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3

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13

14 **Running Title**: Tobramycin and polymyxin-E against *P. aeruginosa*

15

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20 **ABSTRACT**

21 Indwelling medical devices have become a major source of nosocomial infections;

22 especially *Pseudomonas aeruginosa* (*P. aeruginosa*) infection, which remain the most

23 common cause of ventilator associated pneumonia (VAP) in neonates and children. Using

24 medical grade polyvinyl chloride endotracheal tubes (ETTs), the activity of tobramycin and

25 polymyxin-E was quantified in a simulated prevention and treatment static time kill model

26 using biofilm forming *P. aeruginosa.* The model simulated three clinical conditions: 1)

27 planktonic bacteria in the presence of antibiotics, tobramycin and polymyxin-E, without

28 ETTs, 2) planktonic bacteria grown in the presence of *P. aeruginosa,* antibiotic and ETTs

29 (simulating prevention) and 3) a 24h formed *P. aeruginosa* biofilm on ETTs prior to

30 antibiotic exposure (simulating treatment). In the model simulating "prevention"

31 (conditions 1 and 2 above), tobramycin alone or in combination with polymyxin-E was

32 more bactericidal than polymyxin-E alone at 24 hours using a concentration greater than 2

33 times the minimum inhibitory concentration (MIC). However, after a 24h old biofilm was

34 allowed to form on the ETTs, neither monotherapy nor combination therapy over 24 hours

35 exhibited bactericidal or bacteriostatic effects. Against the same pathogens, tobramycin

36 and polymyxin-E, both alone or in combination exhibited bactericidal activity prior to

37 biofilm attachment to the ETTs, however no activity was observed once biofilm formed on

38 ETTs. These findings support surveillance culturing to identify pathogens for a rapid and

39 targeted approach to therapy, especially when *P. aeruginosa* is a potential pathogen.

INTRODUCTION

Indwelling medical devices are a major source of nosocomial infections. In particular,

patients requiring mechanical ventilation (intubation with an endotracheal tube (ETT)) face

a high probability of contracting one of the most prevalent nosocomial infections,

ventilator associated pneumonia (VAP).(1-3) Neonatal and pediatric populations are at

especially high risk for VAP because the current standard of care involves prolonged

intubation without ETT exchange or tracheostomy, both common practice in adult patients.

In neonates and infants, the inner diameter of the ETT is often 2.5-3.5mm (the size of a thin

straw), which complicates suctioning of secretions and confounds attempts to maintain

patency. Despite aggressive bedside hygiene, *Pseudomonas aeruginosa* (*P. aeruginosa*)

remains one of the most common causes of VAP in intubated children.(2, 4, 5)

P. aeruginosa, often found on indwelling devices such as ETTs, forms a biofilm which

serves as an ideal environment for antibiotic resistance, making VAP difficult to treat.(6, 7)

Biofilm on ETTs is considered to be a reservoir for infecting pathogens derived from

oropharyngeal flora and gastric microaspiration, and is highly correlated with lower airway

infection and subsequent VAP.(8-11) To date, few side-by-side studies have compared

killing activity (defined as 99.9% kill) of tobramycin and polymyxin-E against *P.

aeruginosa,* especially in the context of ETT biofilm and VAP.(12-15) The effect of

monotherapy and/or combination therapy (synergistic versus antagonistic activity) must be

assessed when evaluating antimicrobial drug therapy, especially in the presence of medical

grade polyvinyl chloride (PVC) or conventional ETTs. For convenience, most studies

investigating antibiotic susceptibility in formed biofilms have used PVC coupons rather

63   than clinically available medical devices.(16-18) However, most of the coupons made of

64   PVC are not medical grade and, in many cases, do not contain equivalent plasticizer

65   content. These differences result in different texture and flexibility between medical grade

66   PVC products and PVC coupons used in biofilm experiments. Using clinically available

67   ETTs, this study aimed to both assess the efficacy of antibiotics against planktonic vs.

68   biofilm formed *P. aeruginosa*, and to identify which antibiotic, alone or combination,

69   demonstrates the best in vitro activity against *P. aeruginosa* in the context of VAP.

MATERIALS AND METHODS

Bacterial Isolates. American Type Culture Collection (ATCC, Manassas, VA, USA) strain 25668 was obtained. Reference strain PAO1 was obtained from Dr. Thomas Murray, Frank H. Netter MD School of Medicine, Quinnipiac University, North Haven, CT.[19, 20] Prior to use, all bacteria were stored in tryptic soy broth (TSB; Difco laboratories, Sparks, MD) with 15% glycerol and frozen at -80 ℃. Both strains are prolific biofilm producers.[21, 22]

Antimicrobial Agents. Commercially available, chemical grade polymyxin-E (lot# 081M1525V) powder and chemical grade tobramycin (lot# 090M1196V) powder was purchased from Sigma Aldrich (St. Louis, MO). Tobramycin and polymyxin-E powder were stored at $4^{o}C$. Both tobramycin and polymyxin-E were diluted in sterile water and a fresh stock was made each day, and prior to every experiment. Tobramycin and polymyxin-E were tested at one, two, four and eight times their respective minimal inhibitory concentration (MIC) at 0, 4 and 24 hours after inoculation.[23] Cation Adjusted Mueller-Hinton broth (CA-MHB, Difco Laboratories, Sparks, MD) supplemented with 25 mg/L calcium, 12.5 mg/L magnesium and 0.25% dextrose (Fisher Scientific, Pittsburgh, PA, USA) was used to obtain a suspension corresponding to 0.7 - 0.8 McFarland standards to produce an initial starting inocula of 5.5-6.0 x 10^6 colony forming units per milliliter (CFU/mL). Colony counts were determined using tryptic soy agar (TSA, Difco, Becton Dickinson Co., Sparks, MD) plates.

