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

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ABSTRACT

Global trends show an 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

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

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

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

106 (Lee et al., 2006) are well described in the literature. Each of these clinical isolates belongs to
107 one of two major phylogenetic group: group 1, which includes strain PAO1, and group 2, which
108 includes strain PA14 (Freschi et al., 2019). Each phylogenetic group is suspected to have a
109 different effect on the host cells (Hilliam et al., 2020) and the clinical outcomes (Vallas et al.,
110 1996, Borkar et al., 2013, Fleiszig et al., 1996, Lee et al., 2003a). Strain PAO1 is considered to
111 be moderately virulent and forms more structured biofilms on solid surfaces (Goodman et al.,
112 2004) while PA14 is highly virulent, more cytotoxic and forms a weaker biofilm (Wiehlmann
113 et al., 2007, Kasetty et al., 2021, Mikkelsen et al., 2011) called a pellicle that are associated
114 with a stagnant liquid surfaces (Friedman and Kolter, 2004). Kasetty, S. at al. (2021) described
115 differences in biofilm invasion strategies between these two strains in more detail. Genes
116 encoding virulence factors in these strains is regulated by quorum-sensing (QS) systems which
117 are also well described in literature (Girard and Bloemberg, 2008, de Kievit, 2009). In this
118 study we wanted to see if differences between these two strains will be obvious during different
119 stages of infection and after treatments with antibiotics on *ex vivo* porcine keratitis model.
120
121 We tested a range of common antibiotics with various activity against *Pseudomonas* keratitis.
122 We demonstrate that our *ex vivo* porcine keratitis model can be used as a tool to test
123 effectiveness and optimal concentrations of new drugs or preservatives for ocular infections
124 quickly, at lower expense before these treatments are further validated *in vivo*. Our *ex vivo*
125 model could help to select therapeutics that have a greater chance of success in investigations
126 *in vivo*.
127
128 **MATERIALS AND METHODS**
129 **Bacterial strain used**
130 Two wild type strains of *Pseudomonas aeruginosa* (PAO1 and PA14) were a kind gift from
131 Prof. Urs Jenal, University of Basel, Switzerland. Both strains were used to infect *ex vivo*
132 porcine corneas and for establishing MIC and MBEC values.
133
134 **MIC assay**
135 The MIC value for *P. aeruginosa* PAO1 and PA14 was determined according to the EUCAST
136 guidelines (Hasselmann, 2000). The bacterial strains were inoculated in Mueller-Hinton cation
137 adjusted broth (MHB) for 24 hours at 37 °C with agitation at 110 rpm. Before each experiment
138 10 µl of 6-fold dilutions of the inoculum was spot plated on blood agar plates, and the plates
139 were incubated (Infors HT Multitron, UK) overnight at 37 °C in order to enumerate colony

140 forming units in the inoculum. Two hundred microliters of MHB containing an inoculum with
141 $3x10^5$ CFU per well and different concentrations of the test antibiotics was added to each well
142 in a 96-well plate. A concentration of antibiotics ranging from 0.006 to 32 µg/µL was tested.
143 The MIC value was determined as the lowest concentration of an antibiotic which completely
144 inhibits visible bacterial growth after 24 hours at 37 °C in static conditions. In total six
145 antibiotics were tested: gentamicin, meropenem, ciprofloxacin, clindamycin, fusidic acid and
146 chloramphenicol. Clindamycin, fusidic acid and chloramphenicol are normally not used to treat
147 ocular infections caused by *P. aeruginosa* and were used here as a negative control. The optical
148 density at 600 nm was measured using the TECAN Spark plate reader (TECAN, Switzerland)
149 to confirm the growth inhibition. One column of each 96-well plate was designated for growth
150 control and one for sterility control. The procedure was repeated three times across different
151 days for each antibiotic.
152
153 **MBEC assay**
154 Biofilm susceptibility testing assay was performed using a Calgary device (Innovotech,
155 Canada) where the biofilm was grown on a peg (Harrison et al., 2010). First, growth conditions
156 were verified by an equivalence test for biofilm formation (Figure 1 in Supplementary
157 Materials) as described by Harrison et al. (2010). The bacterial strains were streaked out on LB
158 agar plate from cryogenic stock and incubated overnight at 37 °C. A single colony from the
159 agar subculture was used to inoculate 5 mL MHB and the suspension was incubated in a 50
160 mL Falcon tube while shaking at 110 rpm for 24 hours at 37 °C (Infors HT Multitron, UK).
161 The bacterial suspension was centrifuged at 4000 g in Eppendorf 5710R (Thermo Fisher, UK)
162 for 5 minutes. After discarding the supernatant, the pellet was re-suspended in 5 mL of sterile
163 MHB. The inoculum was prepared in a fresh centrifuge tube by diluting the suspension of
164 bacteria to optical density (OD) of 0.05 at 600 nm. The OD_{600nm} was measured using
165 spectrophotometer Jenway (VWR, UK). The inoculum was pipetted in a 96-well plate with a
166 final concentration $8x10^6$ CFU of *P. aeruginosa* PAO1 or PA14 per well (150 µl inoculum in
167 each well). One column in a 96-well plate was used as a control and contained media without
168 bacteria added. Pegs from Calgary Device were immersed in the inoculum. The 96-well plate
169 was double sealed with parafilm, placed inside a plastic box to reduce evaporation and
170 incubated (statically) overnight at 37^0C with 70% humidity in the incubator (Infors HT
171 Multitron, UK) to allow biofilm formation on pegs. Before each experiment 10 µl of 6-fold
172 dilutions of the inoculum was spot plated on blood agar plates, and the plates were incubated
173 overnight at 37 °C in order to enumerate colony forming units (CFU) in the inoculum. After

