

2013

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Tarquinio, Keiko et al. "Activity of tobramycin and polymyxin-E against *Pseudomonas aeruginosa* biofilm coated medical grade endotracheal tubes." *Antimicrob Agents Chemother*. 2013 Dec 30. DOI: 10.1128/AAC.01178-13. Article can be found at: <http://aac.asm.org/content/early/2013/12/24/AAC.01178-13.long>

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1 **Activity of tobramycin and polymyxin-E against *Pseudomonas aeruginosa* biofilm**
2 **coated medical grade endotracheal tubes**

3

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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.

40 **INTRODUCTION**

41 Indwelling medical devices are a major source of nosocomial infections. In particular,
42 patients requiring mechanical ventilation (intubation with an endotracheal tube (ETT)) face
43 a high probability of contracting one of the most prevalent nosocomial infections,
44 ventilator associated pneumonia (VAP).(1-3) Neonatal and pediatric populations are at
45 especially high risk for VAP because the current standard of care involves prolonged
46 intubation without ETT exchange or tracheostomy, both common practice in adult patients.
47 In neonates and infants, the inner diameter of the ETT is often 2.5-3.5mm (the size of a thin
48 straw), which complicates suctioning of secretions and confounds attempts to maintain
49 patency. Despite aggressive bedside hygiene, *Pseudomonas aeruginosa* (*P. aeruginosa*)
50 remains one of the most common causes of VAP in intubated children.(2, 4, 5)

51

52 *P. aeruginosa*, often found on indwelling devices such as ETTs, forms a biofilm which
53 serves as an ideal environment for antibiotic resistance, making VAP difficult to treat.(6, 7)
54 Biofilm on ETTs is considered to be a reservoir for infecting pathogens derived from
55 oropharyngeal flora and gastric microaspiration, and is highly correlated with lower airway
56 infection and subsequent VAP.(8-11) To date, few side-by-side studies have compared
57 killing activity (defined as 99.9% kill) of tobramycin and polymyxin-E against *P.*
58 *aeruginosa*, especially in the context of ETT biofilm and VAP.(12-15) The effect of
59 monotherapy and/or combination therapy (synergistic versus antagonistic activity) must be
60 assessed when evaluating antimicrobial drug therapy, especially in the presence of medical
61 grade polyvinyl chloride (PVC) or conventional ETTs. For convenience, most studies
62 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.

70 **MATERIALS AND METHODS**

71 **Bacterial Isolates.** American Type Culture Collection (ATCC, Manassas, VA, USA) strain
72 25668 was obtained. Reference strain PAO1 was obtained from Dr. Thomas Murray,
73 Frank H. Netter MD School of Medicine, Quinnipiac University, North Haven, CT.(19, 20)
74 Prior to use, all bacteria were stored in tryptic soy broth (TSB; Difco laboratories, Sparks,
75 MD) with 15% glycerol and frozen at -80 °C. Both strains are prolific biofilm
76 producers.(21, 22)

77

78 **Antimicrobial Agents.** Commercially available, chemical grade polymyxin-E (lot#
79 081M1525V) powder and chemical grade tobramycin (lot# 090M1196V) powder was
80 purchased from Sigma Aldrich (St. Louis, MO). Tobramycin and polymyxin-E powder
81 were stored at 4°C. Both tobramycin and polymyxin-E were diluted in sterile water and a
82 fresh stock was made each day, and prior to every experiment. Tobramycin and
83 polymyxin-E were tested at one, two, four and eight times their respective minimal
84 inhibitory concentration (MIC) at 0, 4 and 24 hours after inoculation.(23) Cation Adjusted
85 Mueller-Hinton broth (CA-MHB, Difco Laboratories, Sparks, MD) supplemented with 25
86 mg/L calcium, 12.5 mg/L magnesium and 0.25% dextrose (Fisher Scientific, Pittsburgh,
87 PA, USA) was used to obtain a suspension corresponding to 0.7 - 0.8 McFarland standards
88 to produce an initial starting inocula of $5.5-6.0 \times 10^6$ colony forming units per milliliter
89 (CFU/mL). Colony counts were determined using tryptic soy agar (TSA, Difco, Becton
90 Dickinson Co., Sparks, MD) plates.