92   **Susceptibility Testing.** MIC tests were performed in triplicate using broth microdilution in

93   accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines.(24, 25)

94   The MIC was defined as the minimum concentration of antibiotic that will inhibit the

95   visual growth of the isolated organism. Minimum bactericidal concentrations (MBC) were

96   also determined in triplicate for each antimicrobial agent using CSLI guidelines.(25)

97   Bacteria were quantified using CFU/mL, and 5 microliter aliquots were used for

98   determination of MBC after 24 hours incubation at $37^{o}C$ using TSA.(26)

99

100   **Endotracheal Tubes (ETTs).** Commercially available Sheridan® 6.0 mm ID, uncuffed

101   ETTs (Hudson RIC, Temecula, CA, USA) were obtained. Each ETT was cut into 0.6 cm

102   by 0.3 cm rectangular pieces (ETT chips) using a ¼ rectangle hand puncher (Fiskars

103   Corporation, Helsinki, Finland), and sterilized with ethylene oxide gas prior to use in pre-

104   formed and formed biofilm time kills experiments.(22) For comparison, we also tested

105   commercially available PVC coupons (part Number RD 128-PVC, Biosurface

106   Technologies, Corp, Bozeman, MT) for pre-formed biofilm *P. aeruginosa* PAO1.(16-18)

107

108   **Biofilm Formation.** Sterile ETT chips were placed in each well of a 24-well plate (BD

109   Biosciences, San Jose, CA). The ETT chip was submerged with 2 mL of a final bacteria

110   inoculum, either PAO1 or ATCC 25668, obtained as described above using TSB

111   supplemented with 1% dextrose, 2% NaCl and 25 mg/L calcium (STSB) using modified

112   Growing and Analyzing Static Biofilms.(27) The well plate was incubated at $37^{o}C$ under

113   static conditions for 24 hours to promote biofilm formation on ETT chips. After 24 hours,

114   each ETT chip was gently rinsed three times in sterile phosphate buffered saline (PBS)

115   (Fisher Scientific, Pittsburgh, PA).

116

117   **Time Kill Study.** Using a 24h time kill study, three clinical conditions were modeled using

118   *P. aeruginosa* strains PAO1 and 25668: 1) planktonic bacteria in the presence of the

119   antibiotics tobramycin and polymyxin-E, without ETTs, 2) planktonic bacteria grown in

120   the presence of *P. aeruginosa,* antibiotics and ETTs (simulating prevention) and 3) a 24h

121   formed *P. aeruginosa* biofilm on ETTs prior to antibiotic exposure (simulating treatment).

122   Each time kill experiment was carried out in a minimum of triplicate. All antimicrobial

123   agents were tested at one, two, four and eight times their respective MIC with starting

124   inocula of 5.5–6.0 x 10^6 CFU/mL adjusted to McFarland standards using the Vitek

125   colorimeter (bioMérieux, Inc, Durham, NC).(18, 28)

126

127   Sample aliquots (0.1 mL) were removed from cultures at 0, 4, and 24 hours after vortexing

128   each tube for one minute to remove biofilm growth from the ETT chip.(22) Antimicrobial

129   carryover was accounted for by serial dilution (10 – 10, 000 fold) of plated samples with

130   normal saline or vacuum filtration. This methodology has a lower limit of detection of 2.0

131   $\log_{10}$ CFU/mL.(28) Growth control tubes for each organism were prepared without

132   antibiotic and run in parallel to the antibiotic test tubes.

133

134   For single antimicrobial agents, bactericidal activity (99.9% kill) was defined as a ≥ 3

135   $\log_{10}$CFU/mL reduction at 24h in colony count from the initial inoculum. Bacteriostatic

136   activity was defined as a $< 3 \log_{10}$ CFU/mL reduction at 24h in colony count from the

137   initial inoculum, while inactive was defined as no observed reduction from the initial

138   inoculum.(23) For antibiotics evaluated in combination, synergy was defined as $\geq 2 \log_{10}$

139   CFU/mL decrease, indifference was defined as a 1 to 2 $\log_{10}$ CFU/mL change (increase or

140   decrease), and antagonism was defined as >2 $\log_{10}$ CFU/mL increase in growth compared

141   to the most active single agent.

142

143   **Data analysis**. All statistical analyses were performed using SPSS statistical software

144   (IBM SPSS statistics version 20, IBM Corporation, Armonk, NY USA). After 24 h of

145   exposure to antimicrobial agent(s), the biofilm formation was quantified, bacteria at 4 hour

146   and 24 hour were counted (with a lower limit of detection 2.0 $\log_{10}$ CFU/mL) to compare

147   between antimicrobial groups, concentrations and strains using analysis of variance

148   (ANOVA) followed by Tukey's *post-hoc* analysis. Multiple regressions for the association

149   between substrates and CFU/mL were analyzed. A *p* value of ≤ 0.05 indicated statistical

150   significance.

151 **RESULTS**

152 The MIC for tobramyxin was 0.5 μg/mL and for polymyxin-E was 2 μg/mL for both PAO1 and

153 25668 strains. The MBC for tobramycin was 4 and 32 μg/mL and for polymyxin-E was 16 and

154 64 μg/mL respectively for Pseudomonas PAO1 and ATCC 25668 strains.