174 overnight incubation the pegs were rinsed twice for 1 minute in two 96-well plates with 200 µl
175 of sterile water per well to remove bacteria that did not attach to pegs (planktonic cells).
176
177 For equivalence assay, the pegs were then transferred to a 96-well plate with 200 µl of LB with
178 1% Tween 20 per well, sonicated for 10 minutes at 60 Hz to disrupt bacteria from the biofilm
179 on pegs into a recovery medium. After sonication, 20 µl of the recovery medium with the
180 bacteria was diluted in series up to 10^4 in 180 µl of sterile water. All dilutions were plated out
181 on LB agar plates for CFU count and incubated at 37 °C overnight.
182
183 For MBEC assay the pegs were transferred after rinsing steps to a 96-well plate with antibiotics
184 in MHB. The plate was incubated overnight and then rinsed and sonicated in the same way as
185 equivalence assay plates. Ciprofloxacin, meropenem and gentamicin were tested with
186 concentrations starting from 1µg μL^{-1} to 512 µg μL^{-1}. Minimum biofilm eradication
187 concentration (MBEC) value represents the wells with the lowest concentration of an antibiotic
188 where the biofilm was completely eradicated i.e. there was no growth from biofilms across all
189 replicates. One column of each 96-well plate was designated for untreated control and one for
190 sterility control. The procedure was repeated four times across different days for each antibiotic
191 with four technical replicates each time.
192
193 **Testing antibiotics on *ex vivo* porcine cornea model**
194 In this study, porcine eyes were extracted within four hours from slaughter and transported
195 from the abattoir (R.B. Elliott and Son Abattoir, Calow, England) in a Nalgene container filled
196 with sterile phosphate buffer saline (PBS, Sigma, Germany). The age of pigs varied between
197 26 to 28 weeks. The corneas were excised in the laboratory within two hours from delivery and
198 used for experiments within a week from excision. The pigs were sacrificed for human
199 consumption and not for the purpose of this study.
200
201 Porcine eyes were prepared for infection as described previously (Okurowska et al., 2020).
202 Briefly, the porcine eyes from the abattoir were washed with sterile PBS before and after
203 disinfection with 3% povidone iodine diluted with PBS for 60 seconds. Corneas with scleral
204 rings were dissected, rinsed with 1.5% povidone iodine and then soaked in PBS before
205 transferring to warm Dulbecco's modified Eagle's medium (DMEM, Fisher Scientific, UK)
206 supplemented with growth factors and antibiotics. The composition of the culture medium was
207 as follows: DMEM: Ham's (Sigma, Germany) [1:1] supplemented with 5 µg mL^{-1} insulin (SLS,

208 UK) and 10 ng mL^{-1} epidermal growth factor (EGF) (SLS, UK), 10 % fetal calf serum (FCS)
209 (Pan-Biotech, UK), 100 U mL^{-1} penicillin, 100 U mL^{-1}streptomycin (SLS, UK) and 2.5 µg mL^{-}
210 ^{1} amphotericin B (Sigma, UK). The corneas were incubated in medium with antimicrobials for
211 24 hours at 37 °C and then washed once with 2 mL PBS and incubated in antimicrobial-free
212 medium for 48 hours to remove residual antibiotics. The medium was replaced every day
213 during this time. On the infection day porcine corneas were infected with $8x10^{6}$ CFU in 200 µl
214 of PBS and incubated for 6 hours. After the incubation, the PBS along with suspended bacteria
215 were removed with a sterile 1 mL pipette tip and replaced either with 200 µL of PBS (control
216 corneas) or with a PBS with added antibiotic (treated corneas). The corneas were treated with
217 either 1024 µg mL^{-1} or with MIC concentration of ciprofloxacin, meropenem, gentamicin for
218 18 hours at 37 °C. All corneas were photographed with Dino-lite Xcope camera (AnMo
219 Electronics Corporation, Taiwan). Ninety cornea images were independently scored for opacity
220 by five people using following grading system: 0 – no haze, cornea clear; 1 – faint opacity or
221 cloudiness visible; 2 – cornea looks swollen, white or hazy patch clearly visible. All graphs
222 were plotted using GraphPad Prism version 8.4.1.
223
224 **Statistics**
225 Statistical analysis of viable cell counts for experiment comparing two strains of *Pseudomonas*
226 was carried out by unpaired t-tests with Holm-Sidak correction while effect of treatment versus
227 placebo was calculated using Kruskal-Wallis multiple comparisons test, using GraphPad Prism
228 version 8.4.1. *P*-values lower than 0.05 were considered significant.
229
230 **Data availability**
231 All supporting data are provided in the Supplementary Materials file.
232
233 **RESULTS**
234
235 ## MIC assay
236 MIC assays revealed that both strains of *P. aeruginosa* used in this study were sensitive to
237 gentamicin, meropenem and had intermediate resistance to ciprofloxacin (Table 1) while they
238 were resistant to clindamycin, fusidic acid and chloramphenicol (MIC > 32 µg mL^{-1}). The MIC
239 values for gentamicin were identical for both strains (2 - 4 µg mL^{-1}). However, some small
240 differences were observed between strains treated with meropenem and ciprofloxacin. Strain
241 PA14 was marginally more susceptible to meropenem (0.25 µg mL^{-1}) while strain PAO1 was
242 marginally more susceptible to ciprofloxacin (0.125-0.25 µg mL^{-1}).

243

244 **Table 1. Determination of MIC and MBC of *P. aeruginosa* for PAO1 and PA14 isolates**
245 **against gentamicin, meropenem and ciprofloxacin . Values in the table represent µg mL^{-}**
246 **^{1}.**
247

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**

248

249

250 ## MBEC assay

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

255

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

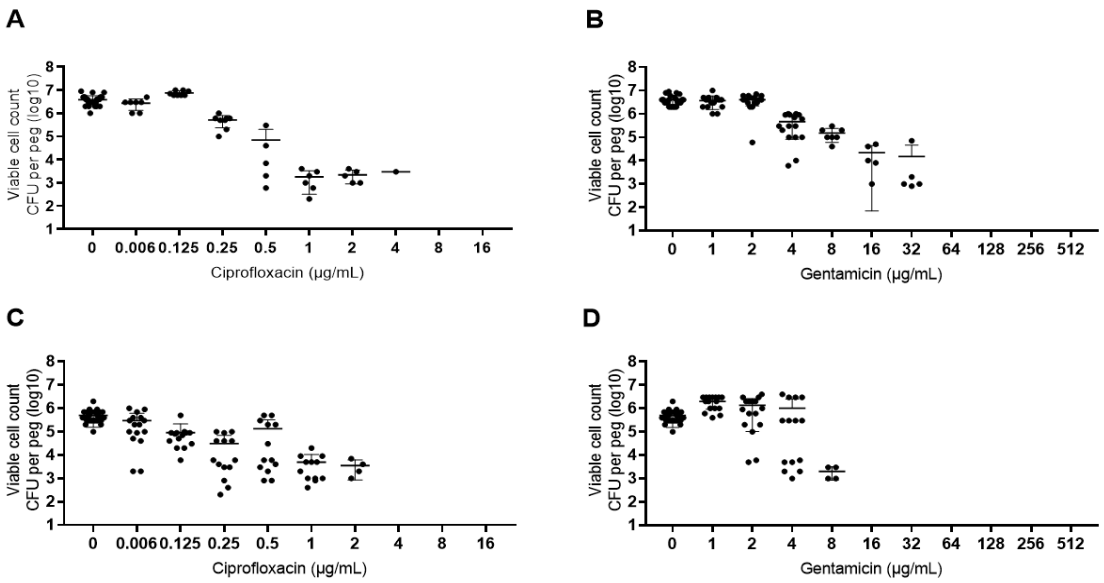


Fig.1. MBEC assay results representing colony forming units of *P. aeruginosa* PAO1 (A, B) and PA14 (C,D) treated with ciprofloxacin and gentamicin.

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.