91

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°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°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 $5.5\text{--}6.0 \times 10^6$ 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 tobramycin 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 $3.81 \pm 0.16 \log_{10}$ 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

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 log₁₀ CFU/mL, *p*=
177 0.004) and 8 times (MD= -1.45, 95% CI, -2.5-0.4 log₁₀ 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 log₁₀ 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 log₁₀ 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 log₁₀ CFU/mL) and with
189 greater than 2 times the MIC of polymyxin-E (average decrease of >3.1 log₁₀ 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 log₁₀CFU/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 log₁₀ 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

198 **DISCUSSION**

199 Ventilator associated pneumonia, a common nosocomial infection often caused by bacteria that
200 produce biofilm, results in increased morbidity, medical costs and multi-drug resistant
201 organisms.(2, 3, 29-32) In one study, adult patients with VAP were hospitalized longer (38 vs.
202 13 days, $p<0.01$), mortality rates were higher (50% vs. 34%, $p<0.01$), and hospital costs were
203 greater (\$70,568 vs. \$21,620, $p<0.01$) compared to uninfected ventilated patients, with estimated
204 VAP attributable costs of \$11,897.(32) However, limited diagnostic criteria and modification of
205 ETTs make VAP prevention particularly challenging and difficult especially for neonates and
206 children.(2)

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

214
215 One controversial approach to treatment of VAP is “selective decontamination of the digestive
216 tract” with broad spectrum intravenous (IV) antimicrobials.(37, 38) However, IV prophylaxis is
217 not widely accepted due to fear of creating antibiotic resistant strains among VAP pathogens. In
218 the pediatric population, one of the most common VAP pathogen is *P. aeruginosa*, accounting
219 for 17-25% of VAP.(2, 4, 5) Our model is most consistent with the practice of direct instillation
220 of liquid antimicrobial agents through the ETT as prophylaxis against, or treatment of VAP
221 caused by *P. aeruginosa* compared to inhalation of nebulized antibiotics.(39) Instillation

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.

263 **ACKNOWLEDGEMENTS**

264 This study was funded by PCCSDP (Pediatric Critical Care Scientist Development Program),
265 through NIH 5K12HD047349-07, and supported by Department of Pediatrics, Hasbro Children's
266 Hospital, Rhode Island Hospital.

267 Presented as a poster (E-790) at the Interscience Conference of Antimicrobial Agents and
268 Chemotherapy 2012 meeting, San Francisco CA, September 7-12th 2012.