155

156 In the planktonic time kill study, tobramycin demonstrated bactericidal activity against both

157 Pseudomonas isolates at 24 hours with average decrease of 3.81 ± 0.16 $\log_{10}$ CFU/mL for all

158 concentrations except 1 time the MIC for PAO1 (Fig. 1.a & b). Polymyxin-E demonstrated

159 bacteriostatic activity at 2 and 4 times the MIC (average decrease of 2.16-2.63 $\log_{10}$CFU/mL),

160 and bactericidal activity at 8 times the MIC (average decrease of 3.07-3.56 $\log_{10}$CFU/mL), but

161 inactive at 1 time the MIC for both isolates at 24 hours (Fig. 1.c & d). The combination therapy

162 at 2, 4, and 8 times the MIC demonstrated indifference with >3.44 $\log_{10}$CFU/mL kill for PAO1,

163 and >3.46 $\log_{10}$CFU/mL kill for 25668 at 24 hours (Fig 1. e & f).

164

165 In the pre-formed biofilm time kill studies (simulating prevention) at 24 hours, tobramycin

166 demonstrated bactericidal activity against both Pseudomonas isolates (average decrease of > 3.3

167 $\log_{10}$CFU/mL), except 1 time the MIC for 25668 which showed inactivity (1.02 ± 1.86

168 $\log_{10}$CFU/mL increase; Fig 2.a & b). Similarly, polymyxin-E demonstrated bactericidal activity

169 (average decrease of >3.08 $\log_{10}$CFU/mL) at greater than 2 times the MIC, but bacteriostatic

170 activity at 1 time the MIC for both isolates at 24 hours (Fig. 2.c & d). Tobramycin and

171 polymyxin-E combination demonstrated indifferent activity at all concentrations for both isolates

172 (Fig. 2.e & f).

173

9

174   In formed biofilm time kill studies (simulating treatment) for PAO1, combination therapy at 4

175   times the MIC was significantly more active at 4 hours compared to polymyxin-E alone at 4

176   times (mean difference (MD)= -1.34, 95% confidence interval [CI], -2.4-0.3 log $_{10}$ CFU/mL, $p=$

177   0.004) and 8 times (MD= -1.45, 95% CI, -2.5-0.4 log $_{10}$ CFU/mL, p=0.001) the MIC. Similarly,

178   combination therapy at 8 times the MIC was significantly more active at 4 hours compared to

179   polymyxin-E alone at 8 times the MIC (MD= -1.23, 95% CI, -2.3-0.2 log $_{10}$ CFU/mL, p=0.001).

180   However, indifferent activity was observed at 24 hours. Similarly, for 25668, combination

181   therapy at 8 times the MIC was significantly more active compared to polymyxin-E alone at 4

182   hours (MD= -1.06, 95% CI, -1.7-0.4 log $_{10}$ CFU/mL, $p<$ 0.001). However, indifferent activity

183   was observed at 24 hours. Once biofilm is formed, both single agent and combination antibiotics

184   resulted in inactivity or indifference (Fig. 3. a - f).

185

186   In addition to medical grade PVC ETTs, we assayed time kill using commercially available PVC

187   coupons(16-18). A similar trend of bactericidal activity was demonstrated at 24 hours with

188   greater than 4 times the MIC of tobramycin (average decrease of >3.03 log$_{10}$ CFU/mL) and with

189   greater than 2 times the MIC of polymyxin-E (average decrease of >3.1 log$_{10}$ CFU/mL), but

190   indifference was noted when the combination of tobramycin and polymyxin-E was evaluated at

191   2, 4, and 8 times the MIC (average decrease of > 3.21 log$_{10}$CFU/mL). ANOVA showed that

192   there was a significant difference between substrates and CFU/mL at 4 hours (MD= 0.08, 95%

193   CI, 2.5-3.7 log $_{10}$ CFU/mL, p= 0.041) (Table 1). Multiple regression analysis demonstrated that

194   there was a significant association between CFU/mL with substrate at 4 hours (partial eta

195   squared [eta] =0.493, $p<$ 0.001) and at 24 hours (eta=0.208, p<0.001). The overall model fit was

196   R^2 = 0.954.

197

DISCUSSION

Ventilator associated pneumonia, a common nosocomial infection often caused by bacteria that produce biofilm, results in increased morbidity, medical costs and multi-drug resistant organisms.(2, 3, 29-32) In one study, adult patients with VAP were hospitalized longer (38 vs. 13 days, $p<0.01$), mortality rates were higher (50% vs. 34%, $p<0.01$), and hospital costs were greater ($70,568 vs. $21,620, $p< 0.01$) compared to uninfected ventilated patients, with estimated VAP attributable costs of $11,897.(32) However, limited diagnostic criteria and modification of ETTs make VAP prevention particularly challenging and difficult especially for neonates and children.(2)

In children, re-intubation and tracheostomy insertion create the additional risk of damaging their small and fragile airway; therefore, re-intubation or tracheostomy after a standard duration of intubation is not routinely practiced. Thus, the longer the ETTs remain in patients due to prolonged mechanical ventilation, the more likely biofilms are to develop and adhere.(33-35) This bacterial accumulation of biofilms on ETTs may become dislodged during simple routine care such as suctioning or due to ventilation air flow. Bacteria and biofilm that break off become planktonic and seed further in the airway, causing more complicated pneumonia.(8, 36)

One controversial approach to treatment of VAP is "selective decontamination of the digestive tract" with broad spectrum intravenous (IV) antimicrobials.(37, 38) However, IV prophylaxis is not widely accepted due to fear of creating antibiotic resistant strains among VAP pathogens. In the pediatric population, one of the most common VAP pathogen is *P. aeruginosa*, accounting for 17-25% of VAP.(2, 4, 5) Our model is most consistent with the practice of direct instillation of liquid antimicrobial agents through the ETT as prophylaxis against, or treatment of VAP caused by *P. aeruginosa* compared to inhalation of nebulized antibiotics.(39) Instillation

