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

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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

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

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

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

MATERIALS AND METHODS

Bacterial strain used

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

MIC assay

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

forming units in the inoculum. Two hundred microliters of MHB containing an inoculum with $3x10^5$ CFU per well and different concentrations of the test antibiotics was added to each well in a 96-well plate. A concentration of antibiotics ranging from 0.006 to 32 µg/µL was tested. The MIC value was determined as the lowest concentration of an antibiotic which completely inhibits visible bacterial growth after 24 hours at 37 °C in static conditions. In total six antibiotics were tested: gentamicin, meropenem, ciprofloxacin, clindamycin, fusidic acid and chloramphenicol. Clindamycin, fusidic acid and chloramphenicol are normally not used to treat ocular infections caused by *P. aeruginosa* and were used here as a negative control. The optical density at 600 nm was measured using the TECAN Spark plate reader (TECAN, Switzerland) to confirm the growth inhibition. One column of each 96-well plate was designated for growth control and one for sterility control. The procedure was repeated three times across different days for each antibiotic.

MBEC assay

Biofilm susceptibility testing assay was performed using a Calgary device (Innovotech, Canada) where the biofilm was grown on a peg (Harrison et al., 2010). First, growth conditions were verified by an equivalence test for biofilm formation (Figure 1 in Supplementary Materials) as described by Harrison et al. (2010). The bacterial strains were streaked out on LB agar plate from cryogenic stock and incubated overnight at 37 °C. A single colony from the agar subculture was used to inoculate 5 mL MHB and the suspension was incubated in a 50 mL Falcon tube while shaking at 110 rpm for 24 hours at 37 °C (Infors HT Multitron, UK). The bacterial suspension was centrifuged at 4000 g in Eppendorf 5710R (Thermo Fisher, UK) for 5 minutes. After discarding the supernatant, the pellet was re-suspended in 5 mL of sterile MHB. The inoculum was prepared in a fresh centrifuge tube by diluting the suspension of bacteria to optical density (OD) of 0.05 at 600 nm. The OD_{600nm} was measured using spectrophotometer Jenway (VWR, UK). The inoculum was pipetted in a 96-well plate with a final concentration $8x10^6$ CFU of *P. aeruginosa* PAO1 or PA14 per well (150 µl inoculum in each well). One column in a 96-well plate was used as a control and contained media without bacteria added. Pegs from Calgary Device were immersed in the inoculum. The 96-well plate was double sealed with parafilm, placed inside a plastic box to reduce evaporation and incubated (statically) overnight at 37^0C with 70% humidity in the incubator (Infors HT Multitron, UK) to allow biofilm formation on pegs. Before each experiment 10 µl of 6-fold dilutions of the inoculum was spot plated on blood agar plates, and the plates were incubated overnight at 37 °C in order to enumerate colony forming units (CFU) in the inoculum. After

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

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

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

Testing antibiotics on *ex vivo* porcine cornea model

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

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

UK) and 10 ng mL^{-1} epidermal growth factor (EGF) (SLS, UK), 10 % fetal calf serum (FCS) (Pan-Biotech, UK), 100 U mL^{-1} penicillin, 100 U mL^{-1}streptomycin (SLS, UK) and 2.5 µg mL^{-1} amphotericin B (Sigma, UK). The corneas were incubated in medium with antimicrobials for 24 hours at 37 °C and then washed once with 2 mL PBS and incubated in antimicrobial-free medium for 48 hours to remove residual antibiotics. The medium was replaced every day during this time. On the infection day porcine corneas were infected with $8x10^6$ CFU in 200 µl of PBS and incubated for 6 hours. After the incubation, the PBS along with suspended bacteria were removed with a sterile 1 mL pipette tip and replaced either with 200 µL of PBS (control corneas) or with a PBS with added antibiotic (treated corneas). The corneas were treated with either 1024 µg mL^{-1} or with MIC concentration of ciprofloxacin, meropenem, gentamicin for 18 hours at 37 °C. All corneas were photographed with Dino-lite Xcope camera (AnMo Electronics Corporation, Taiwan). Ninety cornea images were independently scored for opacity by five people using following grading system: 0 – no haze, cornea clear; 1 – faint opacity or cloudiness visible; 2 – cornea looks swollen, white or hazy patch clearly visible. All graphs were plotted using GraphPad Prism version 8.4.1.

