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Differences in biofilm formation and antimicrobial resistance of *Pseudomonas aeruginosa* isolated from airways of mechanically ventilated patients and cystic fibrosis patients

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Abstract

Pseudomonas aeruginosa biofilms exhibit increased antimicrobial resistance compared with planktonic isolates and are implicated in the pathogenesis of both acute and chronic lung infections. Whilst antibiotic choices for both infections are based on planktonic antibiotic susceptibility results, differences in biofilm-forming ability between the two diseases have not previously been explored. The aim of this study was to compare differences in biofilm formation and antibiotic resistance of *P. aeruginosa* isolated from intubated patients and from patients with chronic pulmonary disease associated with cystic fibrosis (CF). The temporal evolution of antibiotic resistance in clonal *P. aeruginosa* strains isolated from CF patients during periods of chronic infection and acute pulmonary exacerbation was also evaluated. Biofilm formation and biofilm antibiotic susceptibilities were determined using a modified microtitre plate assay and were compared with antibiotic susceptibility results obtained using traditional planktonic culture. Clonality was confirmed using random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) analysis. *Pseudomonas aeruginosa* isolates collected from intubated patients produced substantially more biofilms compared with CF isolates. There was considerable heterogeneity in biofilm-forming ability among the CF isolates and this was unrelated to pulmonary status. Biofilm antibiotic resistance developed rapidly among clonal CF isolates over time, whilst traditional antibiotic resistance determined using planktonic cultures remained stable.

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Competing interests

None declared.

Ethical approval

Approval was obtained from the University of California, San Francisco (UCSF) Human Research Protection Program (approval nos. H53004-28671-05 and H1287-21213).

There was a significant positive correlation between imipenem/cilastatin and ceftazidime resistance and biofilm-forming ability. The variability in biofilm-forming ability in *P. aeruginosa* and the rapid evolution of biofilm resistance may require consideration when choosing antibiotic therapy for newly intubated patients and CF patients.

Keywords

Pseudomonas aeruginosa; Bacterial biofilm; Antimicrobial resistance; Mechanical ventilation; Cystic fibrosis

1. Introduction

Pseudomonas aeruginosa is the leading cause of morbidity and mortality in patients with ventilator-associated pneumonia (VAP) and cystic fibrosis (CF). Whilst VAP and CF are notably different pulmonary disease processes, biofilm formation is a unifying process in the pathogenesis both of acute lung infections such as VAP and chronic lung infections such as CF.

Biofilms are structured communities of bacterial cells enclosed in an extracellular secreted polymeric matrix consisting of secreted proteins, exopolysaccharides and nucleic acids that can adhere both to abiotic and living surfaces, serving as a permanent source of infection [1]. Biofilms have been shown to be up to 1000 times more resistant to antibiotics than planktonic, or free-swimming, cells of the same isolate [2]. This is due to a number of mechanisms, including the exopolysaccharide matrix acting as a physical barrier to antibiotic penetration and the creation of an antibiotic gradient throughout the biofilm. Exposure of cells within the biofilm to sublethal concentrations of antibiotics further promotes antimicrobial resistance in cells that may then detach from the biofilm and disseminate infection elsewhere. Oxygen and nutrient depletion may cause the bacteria to enter a non-growing or stationary phase, which increases resistance to antibiotics such as β -lactams [3]. In addition, biofilm-specific antimicrobial resistance genes not expressed during the planktonic phase have been shown to increase resistance of cells in these sessile communities [4].

There are notable differences in the pathophysiology and presentation of VAP and CF. In mechanically ventilated (MV) patients, injury due to insertion of the endotracheal tube (ETT) and the absence of host defences facilitate the entry of bacteria in the tracheal mucosa. Bacterial colonisation and biofilm formation can rapidly occur on the inner lumen of the ETT as early as 12 h after intubation [5–7]. The biofilm can then be dislodged from the ETT with suctioning and disseminated towards the lower respiratory tract, potentially leading to VAP. VAP with *P. aeruginosa* is an invasive and rapidly progressing infection characterised by acute leukocytosis, fever and increased need for ventilator support. In contrast, initial infection with *P. aeruginosa* in CF patients typically occurs in childhood and infection is lifelong and persistent. The thick mucus layer overlaying CF airway epithelial cells is particularly amenable to the formation of biofilms. With the establishment of chronic infection, *P. aeruginosa* may convert to a mucoid phenotype (i.e. hyperproduction of alginate), resulting in biofilms that are virtually impossible to clear by conventional antimicrobial therapy. Whilst chronic colonisation with *P. aeruginosa* biofilms in CF patients is typically subclinical and silent, acute exacerbations can lead to respiratory decompensation, likely due to release of planktonic bacteria from biofilm colonies [8]. The continual persistent infection, in addition to host defences, leads to chronic airway inflammation and eventual airway destruction. In contrast to *P. aeruginosa* VAP, pulmonary

exacerbations in CF patients are episodic with only mild elevations in temperature and white blood cell (WBC) count.

In this study, the biofilm-forming ability and associated antibiotic resistance of *P. aeruginosa* recovered from two distinct patient populations, namely MV patients newly colonised with multidrug-resistant (MDR) *P. aeruginosa* and CF patients during periods of chronic infection and episodes of acute pulmonary exacerbation, were compared. Serial *P. aeruginosa* isolates from CF patients were examined to evaluate the evolution of antimicrobial resistance both in planktonic and biofilm cultures over time.