11

222   treatments pose less risk of systemic toxicity than IV administration because antimicrobial agents

223   can be delivered locally using ETTs or tracheostomy tubes in children and neonates. Moreover,

224   instillation can deliver drug directly to the site of pneumonia whereas nebulized drug may adsorb

225   on the ETT, permeate into the ETT wall, or remain in the proximal airway. Therefore, our study

226   model using ETT chips is useful to help understand the effects of tobramycin and polymyxin-E,

227   alone or in combination, to treat VAP cause by *P. aeruginosa*.

228

229   In our study, we examined *P. aeruginosa* growth with or without the presence of medical grade

230   polyvinyl chloride (PVC) ETT to evaluate the bactericidal effects of two antibiotics in the

231   condition of VAP. We found that in an in vitro condition, the bactericidal effect of tobramycin

232   or polymyxin-E monotherapy required greater than 2 times the MIC at 24 hours for the pre-

233   biofilm condition (prevention). However, antibiotics demonstrated different activity against the

234   two different strains. For PAO1, tobramycin monotherapy was equally active for killing

235   compared to the combination approach.   For 25668, the combination therapy was more active

236   for killing compared to monotherapy at 24 hours (Fig 2.a-f); this finding may be related to the

237   biofilm-forming abilities of each bacterium.

238

239   Our study also demonstrated that two of the antibiotics tested in either monotherapy or in

240   combination showed inactivity or indifference once biofilm was formed on ETTs against both

241   Pseudomonas strains (Fig 3.a - f). This is in contrast to the conclusions drawn by Herrmann et

242   al. using a 96-peg Calgary biofilm device in vitro showing combination therapy with colistin-

243   tobramycin combination was superior to monotherapy against *Pseudomonas* biofilm.(40)

244

245   Many in vitro studies have used commercially available PVC coupons, which have different

246   texture and flexibility (based on the plasticizer content compared to medical grade PVC ETTs).

247   We hypothesized that bacterial colonies would form differently on commercially available PVC

248   coupons compared to medical grade PVC ETTs. To capture *Pseudomonas* growth in relation to

249   different material surfaces more accurately, we studied the same antibiotic therapy against

250   *Pseudomonas* PAO1 using both PVC coupon and PVC ETTs. There was the significant

251   association among CFU/mL and substrate at 4 and 24 hours (Table 1), thus it showed the

252   importance of utilizing same device material to mimic VAP condition to evaluate antibiotic

253   activity on biofilm.

254

255   In conclusion, neither single nor combination therapy with tobramycin and/or polymyxin-E

256   demonstrated killing activity once *Pseudomonas* biofilm was already formed on ETTs, however,

257   no antagonism was noted. Bactericidal effects against pre-formed biofilm (simulating

258   prevention) in the presence of ETTs suggest that surveillance cultures could identify pathogens

259   prior to biofilm formation, and allow prophylactic or targeted approaches to therapy, especially

260   when *Pseudomonas* is a potential pathogen. In addition, this study demonstrated the importance

261   of material choice in vitro time kill study. Further investigation could incorporate wild type

262   strains as well as clinically feasible treatment options for VAP in children.