269

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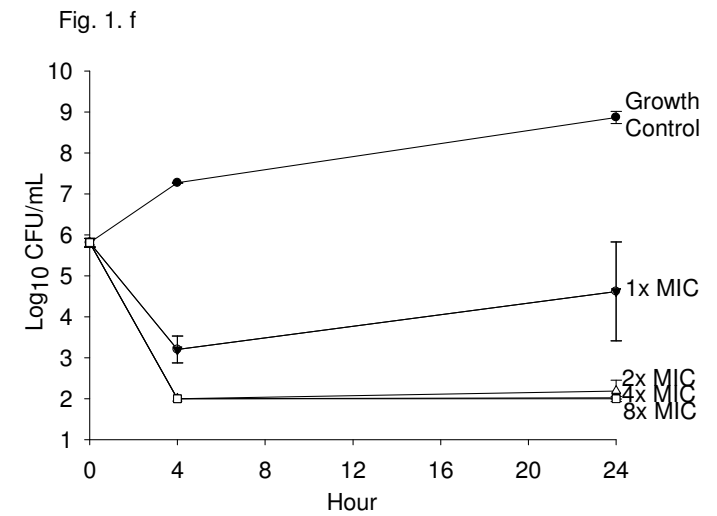
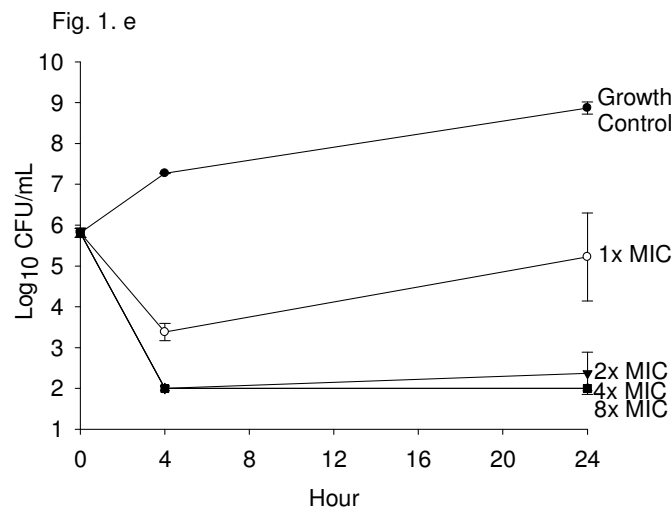
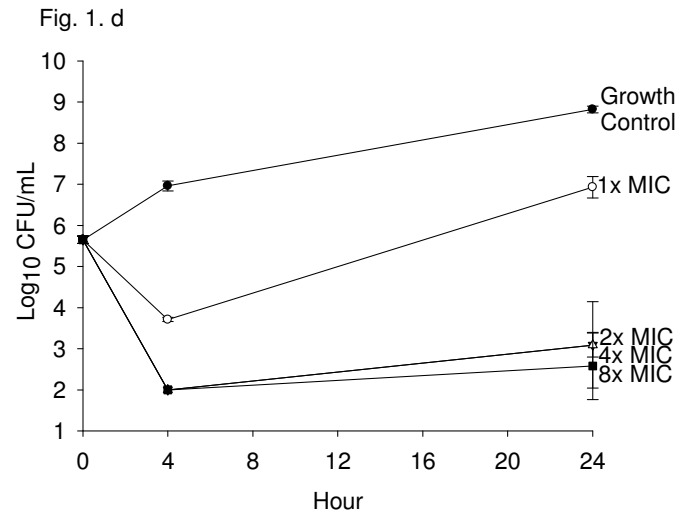