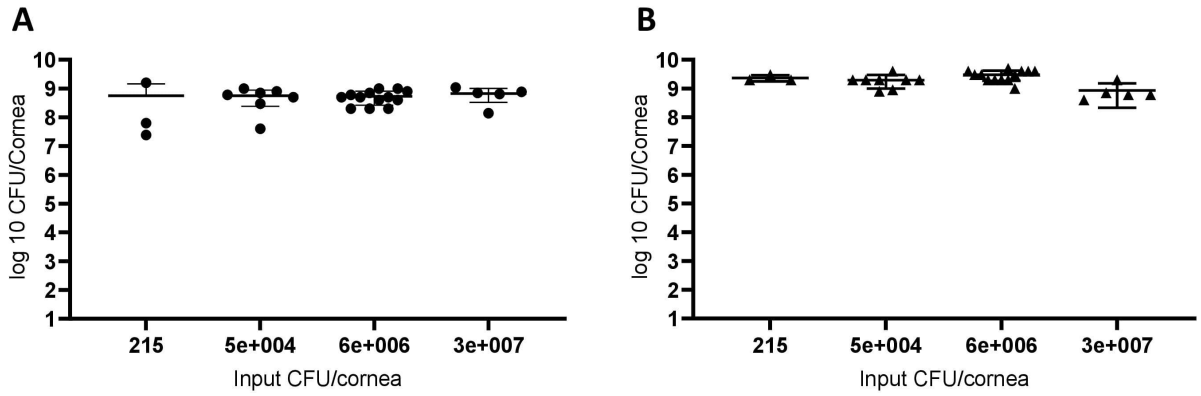


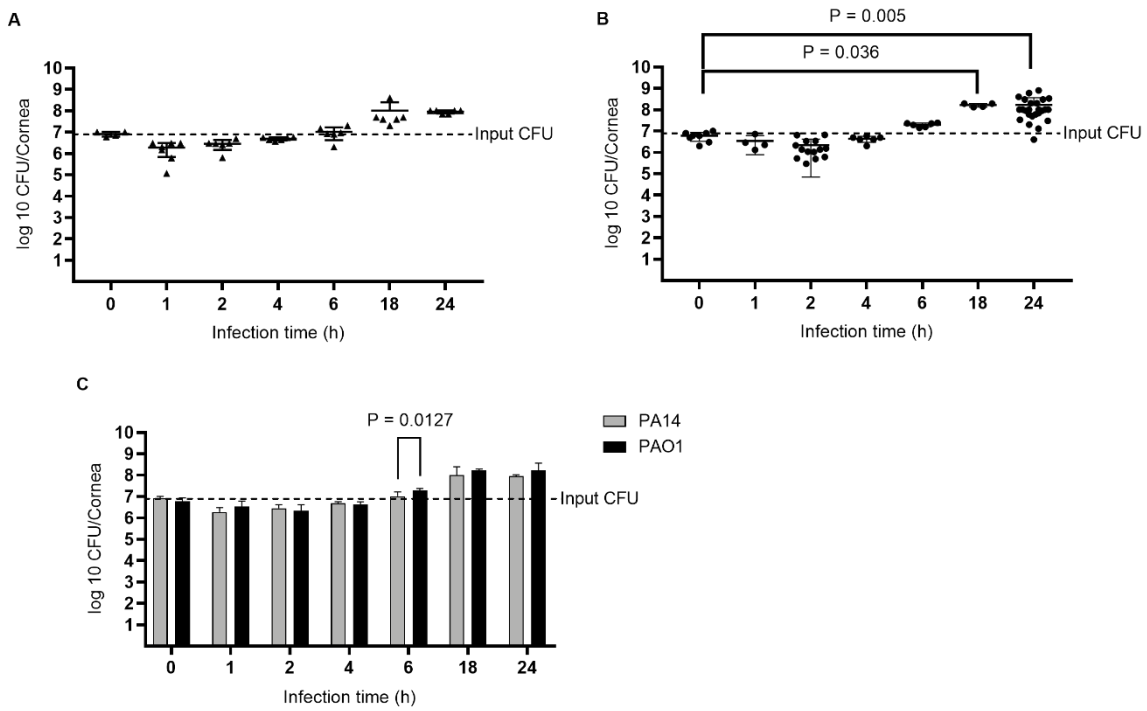
Fig 2. Number of viable *P. aeruginosa* PA14 retrieved from porcine cornea after infection with 215, $5x10^4$, $6x10^6$ and $3x10^7$ CFU per cornea. Corneas were infected for 24 hours (A) and 48 hours (B). Each dot represents results from one cornea. Error bars represent standard deviation. Statistical 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

315 all these time points, the number of CFU retrieved per cornea were lower than the inoculum
316 size (7.9 x 10^6 CFU per cornea). Subsequently the increase in bacteria load in the infected
317 cornea was higher compared to the inoculum size for *P. aeruginosa* PAO1 (Fig. 3C): 2.0 x 10^7
318 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
319 per cornea at 24 hpi (n = 25) (Fig. 3B). Difference in CFU values for PAO1 retrieved at 1hpi,
320 2 hpi and 4hpi in comparison to 18 hpi and 24 hpi was significant ($p<0.05$).
321
322 These data demonstrate that both strains of *P. aeruginosa* were able to initiate and maintain
323 infection on porcine corneas within first few hours of incubation. In both strains, despite
324 inclusion of a washing step, there was a net increase in the number of CFU retrieved after
325 incubation compared to the inoculum which suggests that infection was well established in the
326 model. In the subsequent experiments, antibiotic treatments were added to corneas at 6 hpi
327 because there was a clearly visible increase in CFU counts at this time point in comparison to
328 input of bacteria which indicated that the infection was well-established.

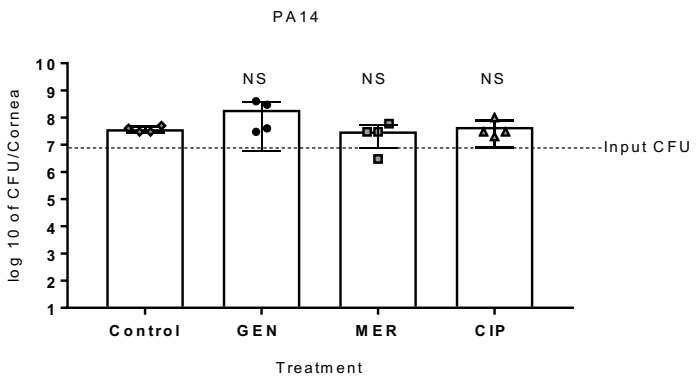


329
330 Fig. 3. Number of viable CFU of *P. aeruginosa* on porcine corneas infected for 1, 2, 4, 6, 18
331 and 24 hours with *P. aeruginosa* PA14 (A) and *P. aeruginosa* PA01 (B). Data from both strains
332 are compared on one graph (C). Inoculum CFU are shown both as CFU at t=0 infection time
333 (x-axis) as well as a dotted line labelled as Input CFU. Each dot on charts A and B represents
334 one cornea. Error bars represent standard deviation. Statistical significance for graph A and B
335 is presented according to Kruskall-Wallis test while for graph C is presented according to the
336 unpaired *t*-test with Holm-Sidak correction **p*-value <0.05. Raw data is available in
337 Supplementary Materials, Data Availability Section.