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385

386

FIGURES

Fig. 1. a

Fig. 1. b

Fig. 1. c

Fig. 1. d

Fig. 1. e

Fig. 1. f
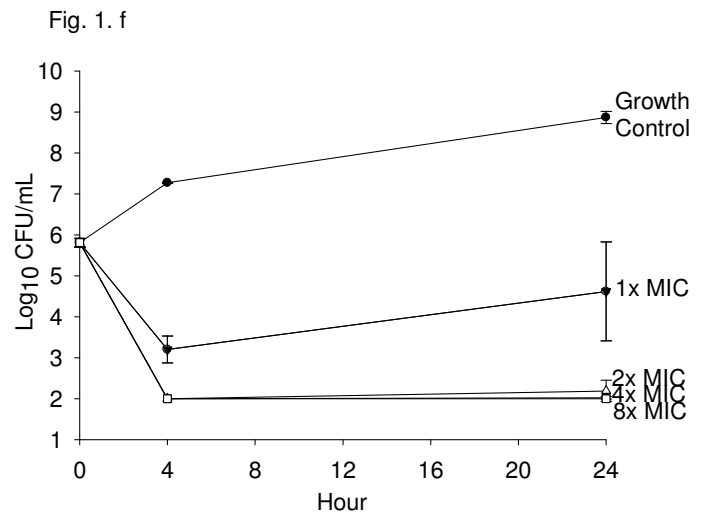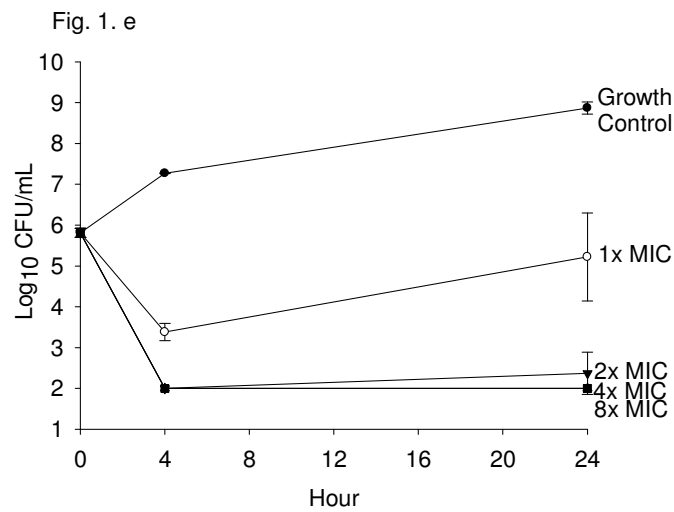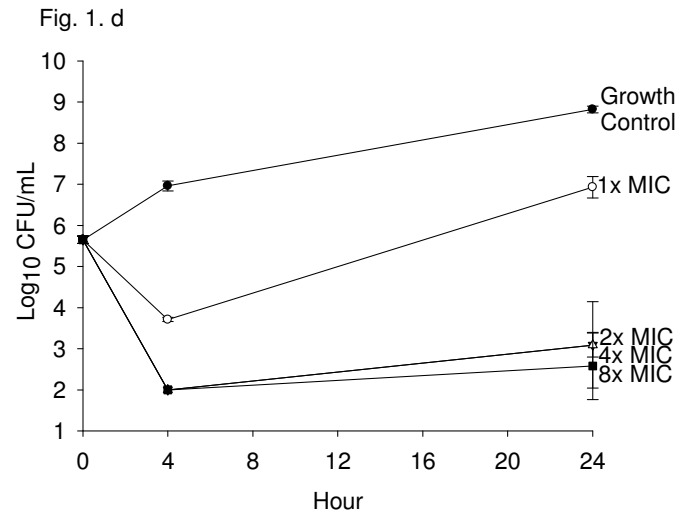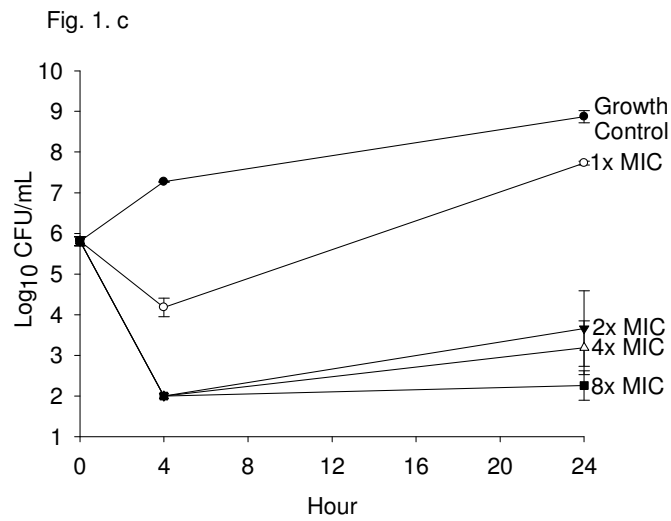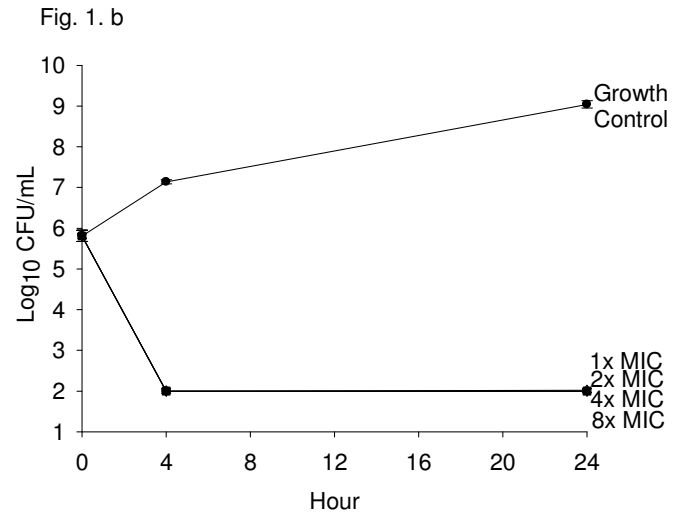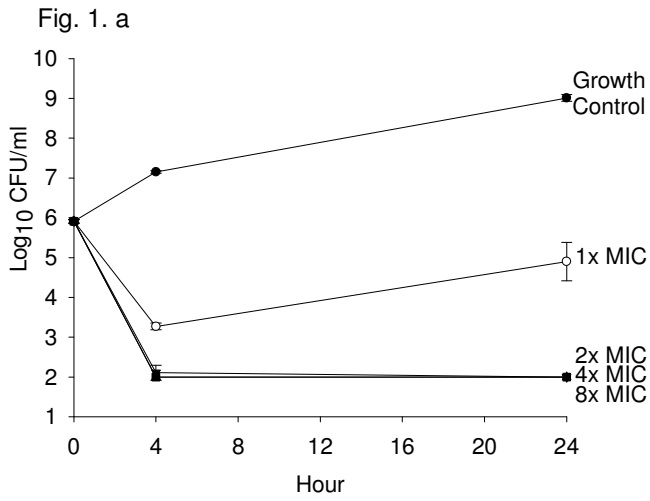
389　**Figure 1.a-f.** Time Kill against planktonic *P. aeruginosa.* Tobramycin against planktonic *P.*

390　*aeruginosa* PAO1 (Fig 1.a), 25668 (Fig 1.b), polymyxin-E against planktonic *P. aeruginosa*

391　PAO1 (Fig 1.c), 25668 (Fig 1.d), combination of tobramycin and polymyxin-E against

392　planktonic *P. aeruginosa* PAO1 (Fig 1.e) and 25668 (Fig 1.f). Results are presented as mean ±

393　standard deviation.