2. Materials and methods

2.1. Collection of *Pseudomonas aeruginosa* isolates from mechanically ventilated and cystic fibrosis patients

Daily endotracheal aspirate (ETA) surveillance cultures were performed on patients ≥ 18 years of age who were intubated for ≥ 48 h in the Intensive Care Unit (ICU) irrespective of signs or symptoms of lung infection [9]. Patients with known infection or risk factors for colonisation with *P. aeruginosa* (CF, bronchiectasis, chronic tracheostomy, previous intubation during the current hospitalisation or a previous culture positive for *P. aeruginosa*) were excluded. Six patients were found to be newly colonised with a MDR *P. aeruginosa* isolate. These six isolates were chosen for evaluation of biofilm-forming ability and associated antimicrobial resistance. Clinical characteristics were noted at the time of positive culture. VAP was defined as any lower respiratory infection that developed after 2 days of mechanical ventilation. Criteria for VAP were presence or progression of a new infiltrate identified by chest radiography plus two of the following three clinical criteria: (i) fever $>38^{\circ}\text{C}$; (ii) WBC count $>12\,000$ cells/ mm^3 or <4000 cells/ mm^3 ; and (iii) purulent secretions and $\geq 10^6$ colony-forming units (CFU)/mL *P. aeruginosa* from the ETA and/or $\geq 10^4$ CFU/mL from a bronchoalveolar lavage [10].

To examine the evolution of antibiotic resistance over time in patients with CF, serial respiratory cultures were collected from three CF patients known to be chronically infected with *P. aeruginosa* over a 3-month period during their regularly scheduled outpatient clinic appointment and when hospitalised for treatment of acute pulmonary exacerbation as part of an ongoing prospective cohort study. Five isolates were collected from Patient 1 on Days 1, 28, 29, 30 and 84, three samples were collected from Patient 2 on Days 1, 2 and 62 and two samples were collected from Patient 3 on Days 1 and 61. Clinical characteristics [i.e. age, sex, forced expiratory volume in 1 s (FEV_1) and current antibiotics] were noted at the time of each sample. Patients were noted to be either chronically infected yet clinically stable or in acute pulmonary exacerbation requiring hospitalisation. The Institutional Review Board of University of California, San Francisco (San Francisco, CA) approved all protocols.

2.2. Random amplified polymorphic DNA (RAPD)

A DNA fingerprint of each isolate was generated by RAPD analysis to determine clonality of the *P. aeruginosa* strains. DNA was extracted from overnight Luria–Bertani (LB) cultures of each strain using the Promega Wizard[®] Genomic DNA Purification Kit (Promega, Madison, WI). RAPD primer 272 was used as described previously [11].

2.3. Antimicrobial agents

Tobramycin (Teva Pharmaceuticals, Irvine, CA), ceftazidime (GlaxoSmithKline, Research Triangle Park, NC), piperacillin/tazobactam (TZP) (Wyeth, Philadelphia, PA), imipenem/cilastatin (Merck and Co, Whitehouse Station, NJ) and levofloxacin (Ortho-McNeil-Janssen,

Raritan, NJ) were used. Fresh stock solutions of each antibiotic were prepared in sterile water. Concentrations tested ranged from 0 µg/mL to 256 µg/mL for each drug.

2.4. Biofilm growth assays

Biofilms of each *P. aeruginosa* isolate were grown according to the method of O'Toole and Kolter [12]. Overnight cultures of all isolates were grown to stationary phase and were diluted to an optical density at 650 nm (OD₆₅₀) of 0.1 in fresh LB medium. Then, 200 µL of the diluted culture was used to inoculate 8 wells per isolate of a clear 96-well microtitre plate (Nunc TSP System; Nunc International, Rochester, NY). Peg lids (TSP lids; Nunc International) were used to cover the plates and plates were statically incubated at 37 °C for 24 h. The biofilms formed on the immersed pegs were stained with 0.1% w/v crystal violet (125 µL) for 10 min, rinsed and allowed to dry for several hours. To solubilise absorbed crystal violet, pegs with stained biofilms were incubated in 70% ethanol (200 µL/well) for an additional 10 min. The OD₆₅₀ was measured on a microtitre plate colorimeter (Molecular Devices, Sunnyvale, CA). Mean readings from eight wells were calculated to determine the biofilm-forming ability of each strain. *Pseudomonas aeruginosa* strains PA01 and PA103 were included as control strains, as PA01 has been shown to form robust biofilms while PA103 forms weak biofilms.

2.5. Planktonic antibiotic susceptibility studies

The minimum inhibitory concentration (MIC) of planktonic *P. aeruginosa* was determined for each antibiotic using standard 96-well microbroth dilution plates (Dade Behring, West Sacramento, CA). Results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines [13].