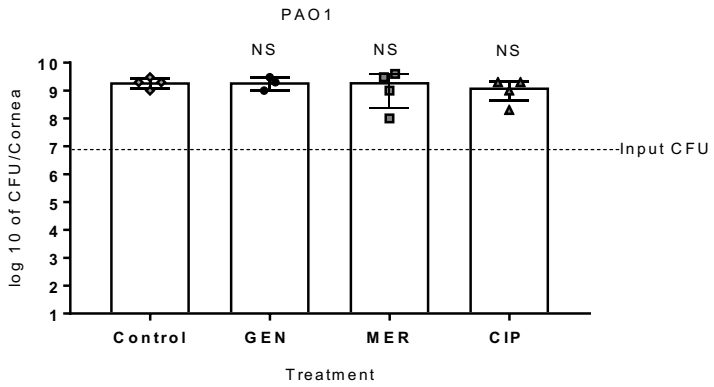
338
339 **Testing antibiotics on the *ex vivo* porcine keratitis model**

340 Next, the effect of MIC concentrations on infected tissue was investigated. *Ex vivo* porcine
341 corneas were infected on average with 1 x 10^7 CFU *P. aeruginosa* PA14 and 9 x 10^6 *P.*
342 *aeruginosa* PAO1 for 6 hours and then MIC concentrations of gentamicin, meropenem and
343 ciprofloxacin were applied for 18 hours. While MIC concentrations of antibiotics successfully
344 inhibited growth of bacteria *in vitro*, these concentrations were ineffective ($p > 0.05$) for both
345 tested strains of *P. aeruginosa* PA14 and PAO1 in *ex vivo* porcine cornea model (Fig. 4). Raw
346 data are presented in Supplementary Materials Table 2. This demonstrates that application of
347 MIC concentrations on *ex vivo* cornea is insufficient to treat ocular infections with *P.*
348 *aeruginosa* despite the fact that the infected tissue was continually exposed to the antibiotic for
349 18 hours.

350 **A**

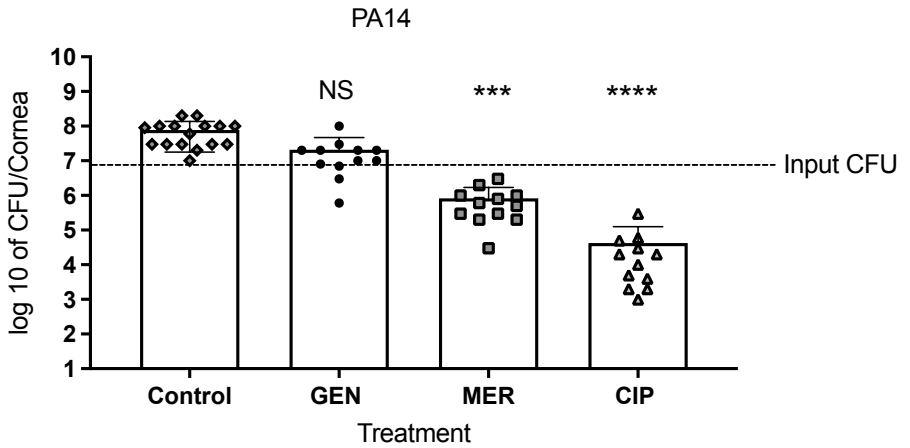


351
352 **B**



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

359 test was performed for the pairwise statistical analysis of treated against control colony forming
360 units for each strain.
361
362 Following previous results, the concentration of antibiotics (gentamicin, meropenem and
363 ciprofloxacin) that were applied on *ex vivo* porcine corneas was increased to 1025 μg mL^{-1}.
364 This concentration is 256 times MIC for gentamicin for strains PAO1 and PA14, respectively.
365 For meropenem, this concentration is 1025 times MIC for strain PAO1 and 4100 times MIC
366 meropenem for PA14. For ciprofloxacin, this concentration is 4100 times MIC for strain PAO1
367 and 2050 times MIC for strain PA14. As this concentration is higher than MIC and MBEC
368 some growth inhibition on *ex vivo* infected tissue was expected. A significant reduction in
369 bacteria load for strain PAO1 in corneas treated with gentamicin ($p = 0.0051$), meropenem (p
370 < 0.0001) and ciprofloxacin ($p < 0.0001$) was observed when compared to controls (Figure 5).
371 There was no significant reduction for corneas infected with strain PA14 and treated with
372 gentamicin ($p = 0.15$). However, treatment with meropenem ($p = 0.0001$) and ciprofloxacin (p
373 <0.0001) had a noticeable reduction in bacteria load.
374
375 Out of three tested antibiotics at concentration 1025 μg mL^{-1}, gentamicin was the least potent,
376 possibly because the concentration of this antibiotic added to corneas in the relation to MIC
377 was much smaller in comparison to meropenem and ciprofloxacin. On average $7x10^7$ CFU (1-
378 log reduction) of strain PAO1 and $2x10^7$ CFU (<1-log reduction) of strain PA14 were recovered
379 from porcine corneas. Meropenem reduced bacteria load approximately by 2-log for both
380 strains ($5x10^6$ CFU per cornea for strain PAO1 and $9x10^5$ CFU per cornea for strain PA14)
381 despite MBEC assay suggesting that biofilms of both strains are tolerant to >512 μg mL^{-1} of
382 this antibiotic. Treatment with ciprofloxacin resulted in an average of $1x10^5$ CFU (5-log
383 reduction) for corneas infected with strain PAO1 and $5x10^4$ CFU (4-log reduction) for corneas
384 infected with strain PA14. Treatment with clindamycin, fusidic acid and chloramphenicol had
385 no effect on bacteria count in comparison to control corneas with added PBS only, and this was
386 expected since MIC results showed that these antibiotics are ineffective on investigated *P.*
387 *aeruginosa* strains (supplementary materials Figure 2).
388 **A**