Fig. 2. a
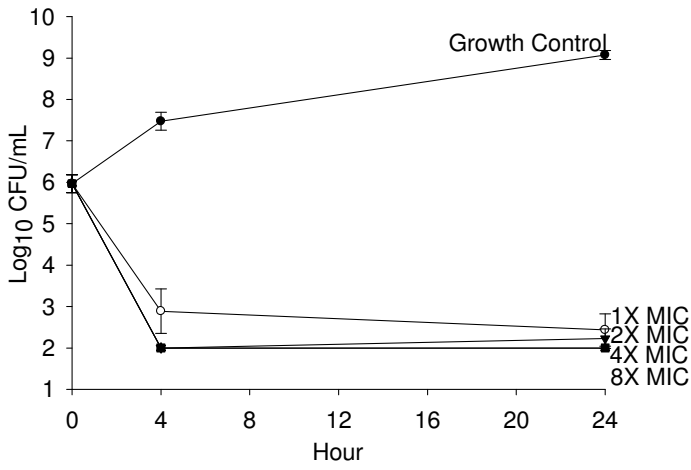
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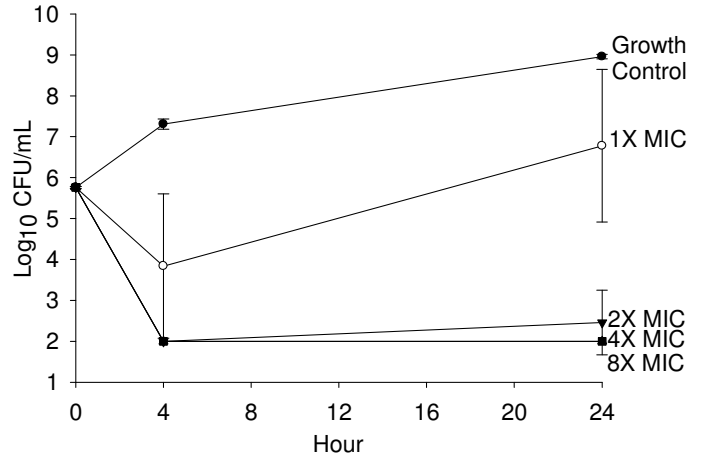
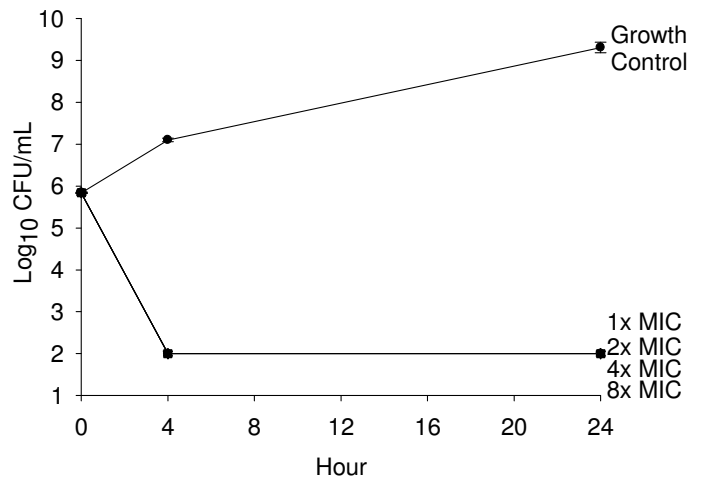Fig. 2. c
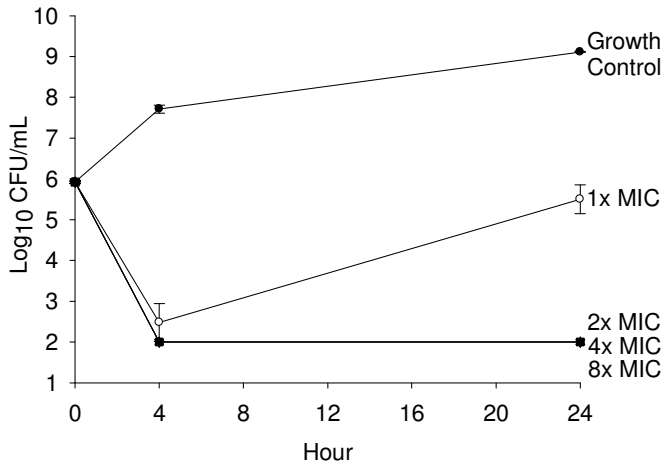
Fig. 2. d
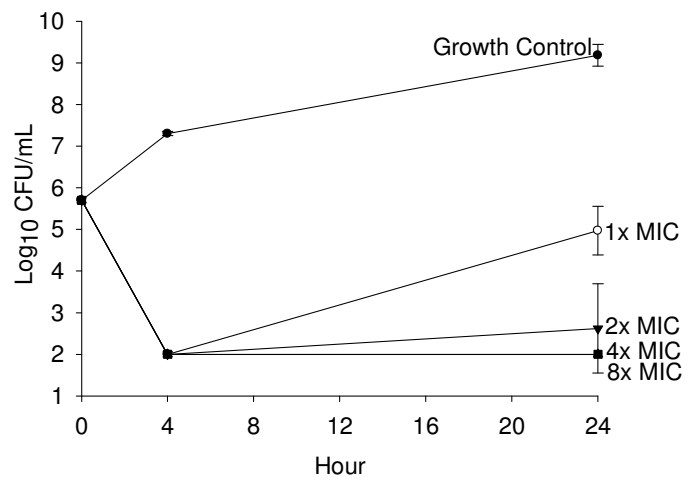
Fig. 2. e
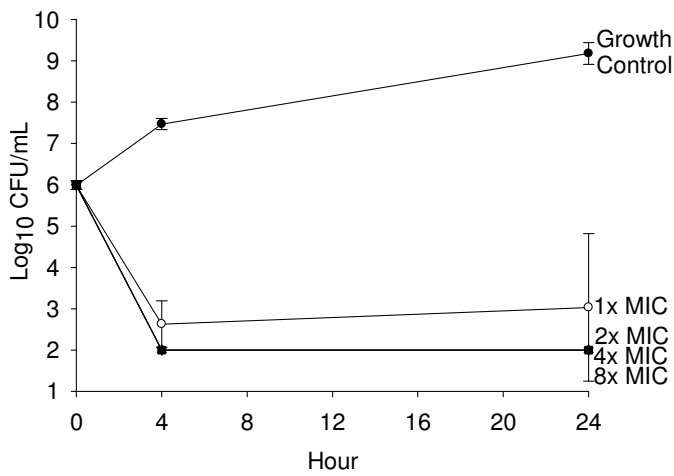
Fig. 2. f