2.6. Biofilm antibiotic susceptibility studies

The biofilm inhibitory concentration (BIC), i.e. the antibiotic concentration needed to inhibit 90% of biofilm viability, was determined using a modified version of the method of Moskowitz et al. [14]. In brief, biofilms were allowed to form on peg lids as described above. Following biofilm formation, peg lids were transferred to a fresh microtitre plate containing 0–256 µg/mL of antibiotic in a total volume of 100 µL/well. Eight replicate wells were used for each concentration tested for each strain. Plates were re-incubated statically at 37 °C for 24 h (antibiotic challenge). Following incubation in antibiotic, peg lids were directly transferred to another 96-well plate containing fresh LB medium prior to centrifugation at 2500 × g for 35 min to remove biofilm cells from the peg lids (biofilm recovery). The peg lid was replaced with a standard lid and residual biofilm viability was measured at each antibiotic concentration as the difference in mean optical density at 600 nm before and after incubation for 6 h at 37 °C (OD₆₀₀ at 6 h – OD₆₀₀ at 0 h). The mean biofilm viability of eight wells per antibiotic concentration tested was calculated.

2.7. Statistical analyses

Regression analyses were performed in the R statistical environment to determine whether a statistically significant correlation between biofilm formation and biofilm resistance for each of the antibiotics tested existed. *P*-values of ≤0.05 were considered statistically significant. Regression plots were constructed using Microsoft Excel (Microsoft Corp., Redmond, WA).

3. Results

3.1. Patient characteristics

The characteristics of the six MV patients and three CF patients are shown in Table 1. Of the MV patients, the mean age at study inclusion was 63 years (range 50–79 years). The mean

time from start of mechanical ventilation to colonisation with MDR *P. aeruginosa* was 17.7 days (range 2–26 days). Four patients died. Carbapenems (e.g. imipenem and meropenem) and linezolid were the most common antibiotics received at the time of colonisation. One patient (Patient 3) was not receiving any antibiotics at the time of colonisation.

Of the three CF patients, the most common antibiotics utilised during episodes of acute pulmonary exacerbation included a β -lactam and either an aminoglycoside or a fluoroquinolone.

3.2. Random amplified polymorphic DNA (RAPD) analysis of strains

RAPD analysis demonstrated that each of the *P. aeruginosa* strains isolated from the MV patients was unique (Fig. 1). Sequential isolates from each of the CF patients were clonal, suggesting that the temporal increase in antimicrobial resistance observed in these strains was due to evolution rather than acquisition of a novel *P. aeruginosa* strain.

3.3. Biofilm formation

Isolates from MV patients exhibited consistently strong biofilm-forming ability compared with strains from CF patients and control strains PA01 and PA103 (Fig. 2). Biofilm formation was comparatively weak for the CF isolates. There was also greater variability in biofilm formation among the CF isolates. There was no correlation between biofilm formation and pulmonary status (e.g. chronic stability versus acute exacerbation).

3.4. Minimum inhibitory concentration analysis

MICs for isolates from the six MV patients and three CF patients are presented in Table 2. Five (83%) of the six MV isolates were resistant to ceftazidime, imipenem/cilastatin and levofloxacin according to CLSI breakpoints. All MV isolates were susceptible to tobramycin. TZP exhibited the most variability in susceptibility, with MICs ranging from 8 μ g/mL to 128 μ g/mL.

In contrast, the MICs of the CF isolates were more susceptible compared with the MV isolates. Ceftazidime, imipenem and levofloxacin susceptibilities remained stable over time in all three CF patients, with either no increase in MIC (ceftazidime and levofloxacin) or a one-fold increase (imipenem, CF1). Interestingly, the Day 30 isolate from Patient 1 exhibited a two-fold decrease in levofloxacin susceptibility. Resistance evolution developed over time for TZP and tobramycin. There was a two-fold increase in the MIC of TZP over time for Patients 1 and 3. Resistance development was greatest with tobramycin and was observed in all three patients over time.

3.5. Biofilm inhibitory concentration analysis

Among the MV patient isolates, the BIC was found to be greater than the MIC for all antibiotics except one (i.e. the BIC for MV5 tested against ceftazidime remained stable) (Table 2). The β -lactam antibiotics exhibited the greatest increase in antibiotic resistance (BICs $\geq$ 256 μ g/mL for five of the six isolates). Levofloxacin BICs were found to be two to five doubling dilutions higher than the corresponding MIC; tobramycin BICs were found to be three to five doubling dilutions higher than the corresponding MIC.

Among the CF isolates, the increase in BIC over MIC was modest compared with the MV isolates. Among the β -lactam antibiotics, the greatest increases were observed with TZP. Levofloxacin and tobramycin exhibited the greatest variability in BICs over time. Tobramycin BICs were found to range from 0.25 $\times$ MIC (CF1, Day 29 isolate) to $\geq$ 8 $\times$ MIC. This result was confirmed with repeat testing. A decrease in BIC was also observed for

levofloxacin (CF2, Day 62). The temporal evolution of biofilm resistance was most pronounced with isolates tested against levofloxacin and tobramycin.

3.6. Correlation between biofilm resistance (BIC) and biofilm formation

Significant positive correlations between BIC and biofilm formation existed for imipenem ($r = 0.83$, $P < 0.001$), levofloxacin ($r = 0.57$, $P < 0.021$) and ceftazidime ($r = 0.62$, $P < 0.01$), suggesting that resistance towards these antimicrobials strongly correlated with biofilm formation (Fig. 3). No significant correlation was observed between biofilm formation and tobramycin ($r = 0.41$, $P < 0.107$). A negative correlation was observed between biofilm formation and TZP, although this was not statistically significant ($r = 0.32$, $P < 0.194$).