B

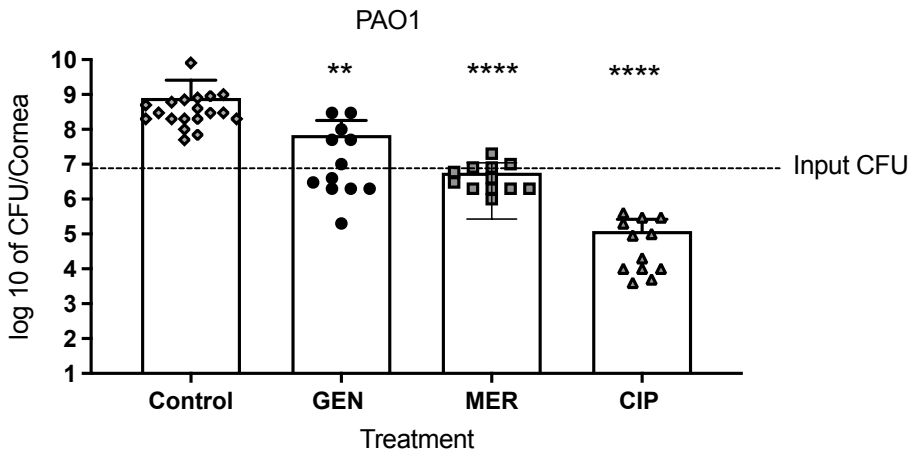
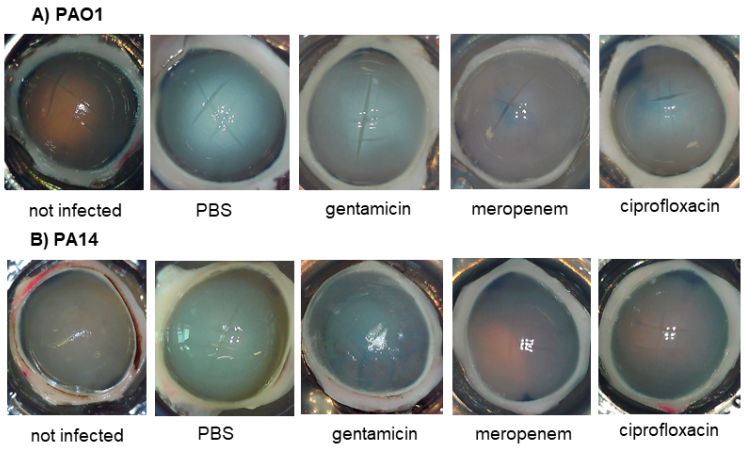


Fig. 5. Colony forming units of *P. aeruginosa* in the *ex vivo* porcine corneas infected for 6 hours with (A) PA14 or (B) PAO1. Control corneas (n = 19 & 16) were immersed in PBS while other corneas were treated with 1025 µg $mL^{-1}$of antibiotic dissolved in PBS. Following antibiotics were applied 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

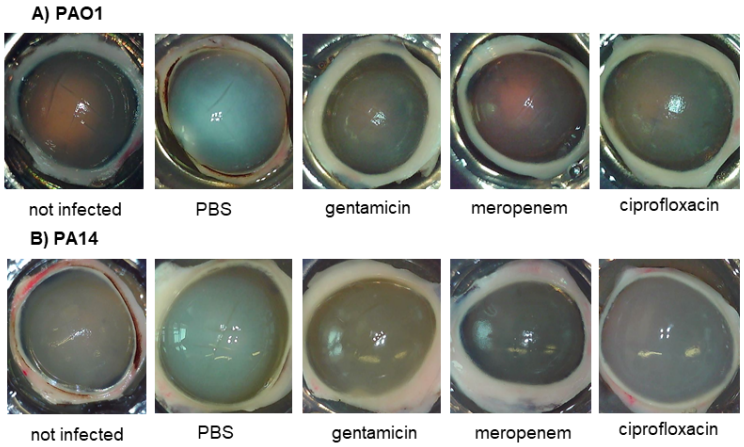
410 with PA14 (Fig. 6B). Corneas infected with both *P. aeruginosa* strains and treated with MIC
411 concentration of gentamicin looked slightly hazier in comparison to meropenem and
412 ciprofloxacin in spite of no reduction in viable colony count across all antibiotics (Fig. 4).



413

414 Fig. 6. Representative images of not infected and infected *ex vivo* porcine corneas without
415 (PBS) treatment and treated with gentamicin, meropenem and ciprofloxacin. Corneas shown
416 here were infected with $6x10^6$ CFU of strain PAO1 (A) and strain PA14 (B) and treated with
417 MIC concentrations of antibiotics after infection had progressed for 6 hours. Corneas were
418 imaged and immediately homogenised for viable counting.
419

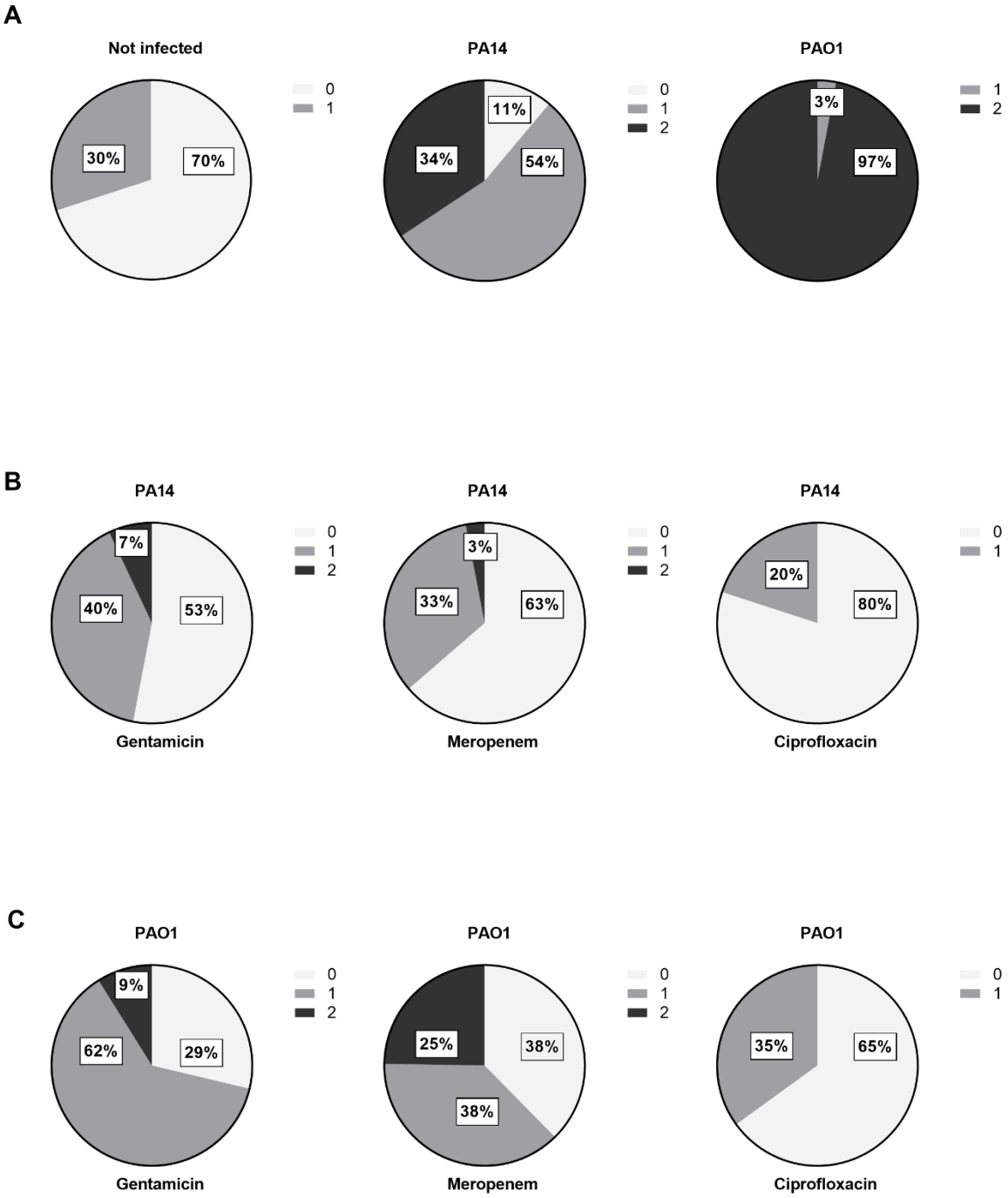
420 Treatment with higher antibiotic concentration (1025 µg mL^{-1}) decreased corneal opacity of
421 infected cornea by preventing development of milky colour (Fig. 7). This effect was especially
422 evident in cornea infected with PA14. Corneas treated with gentamicin, meropenem and
423 ciprofloxacin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