Statistics

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

Data availability

All supporting data are provided in the Supplementary Materials file.

RESULTS

MIC assay

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

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

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

MBEC assay

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

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

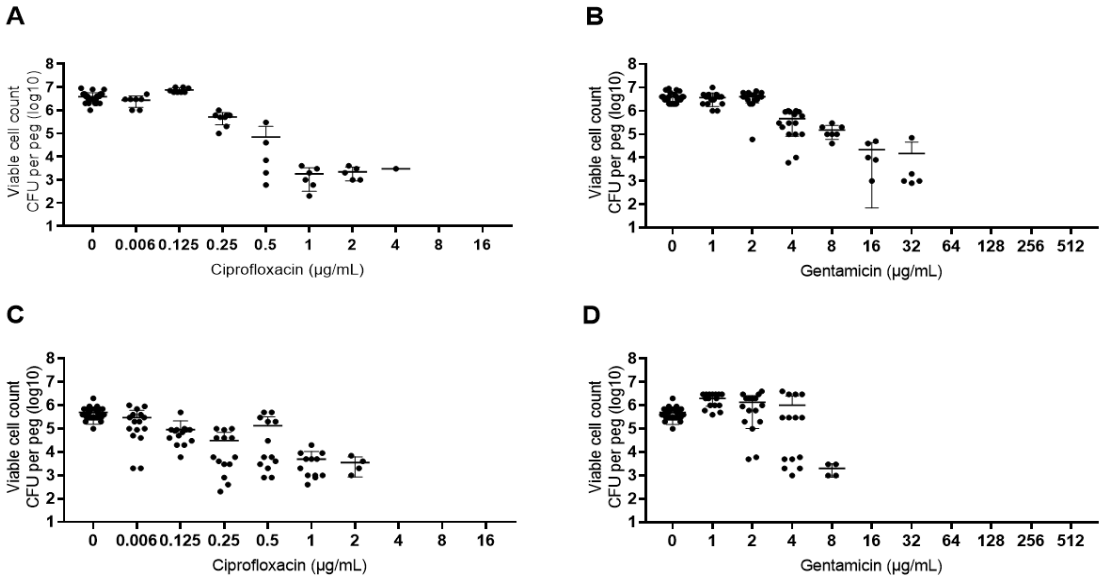


Fig.1. MBEC assay results representing colony forming units of *P. aeruginosa* PAO1 (A, B) and PA14 (C,D) treated with ciprofloxacin and gentamicin.

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 6×10^8 CFUs per cornea were retrieved after 24 hours and 2×10^9 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 1×10^6 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.

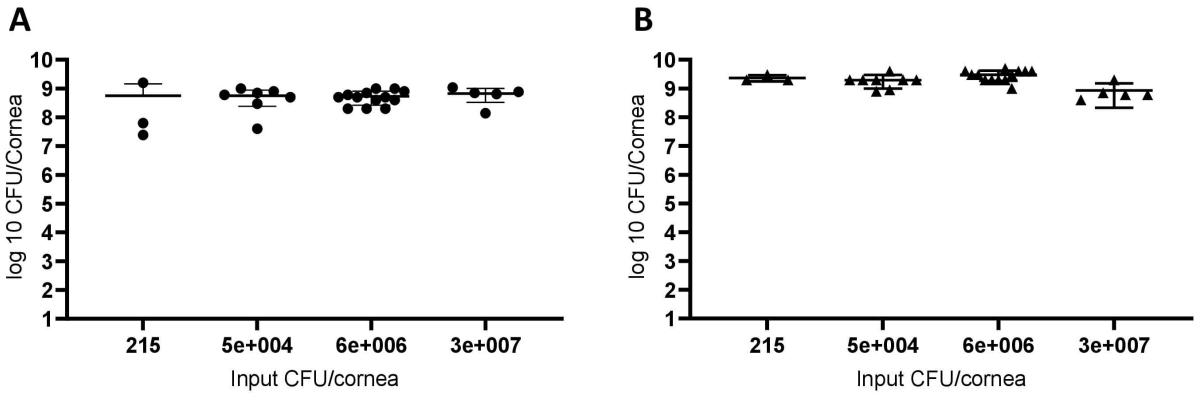


Fig 2. Number of viable *P. aeruginosa* PA14 retrieved from porcine cornea after infection with 215, $5x10^4$, $6x10^6$ and $3x10^7$ CFU per cornea. Corneas were infected for 24 hours (A) and 48 hours (B). Each dot represents results from one cornea. Error bars represent standard deviation. Statistical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

