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

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

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Abstract

Indwelling medical devices have become a major source of nosocomial infections; especially *Pseudomonas aeruginosa (P. aeruginosa)* infection, which remain the most common cause of ventilator associated pneumonia (VAP) in neonates and children. Using medical grade polyvinyl chloride endotracheal tubes (ETTs), the activity of tobramycin and polymyxin-E was quantified in a simulated prevention and treatment static time kill model using biofilm forming *P. aeruginosa*. The model simulated three clinical conditions: 1) planktonic bacteria in the presence of antibiotics, tobramycin and polymyxin-E, without ETTs, 2) planktonic bacteria grown in the presence of *P. aeruginosa*, antibiotic and ETTs (simulating prevention) and 3) a 24h formed *P. aeruginosa* biofilm on ETTs prior to antibiotic exposure (simulating treatment). In the model simulating “prevention” (conditions 1 and 2 above), tobramycin alone or in combination with polymyxin-E was more bactericidal than polymyxin-E alone at 24 hours using a concentration greater than 2 times the minimum inhibitory concentration (MIC). However, after a 24h old biofilm was allowed to form on the ETTs, neither monotherapy nor combination therapy over 24 hours exhibited bactericidal or bacteriostatic effects. Against the same pathogens, tobramycin and polymyxin-E, both alone or in combination exhibited bactericidal activity prior to biofilm attachment to the ETTs, however no activity was observed once biofilm formed on ETTs. These findings support surveillance culturing to identify pathogens for a rapid and 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
than clinically available medical devices. (16-18) However, most of the coupons made of PVC are not medical grade and, in many cases, do not contain equivalent plasticizer content. These differences result in different texture and flexibility between medical grade PVC products and PVC coupons used in biofilm experiments. Using clinically available ETTs, this study aimed to both assess the efficacy of antibiotics against planktonic vs. biofilm formed \textit{P. aeruginosa}, and to identify which antibiotic, alone or combination, demonstrates the best in vitro activity against \textit{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 ºC. 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°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.
**Susceptibility Testing.** MIC tests were performed in triplicate using broth microdilution in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines.(24, 25) The MIC was defined as the minimum concentration of antibiotic that will inhibit the visual growth of the isolated organism. Minimum bactericidal concentrations (MBC) were also determined in triplicate for each antimicrobial agent using CSLI guidelines.(25) Bacteria were quantified using CFU/mL, and 5 microliter aliquots were used for determination of MBC after 24 hours incubation at 37^oC using TSA.(26)

**Endotracheal Tubes (ETTs).** Commercially available Sheridan® 6.0 mm ID, uncuffed ETTs (Hudson RIC, Temecula, CA, USA) were obtained. Each ETT was cut into 0.6 cm by 0.3 cm rectangular pieces (ETT chips) using a ¼ rectangle hand puncher (Fiskars Corporation, Helsinki, Finland), and sterilized with ethylene oxide gas prior to use in pre-formed and formed biofilm time kills experiments.(22) For comparison, we also tested commercially available PVC coupons (part Number RD 128-PVC, Biosurface Technologies, Corp, Bozeman, MT) for pre-formed biofilm P. aeruginosa PAO1.(16-18)

**Biofilm Formation.** Sterile ETT chips were placed in each well of a 24-well plate (BD Biosciences, San Jose, CA). The ETT chip was submerged with 2 mL of a final bacteria inoculum, either PAO1 or ATCC 25668, obtained as described above using TSB supplemented with 1% dextrose, 2% NaCl and 25 mg/L calcium (STSB) using modified Growing and Analyzing Static Biofilms.(27) The well plate was incubated at 37^oC under static conditions for 24 hours to promote biofilm formation on ETT chips. After 24 hours,
each ETT chip was gently rinsed three times in sterile phosphate buffered saline (PBS) (Fisher Scientific, Pittsburgh, PA).

**Time Kill Study.** Using a 24h time kill study, three clinical conditions were modeled using *P. aeruginosa* strains PAO1 and 25668: 1) planktonic bacteria in the presence of the antibiotics tobramycin and polymyxin-E, without ETTs, 2) planktonic bacteria grown in the presence of *P. aeruginosa*, antibiotics and ETTs (simulating prevention) and 3) a 24h formed *P. aeruginosa* biofilm on ETTs prior to antibiotic exposure (simulating treatment).