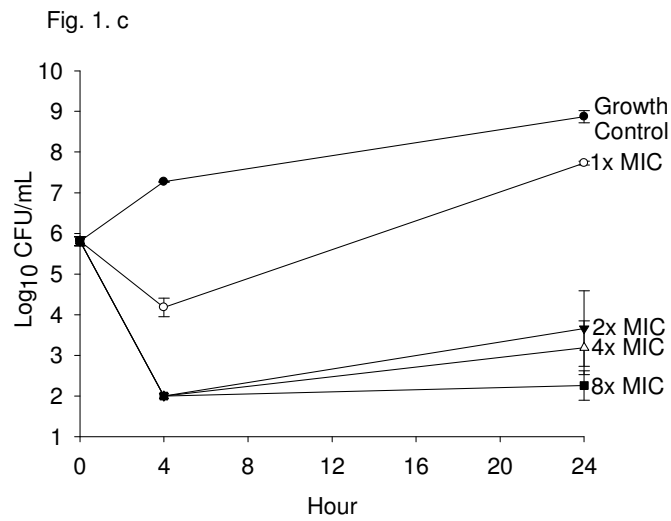
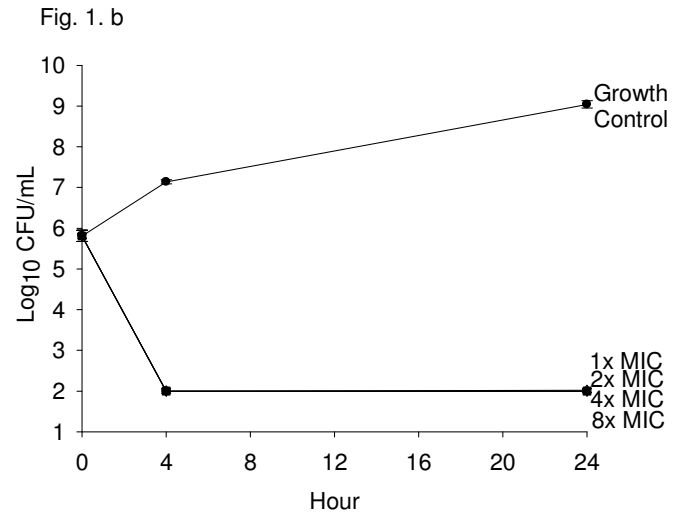
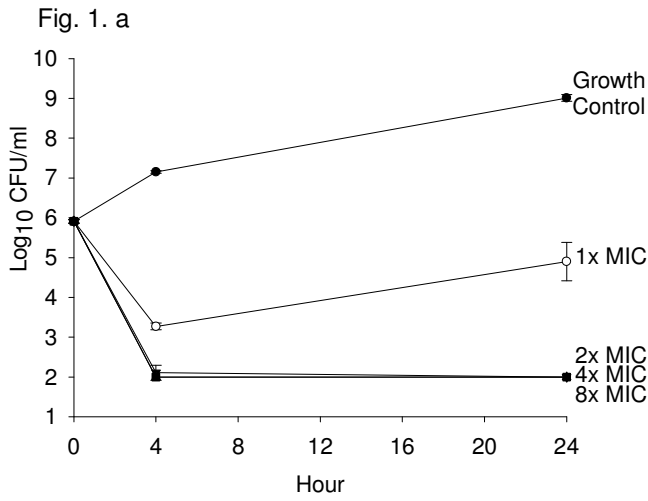
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386