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

429 clindamycin, and chloramphenicol. Corneas shown here were infected with $6x10^6$ CFU of
430 strain PAO1 (A) and strain PA14 (B) 6-hour prior to antibiotic treatment. Corneas were imaged
431 and immediately homogenised for viable counting.
432
433 The presence and intensity of discolouration and opacity for ninety-nine corneas was
434 undertaken by assessing the images to verify if infection or the effect of a treatment could be
435 determined visually. The images of corneas were allocated to the following three grades: 0 –
436 the corneas looked clear and not infected; 1 - corneas looked infected, slightly hazy and cloudy;
437 2 – corneas looked infected, swollen and white/milky in colour. Images of corneas were blind
438 scored by five different people and presented as percentages (Fig. 8).
439
440 Majority of uninfected corneas (70%) were correctly identified while 30% were graded 1 (Fig.
441 8A). This is because the scoring of uninfected corneas could have been affected by swelling
442 that naturally occurs during incubation in media for few days and could make some corneas
443 appear less transparent than normal. Lack of previous experience in scoring, changes in the
444 natural light in the room and quality of some images could also have an effect on cornea
445 grading.
446
447 Some untreated corneas infected with strain PA14 looked clear (11%) whilst majority were
448 correctly identified as infected (54% scored grade 1 and 34% scored grade 2) (Fig. 8A).
449 Majority of untreated corneas (97%) were correctly identified as being infected with strain
450 PAO1 due to the development of an obvious haziness (grade 2) suggesting infection with strain
451 PAO1results in ulceration and severe tissue damage on *ex vivo* porcine cornea (Fig. 8A).
452
453 Overall, corneas infected with strain PA14 and treated with antibiotics and (Fig. 8B) developed
454 less haze in comparison to strain PAO1 (Fig. 8C). Smaller percentage of cornea infected with
455 either strain and treated with 1025 $\mu g\ mL^{-1}$ gentamicin and meropenem were scored grade 2 in
456 comparison to untreated (Fig. 8B & 8C versus Fig. 8A). None of the corneas infected with
457 either strain and treated with ciprofloxacin were graded 2 and majority were graded 0 which
458 suggests that ciprofloxacin had a beneficial effect on preserving corneal transparency.
459
460 Opacity scoring demonstrated visual differences in opacity between infection with *P.*
461 *aeruginosa* cytotoxic strain PA14 and infection with invasive strain PAO1. The PAO1 strain
462 showed the most visually obvious reduction in opacity for both untreated as well as treated
463 corneas. However, higher transparency of corneas treated with antibiotics should not be solely

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



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

473 groups: 0 – corneas clear, no haze or infection visible; 1- corneas looked infected, slightly hazy
474 and cloudy; 2 – corneas looked infected, swollen and white/milky in colour.
475
476
477 **DISCUSSION**
478
479 We previously established an *ex vivo* porcine model of *Pseudomonas aeruginosa* keratitis
480 (Okurowska et al., 2020). In this study we demonstrate that our *ex vivo* porcine cornea model
481 can be used for testing novel treatments against keratitis. We first established MIC and MBEC
482 values for gentamicin, meropenem and ciprofloxacin using cytotoxic (strain PA14) and
483 invasive (strain PAO1) strains of *P. aeruginosa*. Next, we tested the input of bacteria needed
484 to develop an infection and then monitored the development of an infection over time. Finally,
485 we investigated differences in response to antibiotic treatments between cytotoxic (strain
486 PA14) and invasive (strain PAO1) strains of *P. aeruginosa* on *ex vivo* porcine keratitis model.
487
488 Comparing MIC and MBEC results to literature was challenging because of the variance in
489 experimental protocols between research groups (Kowalska-Krochmal and Dudek-Wicher,
490 2021, Schuurmans et al., 2009) but overall, our findings followed the trend in literature.
491 Gentamicin, meropenem and ciprofloxacin were the most effective against both studied strains
492 of *P. aeruginosa*. MBEC values of all tested antibiotics were much higher in comparison to
493 MIC. Our results tie in well with previous studies showing that higher concentrations of
494 antibiotics are needed to eradicate biofilms compared to their planktonic counterparts (Bagge
495 et al., 2004, Brady et al., 2017, Bowler et al., 2012, Ceri et al., 1999).
496
497 Gentamicin was the first antibiotic that we tested for MIC against *P. aeruginosa* because it
498 often is used in early stages of keratitis (Dart and Seal, 1988). We found that MIC results in
499 our study, were in alignment with those found in literature and demonstrated that both strains
500 of *P. aeruginosa* (PA14 and PAO1) were equally sensitive to gentamicin. Our MIC values
501 were either identical (Bowler et al., 2012, Ceri et al., 1999) or similar to those reported by other
502 research groups (1 $\mu g\ mL^{-1}$ and 2 $\mu g\ mL^{-1}$)(Andrews, 2001, Bahari et al., 2017, Pusic et al.,
503 2018). While MIC values did not demonstrate any obvious differences between investigated
504 two strains, MBEC results clearly had shown that PAO1 was more resistant to gentamicin in
505 comparison to PA14. Similar to other studies, MBEC values showed that biofilms were
506 resistant to gentamicin in *P. aeruginosa* (Bowler et al., 2012, Ceri et al., 1999, Billings et al.,
507 2013). These results suggest that whilst gentamicin could be used to eradicate planktonic forms