395   **Figure 2.a-f**. Time Kill against pre-biofilm formed *P. aeruginosa.* Tobramycin against *P.*

396   *aeruginosa* PAO1 (Fig 2.a), 25668 (Fig 2.b), polymyxin-E against *P. aeruginosa* PAO1 (Fig

397   2.c), 25668 (Fig 2.d), combination of tobramycin and polymyxin-E against *P. aeruginosa* PAO1

398   (Fig 2.e) and 25668 (Fig 2.f) with presence of ETT chips. Results are presented as mean ±
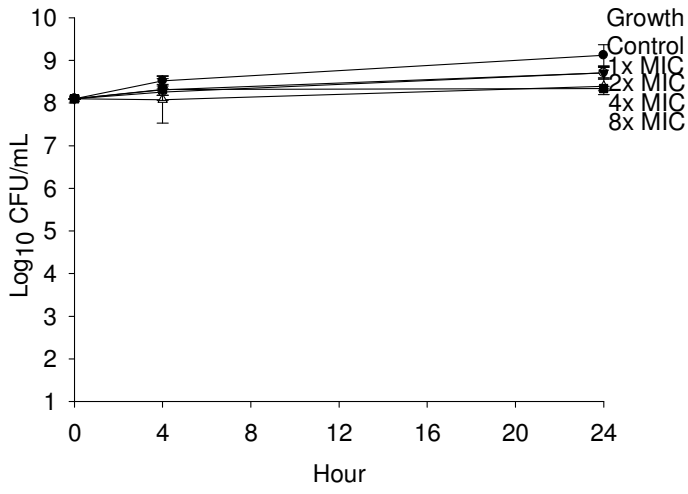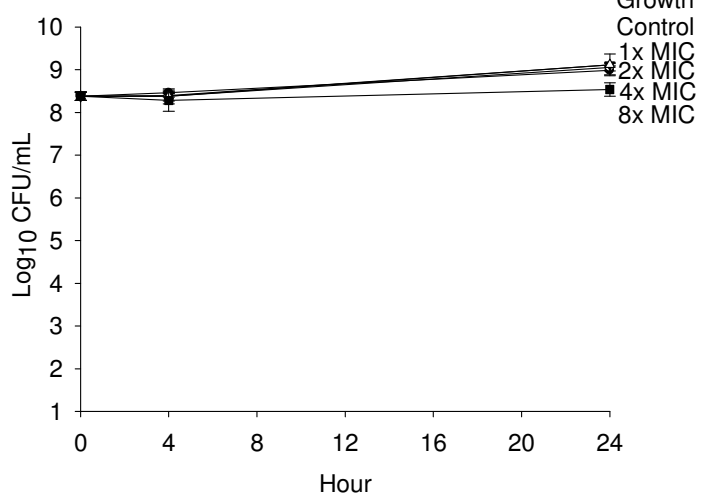
399   standard deviation.

