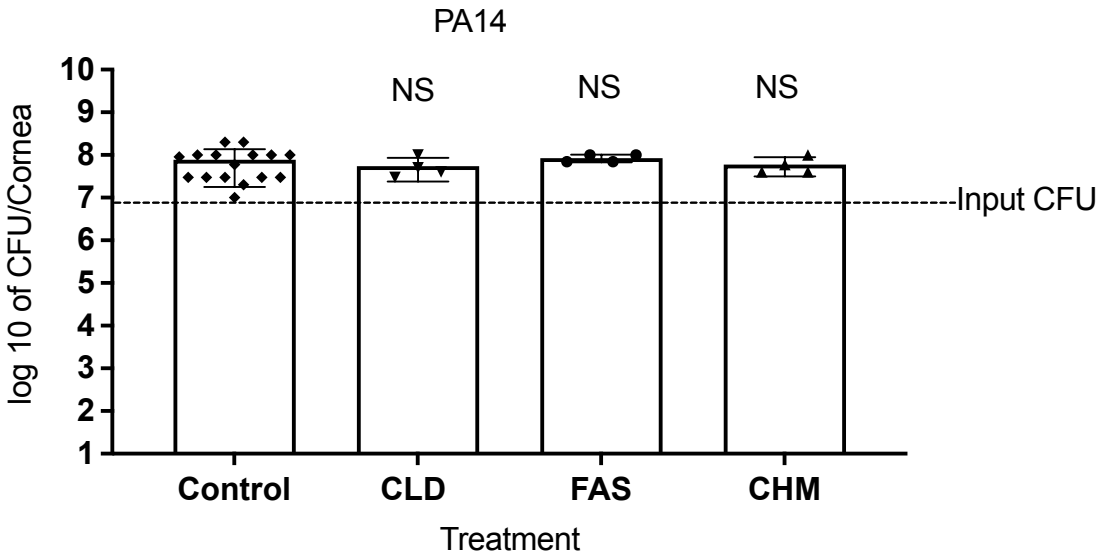


Figure 2. Colony forming units of *P. aeruginosa* in the *ex vivo* porcine corneas infected for 6 hours with (A) PAO1 or (B) PA14. Control corneas were immersed in PBS while other corneas were treated with 1025µg/mL of antibiotic dissolved in PBS. Following antibiotics were applied on infected corneas: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

A



1135
1136 **B**
1137



1138
1139

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

PAO1

Treatment	Concentration µg mL^{-1}	Average	N	SD	% Reduction
PBS	0	3.E+09	4	8.E+08	
Gentamicin	4	2.E+09	4	8.E+08	26.005
Ciprofloxacin	0.25	2.E+09	4	1.E+09	43.625
Meropenem	1	1.E+09	4	9.E+08	53.651

PA14

Treatment	Concentration µg 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

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

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

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

Table 4. Data summary of opacity scoring for *ex vivo* porcine corneas. The table shows a 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 *P. aeruginosa*	**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