4. Discussion

Biofilms have been implicated in virtually every human infection and are particularly recalcitrant to antibiotic compounds and can persist despite sustained host defences. Biofilm formation is an important component of pulmonary colonisation and infection due to *P. aeruginosa* both in acute lung infections such as VAP and in chronic lung infections such as CF. Understanding bacterial physiology and the mechanisms by which *P. aeruginosa* protects itself from lethal concentrations of antibiotics is fundamental to designing interventions against resistant strains. This study compared the biofilm-forming ability of *P. aeruginosa* newly colonising the airway of MV patients and patients with longstanding CF during periods of chronic stability and acute pulmonary exacerbation and its associated effect on antibiotic susceptibility.

Biofilm-forming ability was greatest among *P. aeruginosa* biofilms from newly colonised MV patients. Enhanced biofilm formation by isolates from ETAs suggests that these strains may have originated from *P. aeruginosa* biofilms formed on the inner lumen of the ETT. Biofilm formation on the ETT is facilitated by multiple factors, including the polyvinylchloride plastic surface of the ETT, the lack of host defence mechanisms in the inner lumen and the accumulation of tracheobronchial secretions. Biofilms can then be dislodged from the ETT during routine suctioning and disseminate into the lower respiratory tract, contributing to the development of VAP. Whilst biofilm formation of *P. aeruginosa* isolates colonising the ETT was high among the MV patients, only 50% of patients went on to develop VAP. Adair et al. [7] showed that while 100% of patients (20/20) with VAP had ETTs covered with biofilm, 30% of patients (6/20) without VAP also had ETTs colonised with biofilms. Thus, while ETT colonisation is an important contributor to VAP, it is only one of many notable risk factors, including the severity of the patient's underlying disease, prior surgery and prior exposure to antibiotics [15,16].

In contrast, biofilm formation was lowest among the CF isolates and was highly variable among sequential clonal isolates. There was no correlation between biofilm formation and pulmonary status (e.g. chronic infection versus acute pulmonary exacerbation). The variability in biofilm formation among *P. aeruginosa* CF isolates with similar genotypic profiles is supported by others [17]. However, biofilm formation in relation to pulmonary status in sequential clonal isolates in CF patients has never been investigated. Differences in host factors and antibiotic selection pressure may account for the variability in biofilm formation and pulmonary status in CF patients. Impaired mucociliary clearance, the thick mucus layer overlying lung epithelium, and the presence of a microaerophilic or anaerobic environment are all factors that encourage the formation of *P. aeruginosa* biofilms in CF [18]. The reason for the heterogeneity in biofilm formation between MV and CF patients is likely multifactorial. Biofilms formed on living tissue have been shown to be morphologically different from biofilms formed on abiotic surfaces such as ETTs. Other

factors include differences in host defences, prior antibiotic exposure and the differential expression of bacterial virulence factors [19].

Comparison of MICs and BICs demonstrated a substantial difference in antibiotic resistance among the MV isolates. The greatest increase in biofilm antibiotic resistance occurred with the β -lactam antibiotics, with the majority of isolates exhibiting BICs $\geq 256 \mu\text{g/mL}$. In contrast, the differences between the BIC and MIC for CF isolates were not as pronounced. At subinhibitory imipenem concentrations, imipenem has been shown to induce *ampC* production, increase biofilm volume and increase alginate production in *P. aeruginosa* PA01 biofilms and to induce biofilm formation in MDR *Acinetobacter baumannii* [20,21]. Receipt of subtherapeutic concentrations of imipenem in MV patients likely contributed to the increased biofilm formation and potentially increased *ampC* expression. This increased *ampC* expression, in turn, may explain the increased ceftazidime and TZP resistance observed in this study. In contrast, the majority of CF isolates were susceptible to imipenem and none of the patients received imipenem for treatment of their acute pulmonary exacerbations, leading to potentially less induction of biofilm formation and *ampC* expression. Tobramycin has also been shown to induce biofilm formation at subinhibitory concentrations in PA01. However, biofilm induction occurred in only approximately one-half of CF clinical isolates in a recent study and was independent of tobramycin susceptibility [22]. This may explain the variability in biofilm-forming ability and BIC observed among the CF isolates in this study. It was also observed that resistance evolution in biofilms occurred rapidly, whilst resistance evolution in the planktonic mode of lifestyle remained relatively unchanged over time.

Regression analysis of biofilm formation with antimicrobial resistance demonstrated statistically significant positive correlations for three of the five antimicrobials tested. This suggests that physiological aspects specific to biofilms, such as exopolysaccharide production, biofilm-specific efflux pump expression or β -lactamase production, played a role in increased biofilm antimicrobial resistance. Interestingly, a negative correlation existed between biofilm formation and TZP resistance. Subinhibitory concentrations of TZP have been shown to lead to a decrease in bacterial adherence and biofilm formation among *P. aeruginosa* clinical isolates [23]. In general, increased antimicrobial resistance and biofilm formation were associated, underlining the importance of this mode of lifestyle in clinical infections and when considering treatment options.