508 of *P. aeruginosa*, treating the biofilm formed by this bacterium would require much higher
509 concentrations. Resistance of *P. aeruginosa* biofilm towards gentamicin could be explained by
510 the fact that gentamicin belongs to aminoglycosides group of antibiotics, that are known to
511 bind to various components in the biofilm matrix (Ciofu and Tolker-Nielsen, 2019) such as
512 exopolysaccharides Psl (Billings et al., 2013) and Pel (Colvin et al., 2011).
513
514 We selected meropenem for our studies because of good corneal penetration, low cytotoxicity
515 (Sueke et al., 2015) and promising results in live rabbit keratitis model. Our MIC values for *P.*
516 *aeruginosa* PAO1 showed sensitivity towards meropenem and were identical (0.5 μg mL^{-1})
517 (Riera et al., 2010, Ocampo-Sosa et al., 2012) or close (1-2 μg mL^{-1}) (Haagensen et al., 2017,
518 Bowler et al., 2012, Monahan et al., 2014) to those found in literature. MIC value for strain
519 PA14 in our study are also in accordance with findings reported by others (0.25 μg mL^{-1})
520 (Hassan et al., 2020, Ocampo-Sosa et al., 2012). MBEC values in literature for *P. aeruginosa*
521 PAO1 biofilm treated with meropenem were consistently much higher than MICs (Haagensen
522 et al., 2017, Bowler et al., 2012). Overall, while MIC results show sensitivity, our MBEC
523 results ties well with previous studies showing resistance in *P. aeruginosa* towards this
524 antibiotic (Bowler et al., 2012, Haagensen et al., 2017). These findings support the notion that
525 meropenem is more effective against actively dividing, planktonic bacteria or early-stage
526 biofilm, while less effective against established biofilms (Bowler et al., 2012).
527
528 Finally, we investigated ciprofloxacin in our studies because it is considered as one of the most
529 effective antibiotics against *P. aeruginosa* and therefore used as a first-line treatment in the UK
530 (Hilliam et al., 2020). Our study demonstrated that ciprofloxacin was certainly the most potent
531 antibiotic against planktonic and biofilm of *P. aeruginosa* not only *in vitro* but also on *ex vivo*
532 cornea model. Our MIC values for ciprofloxacin indicate that both strains were susceptible
533 according to EUCAST, 2022 with PA14 marginally more resistant than PAO1. Overall, the
534 results for MIC were similar to those in literature. Research groups reported MIC values in
535 range from 0.125 μg mL^{-1} (Riera et al., 2010) to 0.25 μg mL^{-1} (Shafiei et al., 2014, Bowler et
536 al., 2012) and 1 μg mL^{-1} (Fernandez-Olmos et al., 2012) for PAO1. Some studies reported MIC
537 0.125 μg mL^{-1} for PA14 (Soares et al., 2019, Bruchmann et al., 2013) while ours was
538 marginally higher (0.25-0.5 μg mL^{-1}). Our MBEC results also match trends in other studies *in*
539 *vitro* which demonstrated that there was small but not significant difference in response to
540 ciprofloxacin between biofilm formed by PA14 and PAO1 (Billings et al., 2013, Benthall et
541 al., 2015).

542

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

566

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

576

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

607

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

610 *in vivo* (Bozkurt et al., 2021) and humans (Pande and Bhailume, 2014) without any side effects.
611 Some studies found that meropenem concentrations of 5 mg mL^{-1} increased cellular activity in
612 corneal epithelial cell lines and the cell viability was still high (96%) after meropenem
613 treatment (Sueke et al., 2014, Sueke et al., 2015). Meropenem reduced the bacterial load in our
614 *ex vivo* corneas similarly to other studies on *ex vivo* rabbits or human (Bozkurt et al., 2021).
615 Low toxicity at high concentrations and reduction of bacteria load in studies *in vivo* suggests
616 that meropenem could be a good alternative drug against keratitis in the future (Pande and
617 Bhailume, 2014, Bozkurt et al., 2021). However, the resistance towards this antibiotic in
618 MBEC data is concerning (Bowler et al., 2012, Haagensen et al., 2017). Haagensen et al. (2017)
619 demonstrated that meropenem was highly effective in early stages of *P. aeruginosa* PAO1
620 biofilm formation. We also achieved good reduction of bacteria load after application of
621 meropenem after 6 hours post infection, during possibly early stages of biofilm formation.
622 More studies need to be conducted to assess the effectiveness of this drug and possibility of its
623 use in clinical practice. Some studies report that combining meropenem and ciprofloxacin can
624 have a synergistic effect against some clinical isolates of *P. aeruginosa* (Erdem et al., 2002,
625 Erdem et al., 2003, Siqueira et al., 2014, Pankuch et al., 2008, GarciaRodriguez et al., 1996)
626 which could be tested on our *ex vivo* porcine keratitis model in the future.
627
628 Ciprofloxacin has a very good tissue penetration property. Exposure to this antibiotic for as
629 short as 10 minutes has been demonstrated to result in concentrations exceeding MIC in human
630 cornea *ex vivo* (Silva et al., 2017, McDermott et al., 1993, Akkan et al., 1997, Ozturk et al.,
631 1999) therefore we suspect that the 18 hour continuous exposure to this antibiotic in our study
632 very likely resulted in MIC concentration in corneal tissue. Ciprofloxacin was very effective
633 in eradicating *P. aeruginosa* at higher concentrations in our experiments which is in line with
634 studies *in vivo*. The treatment was equally effective against cytotoxic and invasive strains of *P.*
635 *aeruginosa*. Several studies found that treating corneas with ciprofloxacin significantly
636 reduced or completely ceased infection with *P. aeruginosa* in live rabbits (Obrien et al., 1988,
637 Aliprandis et al., 2005, Guzek et al., 1994, LaBorwit et al., 2001, Bu et al., 2007, Kowalski et
638 al., 2001, Lauffenburger and Cohen, 1993, Oguz et al., 2005, Rhee et al., 2004) and humans
639 (Levey et al., 1998). Although it was found that phenotypic adaptation towards persistence to
640 this antibiotic happens very early if supra-MIC concentrations are used and as a result
641 ciprofloxacin may fail to eradicate biofilm (Soares et al., 2019). Using higher concentrations
642 of ciprofloxacin (0.3%) can cause crystalline corneal precipitation in humans (Wilhelmus and
643 Abshire, 2003, Wilhelmus et al., 1993, McDonald et al., 2014).

644

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

653

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

674

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

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

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AUTHOR CONTRIBUTIONS

KO and EK conceived the hypothesis and research design, KO performed all the experiments, analysed the data, and wrote the manuscript. EK and PM provided resources and reviewed the manuscript.

CONFLICT OF INTERESTS

The authors confirm no conflict of interest.