387 FIGURES



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 \pm
393 standard deviation.

Fig. 2. a

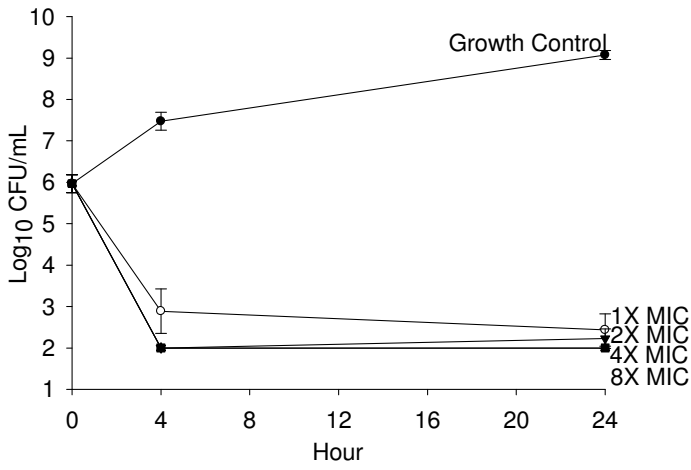


Fig. 2. b

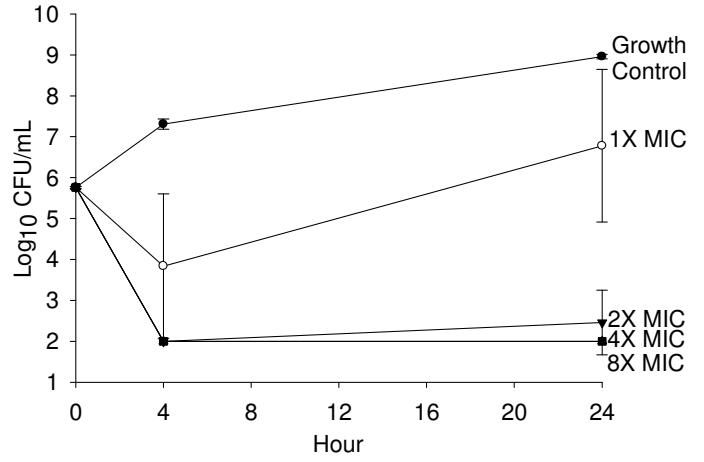


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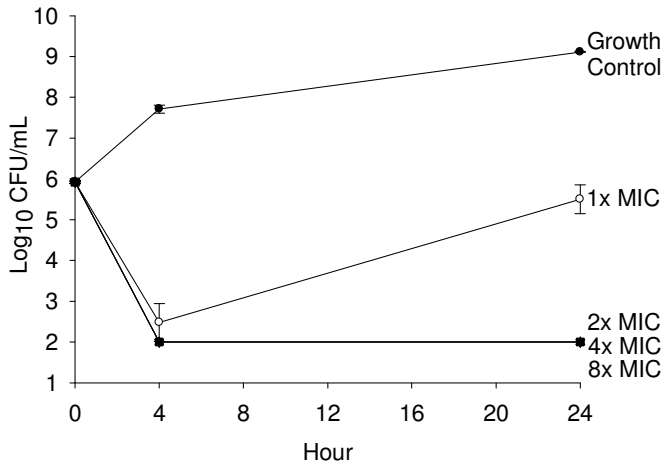


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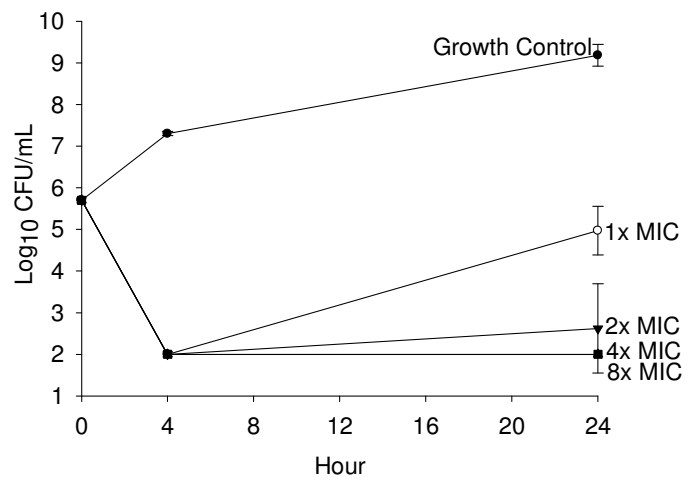


Fig. 2. e

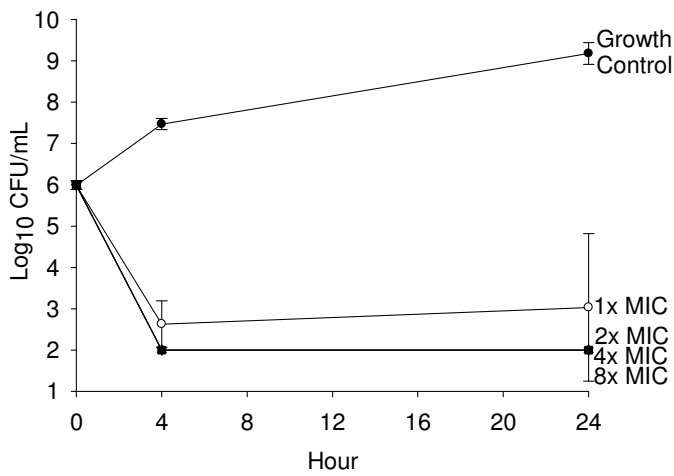
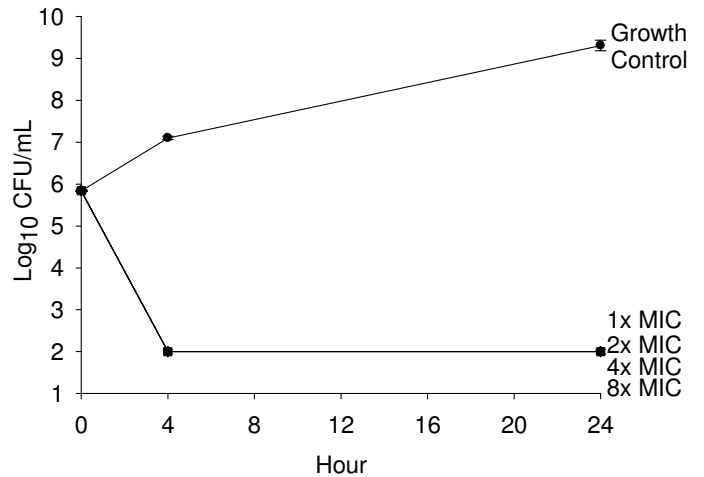