Because of the important role of biofilms in the pathophysiology of VAP and CF, strategies targeting the biofilm have been developed. These include selective decontamination of the ETT and use of antiseptic- or antimicrobial-impregnated ETTs for prevention of VAP. In the North American Silver-Coated Endotracheal Tube (NASCENT) study, Kollef et al. [24] randomised 2003 patients expected to require MV for ≥ 24 h to undergo intubation with either a silver-coated ETT or a conventional uncoated ETT. Compared with the uncoated ETT group, the silver-coated ETT group had a lower incidence of microbiologically confirmed VAP [37/766 (4.8%) vs. 56/743 (7.5%); $P = 0.03$] and a statistically significant delay in the onset of VAP. Other strategies studied include removal of established biofilms and mucus secretions in the ETT using mechanical devices such as the Mucus Shaver. The Mucus Shaver has been shown to be effective in removing biofilm from the inner lumen of the ETT in intubated sheep [25,26]. This technique is promising but further studies will be needed to determine whether removal of the biofilm is effective in preventing VAP. Whilst silver-coated ETTs are currently recommended by some groups, the Mucus Shaver cannot be recommended at this time in the absence of clinical data [27].

Inhaled antibiotics (e.g. tobramycin and colistin) have also been studied as a method to prevent biofilm formation in the ETT and as adjunctive therapy for the treatment of VAP

[28–33]. Compared with systemic administration of antibiotics, inhaled antibiotics can achieve higher pulmonary concentrations, with levels far in excess of planktonic MICs [34,35]. Falagas et al. [36] conducted a meta-analysis of the effect of antibiotics administered via the respiratory tract for the prevention of ICU pneumonia. The incidence of VAP was reduced but there was no overall effect on mortality. Three recently published studies compared adjunctive inhaled colistin or colistimethate plus intravenous (i.v.) antibiotics with i.v. antibiotics alone for the treatment of VAP [29,30,37]. Using a matched retrospective case–control design, Kofteridis et al. [29] found no difference in eradication of Gram-negative organisms, including *P. aeruginosa*, in the treatment group compared with the control group [17/34 patients (50%) vs. 19/42 patients (45%); $P = 0.679$]. There was a trend towards improved clinical cure with inhaled plus i.v. colistin, but no statistically significant differences in either all-cause mortality or VAP-related mortality. Using a retrospective cohort analysis, Korbila et al. [30] found inhaled colistin to be independently associated with VAP cure but also no improvement in mortality. In a recent randomised controlled trial, Rattanaumpawan et al. [37] found that microbiological eradication was significantly improved in the treatment group compared with the control group (60.9% vs. 38.2%, $P = 0.03$), but again no difference in clinical outcome. Thus, inhaled antibiotics may have a favourable effect on microbiological outcome, but this technique has not yet been shown to translate into improved patient outcomes.

The most promising strategy is to dose antibiotics based on biofilm susceptibilities in lieu of planktonic susceptibilities. Keays et al. [38] retrospectively reviewed 110 CF patients who received antibiotic treatment for acute pulmonary exacerbation. Patients who received antibiotic combinations that inhibited bacteria (based on BIC testing) had a decrease in sputum bacterial density and hospital length of stay. Clinical trials examining the effectiveness of antibiotic dosing based on biofilm susceptibilities in CF are currently ongoing [39,40]. Whilst this strategy has not yet been studied for the treatment of VAP, this is an area of potential benefit that will require further investigation.

This study is associated with certain limitations. Previous investigators have suggested that use of more mature biofilms may be more representative of in vivo conditions in CF and exhibit increased antibiotic resistance compared with biofilms formed after only 24 h [41]. However, ETAs were collected from MV patients on a daily basis, thus the biofilm formed on the ETT of these patients would likely be more representative of young biofilm. Use of a 24-h biofilm model allowed direct comparisons between the CF and MV isolates to be made. Owing to the small number of MV and CF patients examined, additional studies examining biofilm formation and resistance in larger cohorts of patients will be needed.

These results indicate that the effect of antibiotics on biofilm formation is highly variable and unpredictable, with some antibiotics inducing biofilm formation while other antibiotics inhibit biofilm formation in these two patient populations. This unpredictability in biofilm formation and BIC make it increasingly difficult for clinicians to choose the most active antibiotic, particularly as biofilm susceptibility testing is not routinely performed as part of clinical care. In addition, the rapid induction and evolution of biofilm resistance in clonal sequential CF isolates, whilst planktonic antibiotic resistance remains stable, is worrisome. Biofilm formation and the associated development of antimicrobial resistance is a multifactorial process dependent on the presence or absence of prosthetic material, host immune status and disease state. These are factors that will require further investigation in order to determine the optimal antibiotic therapy to successfully treat biofilm-mediated infections.