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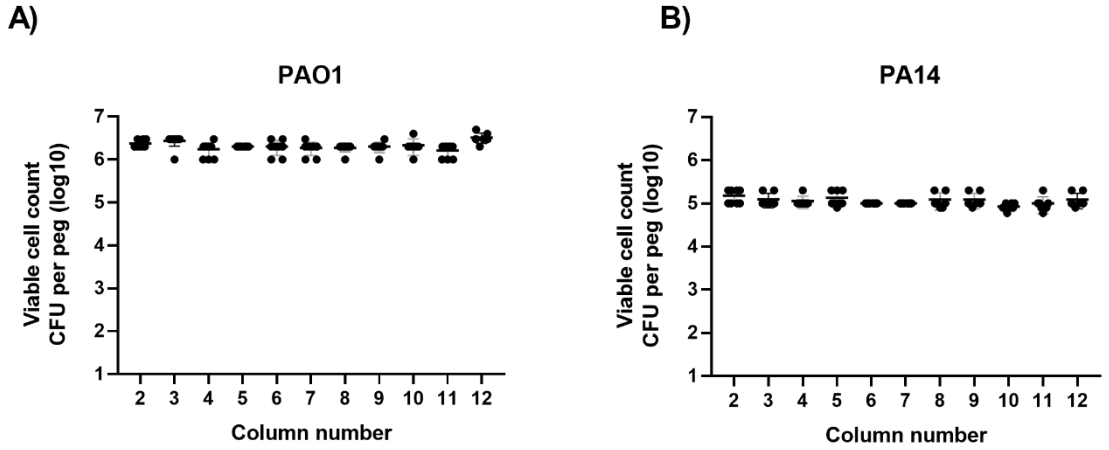
1130

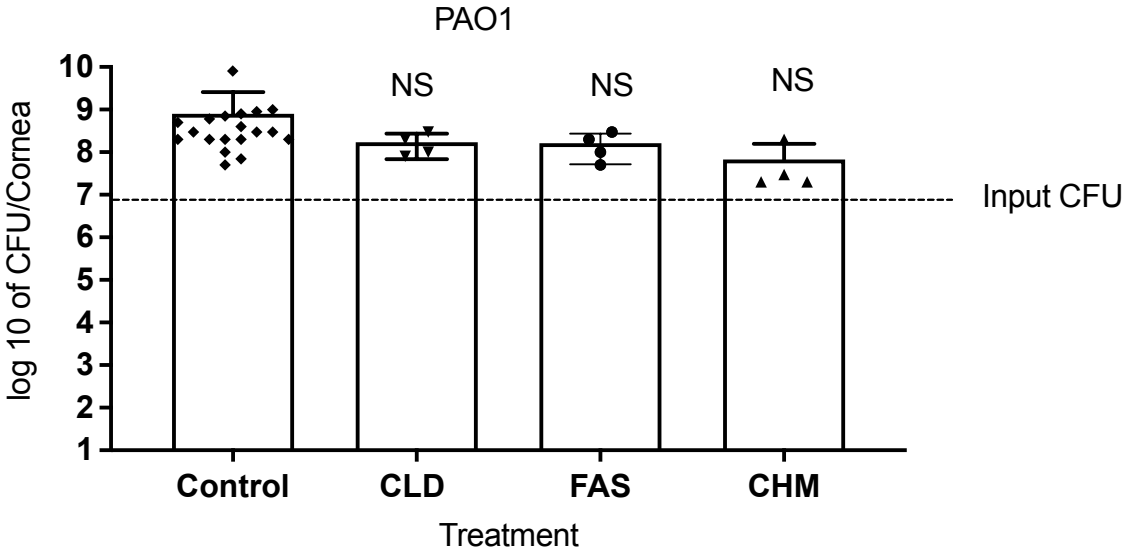
1131 **Supplementary materials**

1132 Figure 1. Equivalence assay results representing colony forming units of *P. aeruginosa* PAO1
1133 (A) and PA14 (C) retrieved from pegs across all columns in a 96-well plate.



1134
1135

1136 Figure 2. Colony forming units of *P. aeruginosa* in the *ex vivo* porcine corneas infected for 6
1137 hours with (A) PAO1 or (B) PA14. Control corneas were immersed in PBS while other corneas
1138 were treated with 1025µg/mL of antibiotic dissolved in PBS. Following antibiotics were
1139 applied on infected corneas: clindamycin (CLD) (n = 4), fusidic acid (FAS) (n = 4) and
1140 chloramphenicol (CHM) (n = 4). Error bars are means ± SD. Unpaired *t*-tests were performed
1141 for the pairwise statistical analysis of treated against untreated colony forming units for each
1142 strain; significant difference (p value < 0.05) is denoted with *.
1143 **A**
1144


PAO1
log 10 of CFU/Cornea
10
9
8
7
6
5
4
3
2
1
NS
NS
NS
Input CFU
Control
CLD
FAS
CHM
Treatment

B

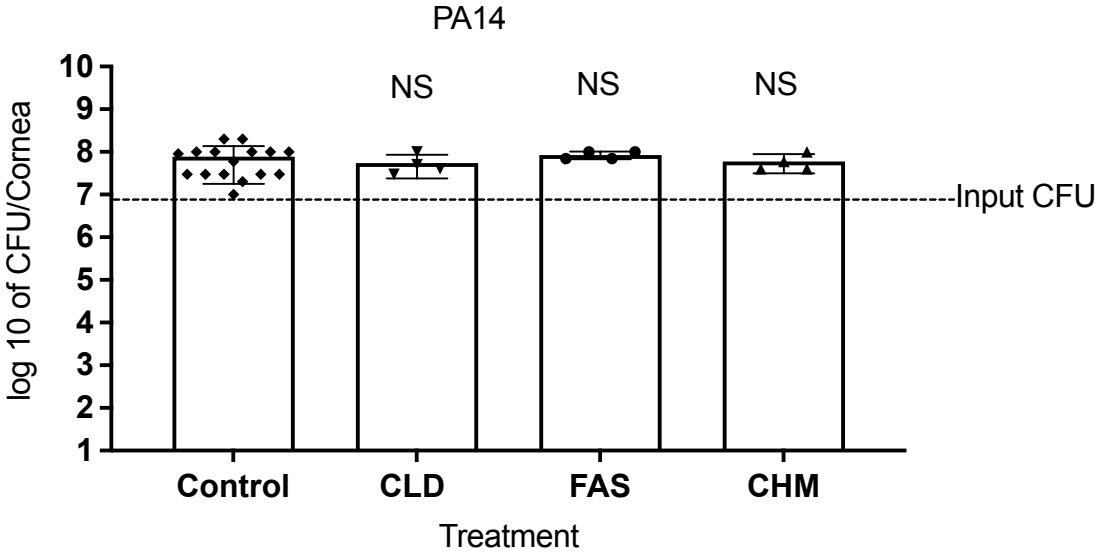

PA14
log 10 of CFU/Cornea
10
9
8
7
6
5
4
3
2
1
NS
NS
NS
Input CFU
Control
CLD
FAS
CHM
Treatment

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

1172 corneas infected with either cytotoxic strain PA14, or invasive strain PAO1 of *P. aeruginosa*
1173 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

1174 • - corneas treated with MIC concentration of an antibiotic