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

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

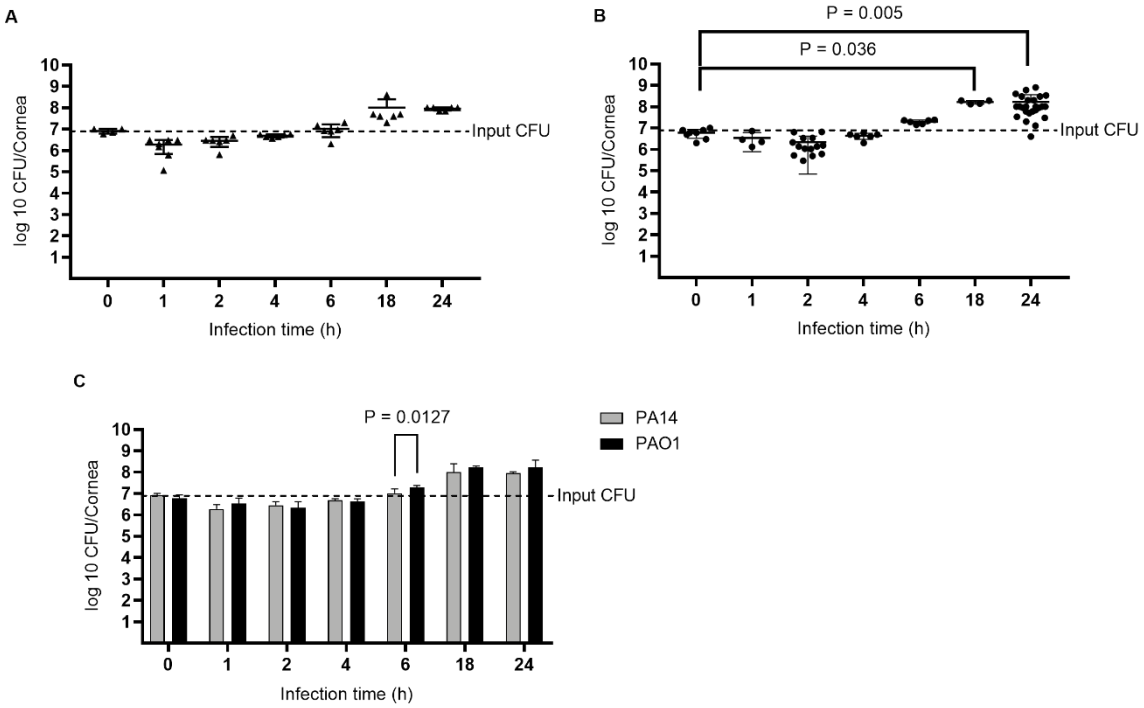
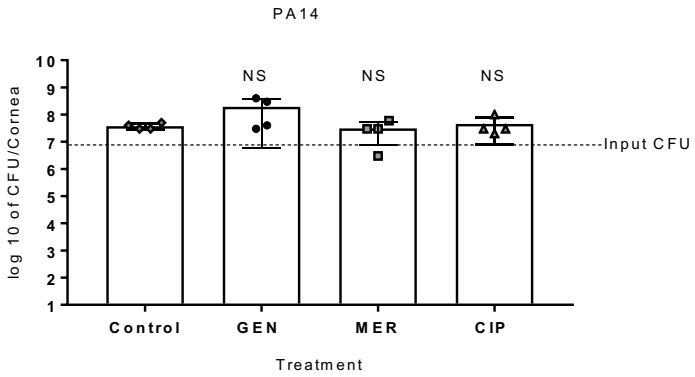


Fig. 3. Number of viable CFU of *P. aeruginosa* on porcine corneas infected for 1, 2, 4, 6, 18 and 24 hours with *P. aeruginosa* PA14 (A) and *P. aeruginosa* PA01 (B). Data from both strains are compared on one graph (C). Inoculum CFU are shown both as CFU at t=0 infection time (x-axis) as well as a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