Each time kill experiment was carried out in a minimum of triplicate. All antimicrobial agents were tested at one, two, four and eight times their respective MIC with starting inocula of 5.5–6.0 x 10^6 CFU/mL adjusted to McFarland standards using the Vitek colorimeter (bioMérieux, Inc, Durham, NC).(18, 28)

Sample aliquots (0.1 mL) were removed from cultures at 0, 4, and 24 hours after vortexing each tube for one minute to remove biofilm growth from the ETT chip.(22) Antimicrobial carryover was accounted for by serial dilution (10 – 10,000 fold) of plated samples with normal saline or vacuum filtration. This methodology has a lower limit of detection of 2.0 log_{10} CFU/mL.(28) Growth control tubes for each organism were prepared without antibiotic and run in parallel to the antibiotic test tubes.

For single antimicrobial agents, bactericidal activity (99.9% kill) was defined as a ≥ 3 log_{10} CFU/mL reduction at 24h in colony count from the initial inoculum. Bacteriostatic activity was defined as a < 3 log_{10} CFU/mL reduction at 24h in colony count from the
initial inoculum, while inactive was defined as no observed reduction from the initial
inoculum.(23) For antibiotics evaluated in combination, synergy was defined as \( \geq 2 \log_{10} \) CFU/mL decrease, indifference was defined as a 1 to 2 \( \log_{10} \) CFU/mL change (increase or decrease), and antagonism was defined as \( >2 \log_{10} \) CFU/mL increase in growth compared to the most active single agent.

**Data analysis.** All statistical analyses were performed using SPSS statistical software (IBM SPSS statistics version 20, IBM Corporation, Armonk, NY USA). After 24 h of exposure to antimicrobial agent(s), the biofilm formation was quantified, bacteria at 4 hour and 24 hour were counted (with a lower limit of detection 2.0 \( \log_{10} \) CFU/mL) to compare between antimicrobial groups, concentrations and strains using analysis of variance (ANOVA) followed by Tukey’s *post-hoc* analysis. Multiple regressions for the association between substrates and CFU/mL were analyzed. A *p* value of \( \leq 0.05 \) indicated statistical significance.
RESULTS

The MIC for tobramycin was 0.5 µg/mL and for polymyxin-E was 2 µg/mL for both PAO1 and 25668 strains. The MBC for tobramycin was 4 and 32 µg/mL and for polymyxin-E was 16 and 64 µg/mL respectively for Pseudomonas PAO1 and ATCC 25668 strains.

In the planktonic time kill study, tobramycin demonstrated bactericidal activity against both Pseudomonas isolates at 24 hours with average decrease of 3.81±0.16 log\textsubscript{10} CFU/mL for all concentrations except 1 time the MIC for PAO1 (Fig. 1.a & b). Polymyxin-E demonstrated bacteriostatic activity at 2 and 4 times the MIC (average decrease of 2.16-2.63 log\textsubscript{10} CFU/mL), and bactericidal activity at 8 times the MIC (average decrease of 3.07-3.56 log\textsubscript{10} CFU/mL), but inactive at 1 time the MIC for both isolates at 24 hours (Fig. 1.c & d). The combination therapy at 2, 4, and 8 times the MIC demonstrated indifference with >3.44 log\textsubscript{10} CFU/mL kill for PAO1, and >3.46 log\textsubscript{10} CFU/mL kill for 25668 at 24 hours (Fig 1. e & f).