Fig. 2. f



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 \pm
399 standard deviation.

Fig. 3. a

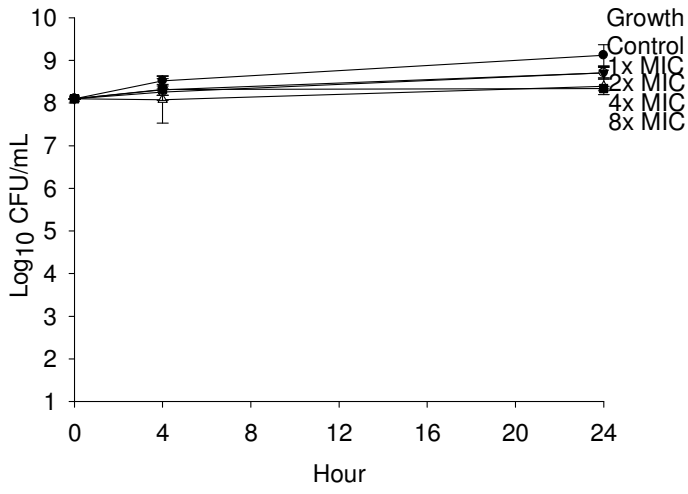


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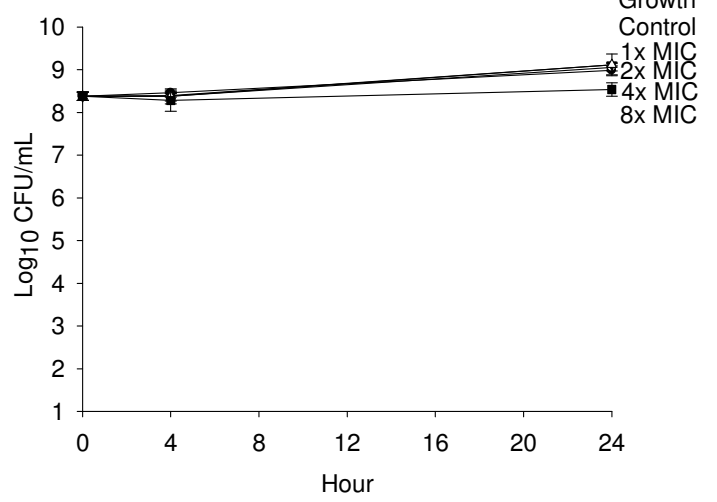


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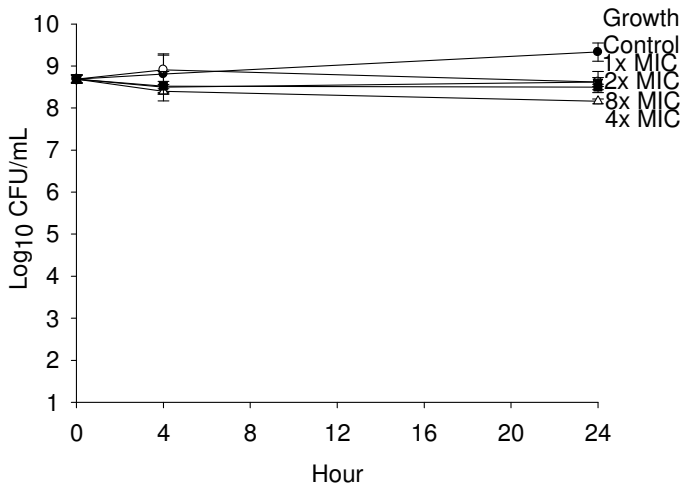