Testing antibiotics on the *ex vivo* porcine keratitis model

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

A



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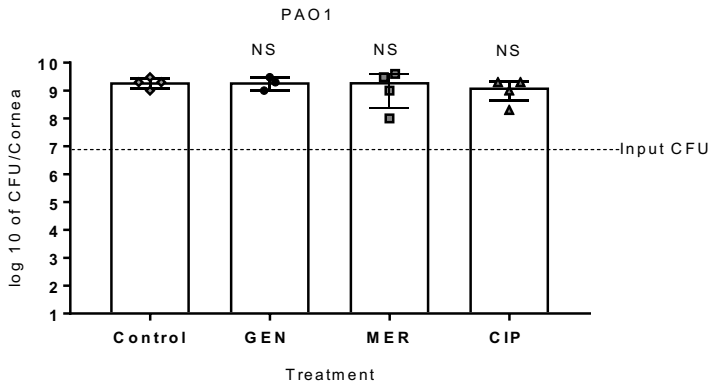


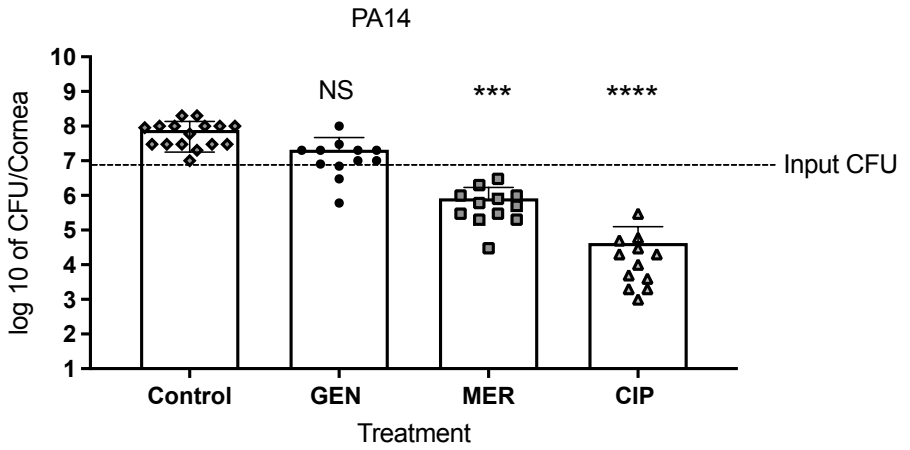
Fig. 4. Colony forming units of *P. aeruginosa* in the *ex vivo* porcine corneas infected for 6 hours with (A) PA14 or (B) PAO1. Control corneas were immersed in PBS while other corneas were treated with MIC concentrations of antibiotic dissolved in PBS. Following antibiotics were applied on infected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

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

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

Out of three tested antibiotics at concentration 1025 μg mL^{-1}, gentamicin was the least potent, possibly because the concentration of this antibiotic added to corneas in the relation to MIC was much smaller in comparison to meropenem and ciprofloxacin. On average $7x10^7$ CFU (1-log reduction) of strain PAO1 and $2x10^7$ CFU (<1-log reduction) of strain PA14 were recovered from porcine corneas. Meropenem reduced bacteria load approximately by 2-log for both strains ($5x10^6$ CFU per cornea for strain PAO1 and $9x10^5$ CFU per cornea for strain PA14) despite MBEC assay suggesting that biofilms of both strains are tolerant to >512 μg mL^{-1} of this antibiotic. Treatment with ciprofloxacin resulted in an average of $1x10^5$ CFU (5-log reduction) for corneas infected with strain PAO1 and $5x10^4$ CFU (4-log reduction) for corneas infected with strain PA14. Treatment with clindamycin, fusidic acid and chloramphenicol had no effect on bacteria count in comparison to control corneas with added PBS only, and this was expected since MIC results showed that these antibiotics are ineffective on investigated *P. aeruginosa* strains (supplementary materials Figure 2).