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References

1. Parsek MR, Greenberg EP. Sociomicrobiology: the connections between quorum sensing and biofilms. *Trends Microbiol.* 2005; 13:27–33. [PubMed: 15639629]
2. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science.* 1999; 284:1318–1322. [PubMed: 10334980]
3. El-Azizi M, Rao S, Kanchanapoom T, Khardori N. In vitro activity of vancomycin, quinupristin/dalfopristin, and linezolid against intact and disrupted biofilms of staphylococci. *Ann Clin Microbiol Antimicrob.* 2005; 4:2. [PubMed: 15638934]
4. Lynch SV, Dixon L, Benoit MR, Brodie EL, Keyhan M, Hu P, et al. Role of the *rapA* gene in controlling antibiotic resistance of *Escherichia coli* biofilms. *Antimicrob Agents Chemother.* 2007; 51:3650–3658. [PubMed: 17664315]
5. Sottile FD, Marrie TJ, Prough DS, Hobgood CD, Gower DJ, Webb LX, et al. Nosocomial pulmonary infection: possible etiologic significance of bacterial adhesion to endotracheal tubes. *Crit Care Med.* 1986; 14:265–270. [PubMed: 3956213]
6. Feldman C, Kassel M, Cantrell J, Kaka S, Morar R, Goolam Mahomed A, et al. The presence and sequence of endotracheal tube colonization in patients undergoing mechanical ventilation. *Eur Respir J.* 1999; 13:546–551. [PubMed: 10232424]
7. Adair CG, Gorman SP, Feron BM, Byers LM, Jones DS, Goldsmith CE, et al. Implications of endotracheal tube biofilm for ventilator-associated pneumonia. *Intensive Care Med.* 1999; 25:1072–1076. [PubMed: 10551961]
8. Kobayashi H, Kobayashi O, Kawai S. Pathogenesis and clinical manifestations of chronic colonization by *Pseudomonas aeruginosa* and its biofilms in the airway tract. *J Infect Chemother.* 2009; 15:125–142. [PubMed: 19554398]
9. Yang K, Zhuo H, Guglielmo BJ, Wiener-Kronish J. Multidrug-resistant *Pseudomonas aeruginosa* ventilator-associated pneumonia: the role of endotracheal aspirate surveillance cultures. *Ann Pharmacother.* 2009; 43:28–35. [PubMed: 19033484]
10. Garnacho-Montero J, Sa-Borges M, Sole-Violan J, Barcenilla F, Escobedo-Ortega A, Ochoa M, et al. Optimal management therapy for *Pseudomonas aeruginosa* ventilator-associated pneumonia: an observational, multicenter study comparing monotherapy with combination antibiotic therapy. *Crit Care Med.* 2007; 35:1888–1895. [PubMed: 17581492]
11. Mahenthiralingam E, Campbell ME, Foster J, Lam JS, Speert DP. Random amplified polymorphic DNA typing of *Pseudomonas aeruginosa* isolates recovered from patients with cystic fibrosis. *J Clin Microbiol.* 1996; 34:1129–1135. [PubMed: 8727889]
12. O'Toole GA, Kolter R. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol Microbiol.* 1998; 30:295–304. [PubMed: 9791175]
13. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; nineteenth informational supplement. Wayne, PA: CLSI; 2009. Document M100-S-19
14. Moskowitz SM, Foster JM, Emerson J, Burns JL. Clinically feasible biofilm susceptibility assay for isolates of *Pseudomonas aeruginosa* from patients with cystic fibrosis. *J Clin Microbiol.* 2004; 42:1915–1922. [PubMed: 15131149]
15. American Thoracic Society; Infectious Diseases Society of America. Guidelines for the management of adults with hospital-acquired, ventilator-associated, and healthcare-associated pneumonia. *Am J Respir Crit Care Med.* 2005; 171:388–416. [PubMed: 15699079]