Fig. 3. a

Log$_{10}$ CFU/mL
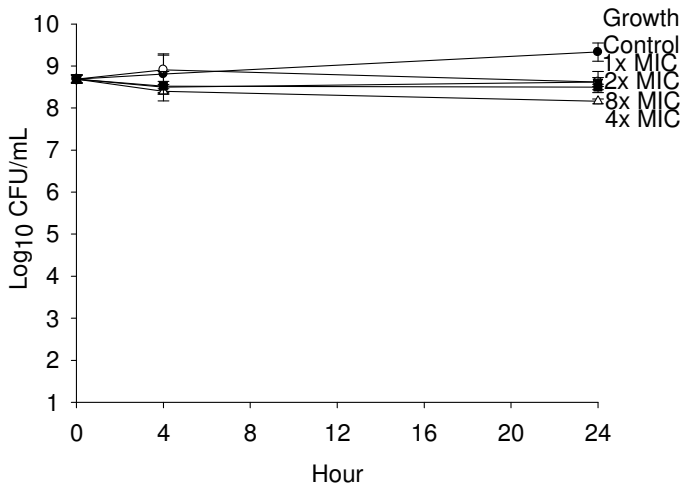
Hour

Growth
Control
1x MIC
2x MIC
4x MIC
8x MIC

Fig. 3. b

Log$_{10}$ CFU/mL

Hour

Growth
Control
1x MIC
2x MIC
4x MIC
8x MIC

Fig. 3. c

Log$_{10}$ CFU/mL

Hour

Growth
Control
1x MIC
2x MIC
8x MIC
4x MIC

Fig. 3. d

Log$_{10}$ CFU/mL

Hour

Growth
Control
2x MIC
1x MIC
8x MIC
4x MIC

Fig. 3. e

Log$_{10}$ CFU/mL

Hour

Growth
Control
1x MIC
2x MIC
4x MIC
8x MIC

Fig. 3. f

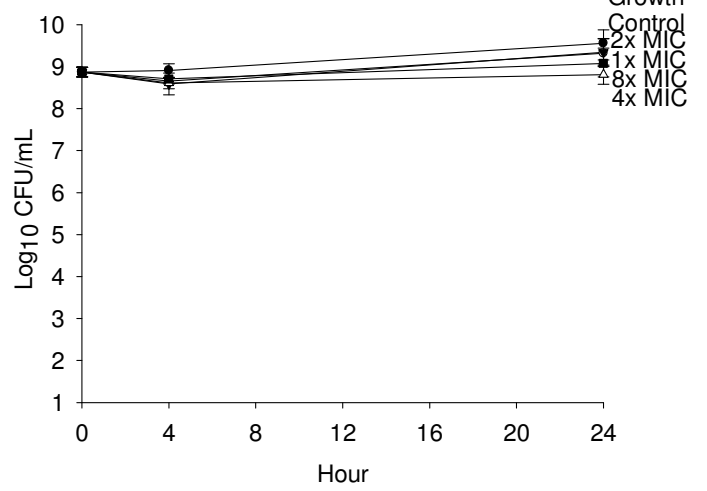Log$_{10}$ CFU/mL
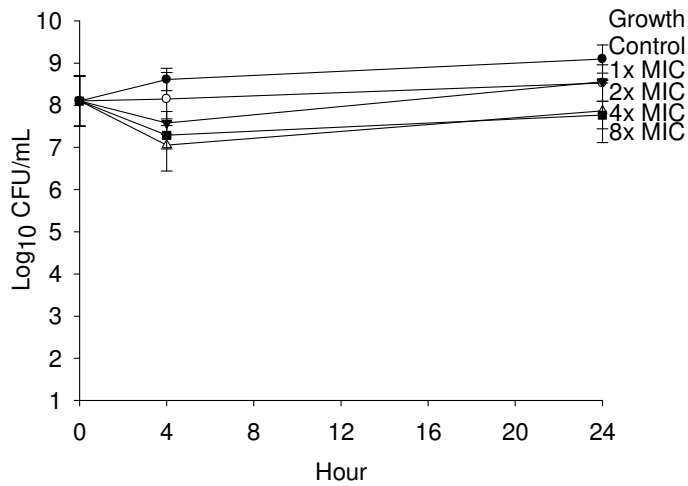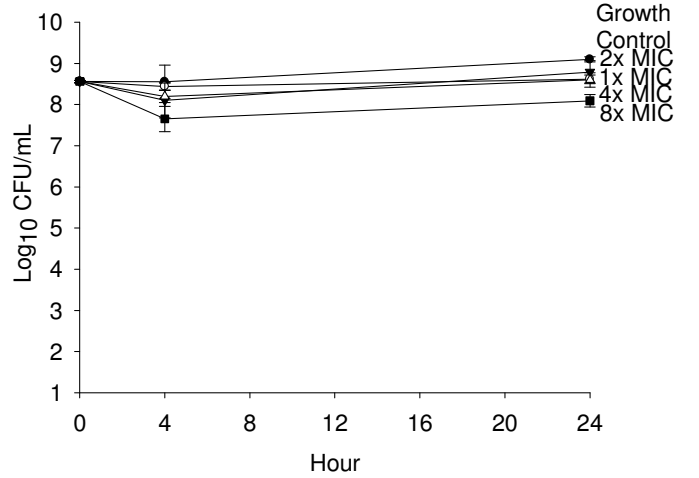
Hour

Growth
Control
2x MIC
1x MIC
4x MIC
8x MIC

400

24

401 **Figure 3. a-f.** Time Kill against biofilm formed *P. aeruginosa.* Tobramycin against *P.*

402 *aeruginosa* PAO1 (Fig 3.a), 25668 (Fig 3.b), polymyxin-E against *P. aeruginosa* PAO1 (Fig

403 3.c), 25668 (Fig 3.d), combination of tobramycin and polymyxin-E against *P. aeruginosa* PAO1

404 (Fig 3.e) and 25668 (Fig 3.f). Results are presented as mean ± standard deviation.

405 **TABLE**

406 **Table 1**: Comparison of $\log_{10}$ colony forming unit/mL (CFU/mL) change at 4 hours from 0 hr

407 growth control between endotracheal tube (ETT) and polyvinyl chloride (PVC) coupons.

	Tobramycin		Polymyxin-E		Tobramycin + Polymyxin-E	
	ETT	PVC coupon	ETT	PVC coupon	ETT	PVC coupon
1 x MIC	-2.81± 0.04	-2.82±0.12	-3.44±0.43	-1.77±0.04	-3.36± 0.46	-4.09±0.10
2 x MIC	-3.97 ±0.21	-0.71±0.01	-3.92± 0.03	-2.86±0.10	-3.99±0.10	-4.24±0.03
4 x MIC	-3.97 ±0.21	-1.23±0.03	-3.92±0.03	-3.16±1.2	-3.99±0.10	-4.24±0.03
8 x MIC	-3.97 ±0.21	-1.06±0.03	-3.92±0.03	-3.49±1.28	-3.99±0.10	-4.24±0.03

408 Average change ± standard deviation.