In the pre-formed biofilm time kill studies (simulating prevention) at 24 hours, tobramycin demonstrated bactericidal activity against both Pseudomonas isolates (average decrease of > 3.3 log\textsubscript{10} CFU/mL), except 1 time the MIC for 25668 which showed inactivity (1.02±1.86 log\textsubscript{10} CFU/mL increase; Fig 2.a & b). Similarly, polymyxin-E demonstrated bactericidal activity (average decrease of >3.08 log\textsubscript{10} CFU/mL) at greater than 2 times the MIC, but bacteriostatic activity at 1 time the MIC for both isolates at 24 hours (Fig. 2.c & d). Tobramycin and polymyxin-E combination demonstrated indifferent activity at all concentrations for both isolates (Fig. 2.e & f).
Informed biofilm time kill studies (simulating treatment) for PAO1, combination therapy at 4 times the MIC was significantly more active at 4 hours compared to polymyxin-E alone at 4 times (mean difference (MD)= -1.34, 95% confidence interval [CI], -2.4-0.3 log_{10} CFU/mL, \(p=0.004\)) and 8 times (MD= -1.45, 95% CI, -2.5-0.4 log_{10} CFU/mL, \(p=0.001\)) the MIC. Similarly, combination therapy at 8 times the MIC was significantly more active at 4 hours compared to polymyxin-E alone at 8 times the MIC (MD= -1.23, 95% CI, -2.3-0.2 log_{10} CFU/mL, \(p=0.001\)). However, indifferent activity was observed at 24 hours. Similarly, for 25668, combination therapy at 8 times the MIC was significantly more active compared to polymyxin-E alone at 4 hours (MD= -1.06, 95% CI, -1.7-0.4 log_{10} CFU/mL, \(p<0.001\)). However, indifferent activity was observed at 24 hours. Once biofilm is formed, both single agent and combination antibiotics resulted in inactivity or indifference (Fig. 3. a - f).

In addition to medical grade PVC ETTs, we assayed time kill using commercially available PVC coupons(16-18). A similar trend of bactericidal activity was demonstrated at 24 hours with greater than 4 times the MIC of tobramycin (average decrease of >3.03 log_{10} CFU/mL) and with greater than 2 times the MIC of polymyxin-E (average decrease of >3.1 log_{10} CFU/mL), but indifference was noted when the combination of tobramycin and polymyxin-E was evaluated at 2, 4, and 8 times the MIC (average decrease of > 3.21 log_{10}CFU/mL). ANOVA showed that there was a significant difference between substrates and CFU/mL at 4 hours (MD= 0.08, 95% CI, 2.5-3.7 log_{10} CFU/mL, \(p= 0.041\)) (Table 1). Multiple regression analysis demonstrated that there was a significant association between CFU/mL with substrate at 4 hours (partial eta squared [\(\eta^2\] =0.493, \(p<0.001\)) and at 24 hours (\(\eta=0.208, p<0.001\)). The overall model fit was \(R^2 = 0.954\).
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. 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. However, limited diagnostic criteria and modification of ETTs make VAP prevention particularly challenging and difficult especially for neonates and children. 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. 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.

One controversial approach to treatment of VAP is “selective decontamination of the digestive tract” with broad spectrum intravenous (IV) antimicrobials. 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. 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. Instillation
treatments pose less risk of systemic toxicity than IV administration because antimicrobial agents can be delivered locally using ETTs or tracheostomy tubes in children and neonates. Moreover, instillation can deliver drug directly to the site of pneumonia whereas nebulized drug may adsorb on the ETT, permeate into the ETT wall, or remain in the proximal airway. Therefore, our study model using ETT chips is useful to help understand the effects of tobramycin and polymyxin-E, alone or in combination, to treat VAP caused by *P. aeruginosa*.

In our study, we examined *P. aeruginosa* growth with or without the presence of medical grade polyvinyl chloride (PVC) ETT to evaluate the bactericidal effects of two antibiotics in the condition of VAP. We found that in an in vitro condition, the bactericidal effect of tobramycin or polymyxin-E monotherapy required greater than 2 times the MIC at 24 hours for the pre-biofilm condition (prevention). However, antibiotics demonstrated different activity against the two different strains. For PAO1, tobramycin monotherapy was equally active for killing compared to the combination approach. For 25668, the combination therapy was more active for killing compared to monotherapy at 24 hours (Fig 2.a-f); this finding may be related to the biofilm-forming abilities of each bacterium.