16. Craven DE, Steger KA. Nosocomial pneumonia in mechanically ventilated adult patients: epidemiology and prevention in 1996. *Semin Respir Infect.* 1996; 11:32–53. [PubMed: 8885061]
17. Deligianni E, Pattison S, Berrar D, Ternan NG, Haylock RW, Moore JE, et al. *Pseudomonas aeruginosa* cystic fibrosis isolates of similar RAPD genotype exhibit diversity in biofilm forming ability in vitro. *BMC Microbiol.* 2010; 10:38. [PubMed: 20141637]
18. Davies JC, Bilton D. Bugs, biofilms, and resistance in cystic fibrosis. *Respir Care.* 2009; 54:628–640. [PubMed: 19393107]
19. Sadikot RT, Blackwell TS, Christman JW, Prince AS. Pathogen–host interactions in *Pseudomonas aeruginosa* pneumonia. *Am J Respir Crit Care Med.* 2005; 171:1209–1223. [PubMed: 15695491]
20. Bagge N, Schuster M, Hentzer M, Ciofu O, Givskov M, Greenberg EP, et al. *Pseudomonas aeruginosa* biofilms exposed to imipenem exhibit changes in global gene expression and β -lactamase and alginate production. *Antimicrob Agents Chemother.* 2004; 48:1175–1187. [PubMed: 15047518]
21. Nucleo E, Steffanoni L, Fugazza G, Migliavacca R, Giacobone E, Navarra A, et al. Growth in glucose-based medium and exposure to subinhibitory concentrations of imipenem induce biofilm formation in a multidrug-resistant clinical isolate of *Acinetobacter baumannii*. *BMC Microbiol.* 2009; 9:270. [PubMed: 20028528]
22. Elliott D, Burns JL, Hoffman LR. Exploratory study of the prevalence and clinical significance of tobramycin-mediated biofilm induction in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *Antimicrob Agents Chemother.* 2010; 54:3024–3026. [PubMed: 20404125]
23. Fonseca AP, Extremina C, Fonseca AF, Sousa JC. Effect of subinhibitory concentration of piperacillin/tazobactam on *Pseudomonas aeruginosa*. *J Med Microbiol.* 2004; 53:903–910. [PubMed: 15314198]
24. Kollef MH, Afessa B, Anzueto A, Veremakis C, Kerr KM, Margolis BD, et al. Silver-coated endotracheal tubes and incidence of ventilator-associated pneumonia: the NASCENT randomized trial. *JAMA.* 2008; 300:805–813. [PubMed: 18714060]
25. Kolobow T, Berra L, Li Bassi G, Curto F. Novel system for complete removal of secretions within the endotracheal tube: the Mucus Shaver. *Anesthesiology.* 2005; 102:1063–1065. [PubMed: 15851896]
26. Berra L, Curto F, Li Bassi G, Laquerriere P, Baccarelli A, Kolobow T. Antibacterial-coated tracheal tubes cleaned with the Mucus Shaver: a novel method to retain long-term bactericidal activity of coated tracheal tubes. *Intensive Care Med.* 2006; 32:888–893. [PubMed: 16601964]
27. Lorente L, Blot S, Rello J. New issues and controversies in the prevention of ventilator-associated pneumonia. *Am J Respir Crit Care Med.* 2010; 182:870–876. [PubMed: 20448095]
28. Adair CG, Gorman SP, Byers LM, Jones DS, Feron B, Crowe M, et al. Eradication of endotracheal tube biofilm by nebulised gentamicin. *Intensive Care Med.* 2002; 28:426–431. [PubMed: 11967596]
29. Kofteridis DP, Alexopoulou C, Valachis A, Maraki S, Dimopoulou D, Georgopoulos D, et al. Aerosolized plus intravenous colistin versus intravenous colistin alone for the treatment of ventilator-associated pneumonia: a matched case–control study. *Clin Infect Dis.* 2010; 51:1238–1244. [PubMed: 20973727]
30. Korbila IP, Michalopoulos A, Rafailidis PI, Nikita D, Samonis G, Falagas ME. Inhaled colistin as adjunctive therapy to intravenous colistin for the treatment of microbiologically documented ventilator-associated pneumonia: a comparative cohort study. *Clin Microbiol Infect.* 2010; 16:1230–1236. [PubMed: 19732088]
31. Kwa AL, Loh C, Low JG, Kurup A, Tam VH. Nebulized colistin in the treatment of pneumonia due to multidrug-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. *Clin Infect Dis.* 2005; 41:754–757. [PubMed: 16080101]
32. Michalopoulos A, Fotakis D, Virtzili S, Vletsas C, Raftopoulou S, Mastora Z, et al. Aerosolized colistin as adjunctive treatment of ventilator-associated pneumonia due to multidrug-resistant Gram-negative bacteria: a prospective study. *Respir Med.* 2008; 102:407–412. [PubMed: 18060758]

33. Michalopoulos A, Kasiakou SK, Mastora Z, Rellos K, Kapaskelis AM, Falagas ME. Aerosolized colistin for the treatment of nosocomial pneumonia due to multidrug-resistant Gram-negative bacteria in patients without cystic fibrosis. *Crit Care*. 2005; 9:R53–R59. [PubMed: 15693967]
34. Palmer LB, Smaldone GC, Simon SR, O'Riordan TG, Cuccia A. Aerosolized antibiotics in mechanically ventilated patients: delivery and response. *Crit Care Med*. 1998; 26:31–39. [PubMed: 9428540]
35. Geller DE, Pitlick WH, Nardella PA, Tracewell WG, Ramsey BW. Pharmacokinetics and bioavailability of aerosolized tobramycin in cystic fibrosis. *Chest*. 2002; 122:219–226. [PubMed: 12114362]
36. Falagas ME, Siempos II, Bliziotis IA, Michalopoulos A. Administration of antibiotics via the respiratory tract for the prevention of ICU-acquired pneumonia: a meta-analysis of comparative trials. *Crit Care*. 2006; 10:R123. [PubMed: 16934148]
37. Rattanaumpawan P, Lorsutthitham J, Ungprasert P, Angkasekwinai N, Thamlikitkul V. Randomized controlled trial of nebulized colistimethate sodium as adjunctive therapy of ventilator-associated pneumonia caused by Gram-negative bacteria. *J Antimicrob Chemother*. 2010; 65:2645–2649. [PubMed: 20876621]
38. Keays T, Ferris W, Vandemheen KL, Chan F, Yeung SW, Mah TF, et al. A retrospective analysis of biofilm antibiotic susceptibility testing: a better predictor of clinical response in cystic fibrosis exacerbations. *J Cyst Fibros*. 2009; 8:122–127. [PubMed: 19064337]
39. Bethesda, MD: Use of a biofilm antimicrobial susceptibility assay to guide antibiotic therapy. ClinicalTrials.gov. Identifier NCT00786513 <http://www.clinicaltrials.gov/ct2/show/NCT00786513>
40. Bethesda, MD: Standard vs. biofilm susceptibility testing in cystic fibrosis (CF). ClinicalTrials.gov. Identifier NCT00153634 <http://www.clinicaltrials.gov/ct2/show/NCT00153634>
41. Tre-Hardy M, Mace C, El Manssouri N, Vanderbist F, Traore H, Devleeschouwer MJ. Effect of antibiotic co-administration on young and mature biofilms of cystic fibrosis clinical isolates: the importance of the biofilm model. *Int J Antimicrob Agents*. 2009; 33:40–45. [PubMed: 18801647]