A



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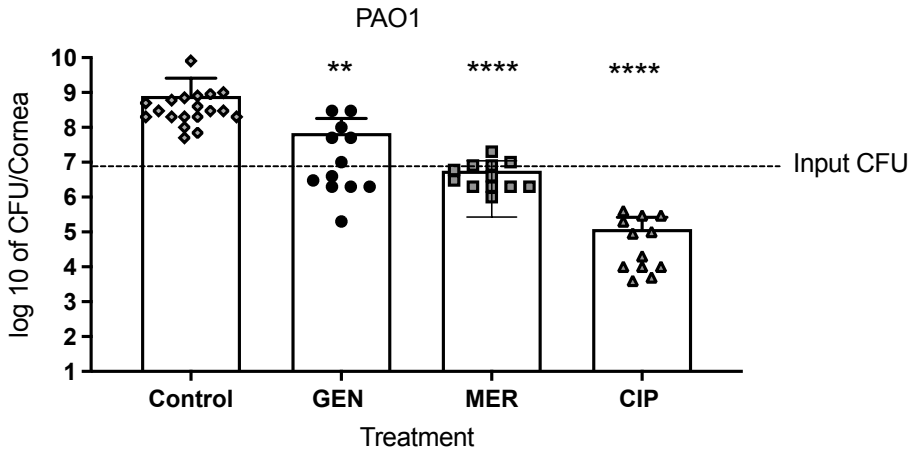
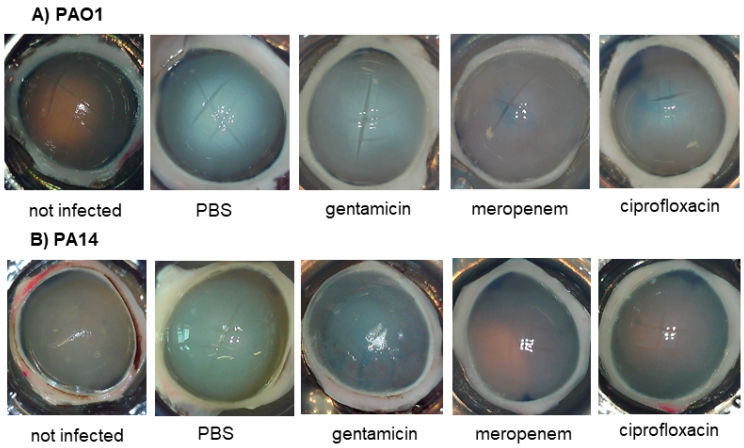


Fig. 5. Colony forming units of *P. aeruginosa* in the *ex vivo* porcine corneas infected for 6 hours with (A) PA14 or (B) PAO1. Control corneas (n = 19 & 16) were immersed in PBS while other corneas were treated with 1025 µg $mL^{-1}$of antibiotic dissolved in PBS. Following antibiotics were applied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

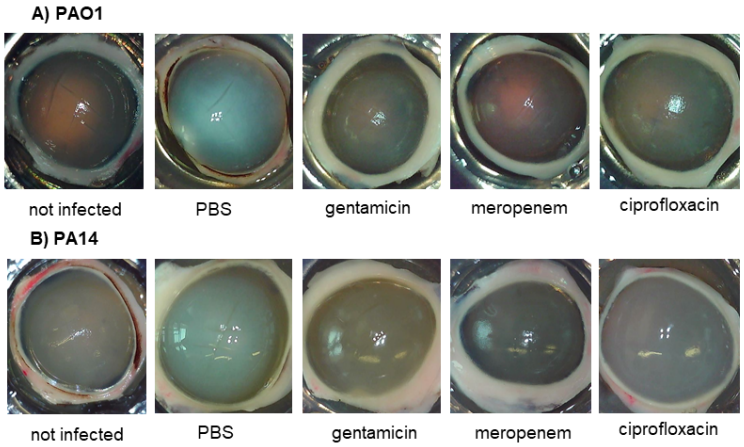
409 with PA14 (Fig. 6B). Corneas infected with both *P. aeruginosa* strains and treated with MIC
410 concentration of gentamicin looked slightly hazier in comparison to meropenem and
411 ciprofloxacin in spite of no reduction in viable colony count across all antibiotics (Fig. 4).



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,

clindamycin, and chloramphenicol. Corneas shown here were infected with $6x10^6$ CFU of strain PAO1 (A) and strain PA14 (B) 6-hour prior to antibiotic treatment. Corneas were imaged and immediately homogenised for viable counting.