Our study also demonstrated that two of the antibiotics tested in either monotherapy or in combination showed inactivity or indifference once biofilm was formed on ETTs against both *Pseudomonas* strains (Fig 3.a-f). This is in contrast to the conclusions drawn by Herrmann et al. using a 96-peg Calgary biofilm device in vitro showing combination therapy with colistin-tobramycin combination was superior to monotherapy against *Pseudomonas* biofilm. (40)
Many in vitro studies have used commercially available PVC coupons, which have different texture and flexibility (based on the plasticizer content compared to medical grade PVC ETTs). We hypothesized that bacterial colonies would form differently on commercially available PVC coupons compared to medical grade PVC ETTs. To capture *Pseudomonas* growth in relation to different material surfaces more accurately, we studied the same antibiotic therapy against *Pseudomonas* PAO1 using both PVC coupon and PVC ETTs. There was the significant association among CFU/mL and substrate at 4 and 24 hours (Table 1), thus it showed the importance of utilizing same device material to mimic VAP condition to evaluate antibiotic activity on biofilm.

In conclusion, neither single nor combination therapy with tobramycin and/or polymyxin-E demonstrated killing activity once *Pseudomonas* biofilm was already formed on ETTs, however, no antagonism was noted. Bactericidal effects against pre-formed biofilm (simulating prevention) in the presence of ETTs suggest that surveillance cultures could identify pathogens prior to biofilm formation, and allow prophylactic or targeted approaches to therapy, especially when *Pseudomonas* is a potential pathogen. In addition, this study demonstrated the importance of material choice in vitro time kill study. Further investigation could incorporate wild type strains as well as clinically feasible treatment options for VAP in children.
ACKNOWLEDGEMENTS

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Figure 1.a-f. Time Kill against planktonic *P. aeruginosa*. Tobramycin against planktonic *P. aeruginosa* PAO1 (Fig 1.a), 25668 (Fig 1.b), polymyxin-E against planktonic *P. aeruginosa* PAO1 (Fig 1.c), 25668 (Fig 1.d), combination of tobramycin and polymyxin-E against planktonic *P. aeruginosa* PAO1 (Fig 1.e) and 25668 (Fig 1.f). Results are presented as mean ± standard deviation.
Figure 2.a-f. Time Kill against pre-biofilm formed *P. aeruginosa*. Tobramycin against *P. aeruginosa* PAO1 (Fig 2.a), 25668 (Fig 2.b), polymyxin-E against *P. aeruginosa* PAO1 (Fig 2.c), 25668 (Fig 2.d), combination of tobramycin and polymyxin-E against *P. aeruginosa* PAO1 (Fig 2.e) and 25668 (Fig 2.f) with presence of ETT chips. Results are presented as mean ± standard deviation.
Figure 3. a-f. Time Kill against biofilm formed *P. aeruginosa*. Tobramycin against *P. aeruginosa* PAO1 (Fig 3.a), 25668 (Fig 3.b), polymyxin-E against *P. aeruginosa* PAO1 (Fig 3.c), 25668 (Fig 3.d), combination of tobramycin and polymyxin-E against *P. aeruginosa* PAO1 (Fig 3.e) and 25668 (Fig 3.f). Results are presented as mean ± standard deviation.
**Table 1**: Comparison of log$_{10}$ colony forming unit/mL (CFU/mL) change at 4 hours from 0 hr growth control between endotracheal tube (ETT) and polyvinyl chloride (PVC) coupons.

<table>
<thead>
<tr>
<th></th>
<th>Tobramycin</th>
<th>Polymyxin-E</th>
<th>Tobramycin + Polymyxin-E</th>
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</thead>
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<tr>
<td></td>
<td>ETT</td>
<td>PVC coupon</td>
<td>ETT</td>
</tr>
<tr>
<td>1 x MIC</td>
<td>-2.81± 0.04</td>
<td>-2.82±0.12</td>
<td>-3.44±0.43</td>
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<td>2 x MIC</td>
<td>-3.97±0.21</td>
<td>-0.71±0.01</td>
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<td>4 x MIC</td>
<td>-3.97±0.21</td>
<td>-1.23±0.03</td>
<td>-3.92±0.03</td>
</tr>
<tr>
<td>8 x MIC</td>
<td>-3.97±0.21</td>
<td>-1.06±0.03</td>
<td>-3.92±0.03</td>
</tr>
</tbody>
</table>

*Average change ± standard deviation.*