Fig. 1. Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) analysis of *Pseudomonas aeruginosa* strains from newly colonised mechanically ventilated patients and from cystic fibrosis (CF) patients during periods of clinical stability and acute pulmonary exacerbation. Codes for CF strains indicate patient number and day of isolation.

Fig. 2. Biofilm-forming ability of isolates from six ventilated patients newly colonised with multidrug-resistant *Pseudomonas aeruginosa* and sequential isolates from three cystic fibrosis (CF) patients. Codes for CF strains indicate patient number and day of isolation. CS, clinical stability; AE, acute pulmonary exacerbation.

Fig. 3.
Regression analysis of biofilm formation and antimicrobial resistance [biofilm inhibitory concentration (BIC)] for each of the five antibiotics tested in the study.

Clinical characteristics of mechanically ventilated and cystic fibrosis patients at the time *Pseudomonas aeruginosa* isolates were obtained

Table 1

Mechanically ventilated patients										
Patient	Age (years)	Gender	Admitting diagnosis	APACHE II score at ICU admission	Days of ventilation prior to colonisation	ICU days	VAP	Died	Cause of death	Antibiotics at time of colonisation
MV1	53	M	ARDS	26	2	10	N	Y	Sepsis/ARDS	Tobramycin, imipenem
MV2	65	F	Acute renal failure, urosepsis/acute pancreatitis	28	25	80	Y	N		Meropenem, linezolid
MV3	79	M	Cardiogenic shock	29	18	29	Y	N		None
MV4	64	M	Acute pancreatitis	15	22	43	N	Y	Sepsis	Meropenem
MV5	50	F	Liver failure	28	13	18	Y	Y	Multisystem organ failure	Vancomycin, linezolid
MV6	65	M	Liver failure	27	26	31	N	Y	Multisystem organ failure	Linezolid
Cystic fibrosis patients										
Patient	Age (years)	Gender	FEV ₁	Clinical stability (CS) versus acute pulmonary exacerbation (AE)		Antibiotics at time of positive culture				
CF1	30	F								
Day 1			1.5	CS						None
Day 28			1.2	AE						None
Day 29			1.2	AE						TZP, tobramycin
Day 30			1.26	AE						TZP, tobramycin
Day 84			1.5	CS						None
CF2	54	M								
Day 1			1.24	AE						Ceftazidime, levofloxacin, TMP/SMX
Day 2			1.3	AE						Ceftazidime, levofloxacin, TMP/SMX
Day 62			1.3	CS						None
CF3	28	F								
Day 1			1.2	CS						None
Day 61			0.99	AE						Ceftazidime, tobramycin, TMP/SMX

APACHE, Acute Physiology and Chronic Health Evaluation; ICU, Intensive Care Unit; VAP, ventilator-associated pneumonia; ARDS, acute respiratory distress syndrome; FEV₁, forced expiratory volume in 1 s; TZP, piperacillin/tazobactam; TMP/SMX, trimethoprim/sulfamethoxazole.

Minimum inhibitory concentration (MIC; in µg/mL) and biofilm inhibitory concentration (BIC; in µg/mL) of *Pseudomonas aeruginosa* isolates from mechanically ventilated and cystic fibrosis patients

Table 2

Patient isolate	Ceftazidime		TZP		Imipenem		Levofloxacin		Tobramycin	
	MIC	BIC	MIC	BIC	MIC	BIC	MIC	BIC	MIC	BIC
Mechanically ventilated patient isolates										
MV1	4	256	8	256	16	>256	2	8	2	16
MV2	32	256	128	256	16	>256	8	256	4	64
MV3	32	256	8	256	4	>256	8	256	2	32
MV4	64	256	128	256	8	>256	8	256	4	128
MV5	16	16	64	256	8	128	8	128	2	64
MV6	32	256	64	256	16	>256	8	128	4	128
% MV strains BIC>MIC	83		100		100		100		100	
Cystic fibrosis patient isolates										
CF1										
Day 1	4	4	16	128	4	8	8	16	4	16
Day 28	4	4	16	256	4	4	8	32	4	4
Day 29	4	4	16	256	4	8	8	64	4	1
Day 30	4	8	16	256	4	4	2	64	4	32
Day 84	4	8	64	256	8	8	8	128	8	32
CF2										
Day 1	1	1	8	32	4	2	2	8	4	32
Day 2	1	1	8	128	4	32	2	16	2	64
Day 62	1	16	8	>256	4	32	2	2	16	>256
CF3										
Day 1	16	64	32	>256	8	32	4	8	2	4
Day 61	16	>256	128	>256	8	64	4	16	16	64
% CF strains BIC>MIC	50		100		60		90		80	