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

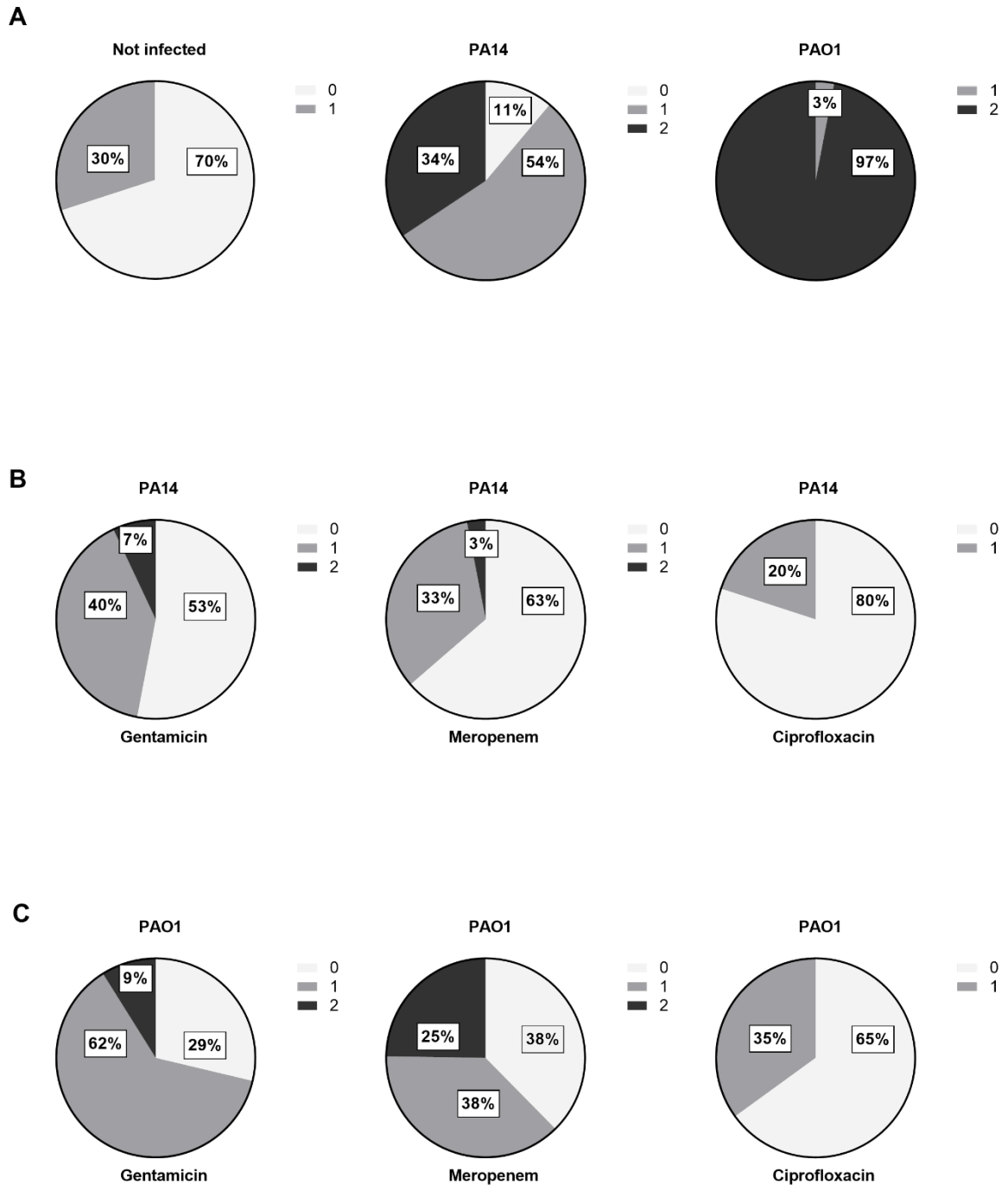
Majority of uninfected corneas (70%) were correctly identified while 30% were graded 1 (Fig. 8A). This is because the scoring of uninfected corneas could have been affected by swelling that naturally occurs during incubation in media for few days and could make some corneas appear less transparent than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

Some untreated corneas infected with strain PA14 looked clear (11%) whilst majority were correctly identified as infected (54% scored grade 1 and 34% scored grade 2) (Fig. 8A). Majority of untreated corneas (97%) were correctly identified as being infected with strain PAO1 due to the development of an obvious haziness (grade 2) suggesting infection with strain PAO1results in ulceration and severe tissue damage on *ex vivo* porcine cornea (Fig. 8A).