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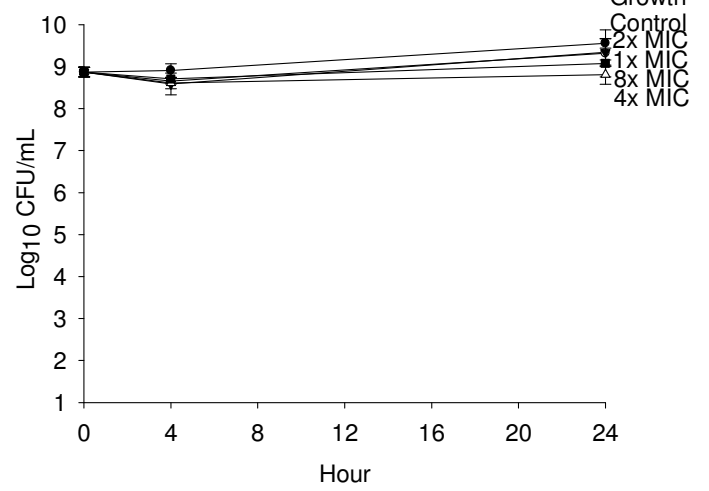


Fig. 3. e

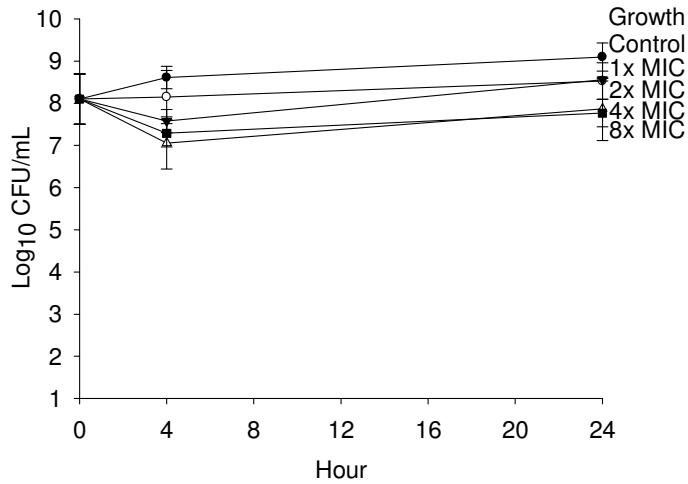
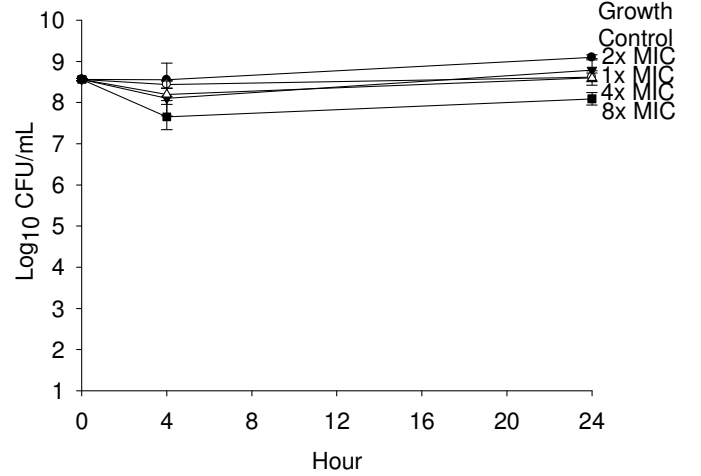


Fig. 3. f



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 \pm standard deviation.

405 **TABLE**

406 **Table 1:** Comparison of log₁₀ 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.