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

Opacity scoring demonstrated visual differences in opacity between infection with *P. aeruginosa* cytotoxic strain PA14 and infection with invasive strain PAO1. The PAO1 strain showed the most visually obvious reduction in opacity for both untreated as well as treated 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

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

DISCUSSION

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

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

Gentamicin was the first antibiotic that we tested for MIC against *P. aeruginosa* because it often is used in early stages of keratitis (Dart and Seal, 1988). We found that MIC results in our study, were in alignment with those found in literature and demonstrated that both strains of *P. aeruginosa* (PA14 and PAO1) were equally sensitive to gentamicin. Our MIC values were either identical (Bowler et al., 2012, Ceri et al., 1999) or similar to those reported by other research groups (1 μg mL^{-1} and 2 μg mL^{-1})(Andrews, 2001, Bahari et al., 2017, Pusic et al., 2018). While MIC values did not demonstrate any obvious differences between investigated two strains, MBEC results clearly had shown that PAO1 was more resistant to gentamicin in comparison to PA14. Similar to other studies, MBEC values showed that biofilms were resistant to gentamicin in *P. aeruginosa* (Bowler et al., 2012, Ceri et al., 1999, Billings et al., 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).

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

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

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

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

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

643

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

652

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

673

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

677 similar to other animal models *in vivo* and to findings in clinical studies on humans. Therefore,
678 our *ex vivo* porcine cornea model is a practical tool for rapidly and cost effectively screening
679 the efficacy of ocular drugs with good sensitivity and reliability. We contend that our *ex vivo*
680 model could be used to reduce and refine use of live animals in keratitis studies. The
681 observations from our *ex vivo* keratitis model could advance discovery of new ocular drugs,
682 facilitate their rapid translation to the market and serve as a guidance for clinical application in
683 the future.
684
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689
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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.

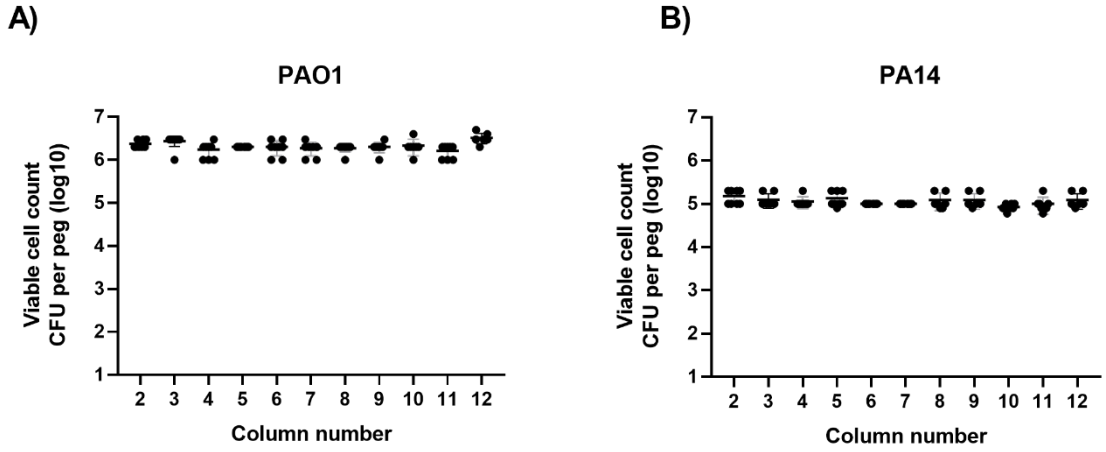
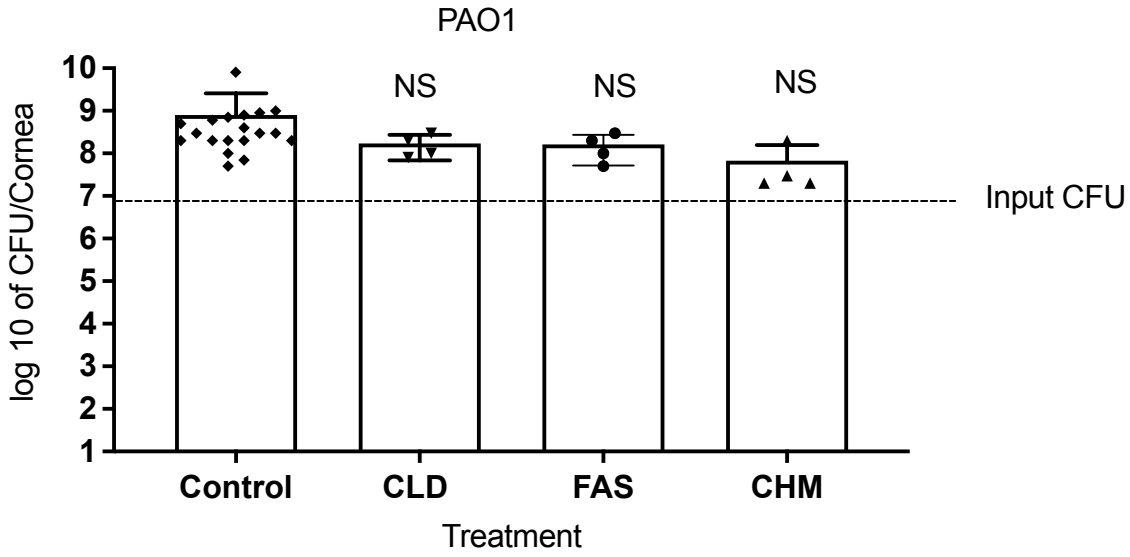


Figure 2. Colony forming units of *P. aeruginosa* in the *ex vivo* porcine corneas infected for 6 hours with (A) PAO1 or (B) PA14. Control corneas were immersed in PBS while other corneas were treated with 1025µg/mL of antibiotic dissolved in PBS. Following antibiotics were applied on infected corneas: 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



B

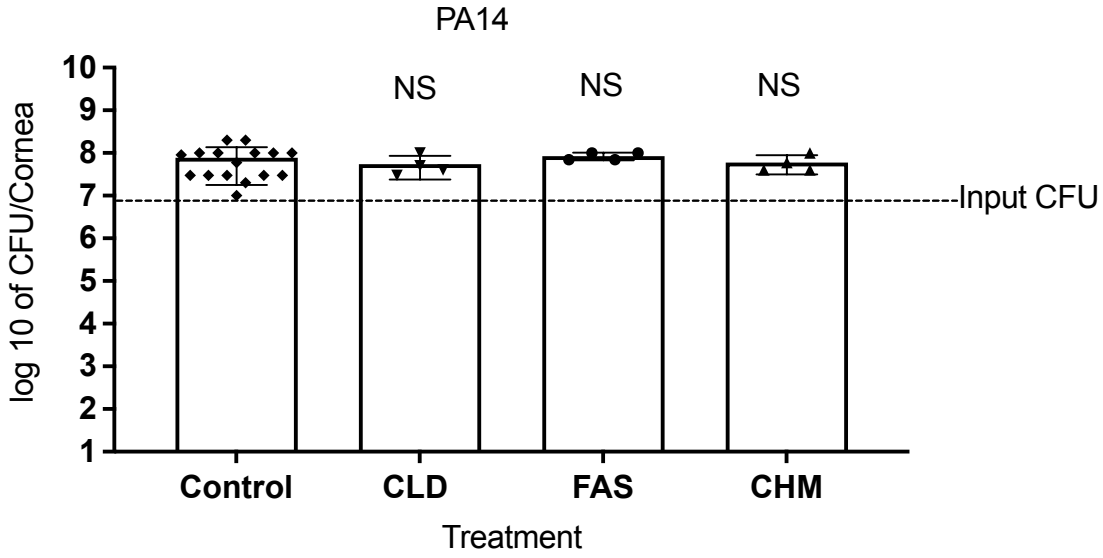


Table 2. Data summary of average colony forming units of *P. aeruginosa* in the ex vivo porcine corneas infected for 6 hours with PAO1 or PA14 and treated with MIC concentrations of gentamicin, ciprofloxacin and meropenem.

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

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

- - corneas treated with MIC concentration of an antibiotic