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A retrospective analysis of biofilm antibiotic susceptibility testing: A better predictor of clinical response in cystic fibrosis exacerbations ☆

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Abstract

Background: Bacteria grow as biofilms within CF airways. However, antibiotic susceptibility testing is routinely performed on planktonically-growing bacteria. This study assessed whether CF patients infected with multiresistant organisms had improved clinical outcomes if given antibiotics that inhibited their biofilm-grown bacteria.
Methods: 110 patients with pulmonary exacerbations were treated with intravenous antibiotics based on susceptibility testing of planktonically-growing bacteria. A retrospective analysis was done using bacterial isolates grown from their sputum at exacerbation. Each isolate was grown as a biofilm and combination antibiotic susceptibility testing was performed. Clinical outcomes in patients treated with biofilm-susceptible antibiotics were compared to those that were not.
Results: 66 of 110 patients (60%) were treated with antibiotic combinations that inhibited all of their planktonically-grown bacterial isolates, however, when the same isolates were grown as biofilms, only 24 patients (22%) had all of their biofilm-grown isolates remaining susceptible to the antibiotics ($P=<0.001$). When patients with at least one biofilm-grown susceptible isolate ($n=61$) were compared to those with none ($n=49$), there was a significant decrease in sputum bacterial density ($P=0.02$) and length of stay ($P=0.04$) and a non-significant decrease in treatment failure. Survival analyses of time to next exacerbation showed non-significant trends favoring patients treated with biofilm-effective antibiotics.
Conclusions: Most patients with CF exacerbations do not receive antibiotics that inhibit all biofilm-grown bacteria from their sputum at exacerbation. Patients treated with biofilm-effective therapy seemed to have improved clinical outcomes.

Keywords: Bacterial biofilms; Clinical outcomes; Cystic fibrosis; Microbiology

1. Introduction

Most adults with cystic fibrosis (CF) suffer from chronic bacterial airway infections. These infections are almost impossible to eradicate due to their multi-drug resistant nature. This resistance is likely caused by bacterial growth in biofilms and selective mutational pressures induced by repeated courses of antibiotic treatment [1]. The refractory nature of these infections poses a challenge to clinicians when deciding which combinations of antibiotics to use in the treatment of CF exacerbations associated with multiresistant bacterial airway infections.

A bacterial biofilm is defined as a community of microorganisms that adhere to a surface, encased within an extracellular polysaccharide matrix [2]. The bacterial cells within the biofilm communicate by a process known as quorum-sensing in order to coordinate formation of the biofilm. Biofilm-grown organisms show increased resistance to antibiotics, either because of decreased penetration of the antibiotics into the

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biofilm or because of the expression of more complex biofilm-specific resistance mechanisms [3–5]. Evidence now exists that bacteria grow as biofilms within CF airways [6,7] and that these biofilm-grown bacteria are less susceptible to antibiotics than planktonically grown bacteria [8–10].

Conventionally, hospital microbiology laboratories have employed standard culture and susceptibility assays where individual antibiotics are tested against planktonically grown bacterial isolates from CF sputum. More recently, combinations of antibiotics have been tested *in vitro* against multiresistant bacteria using antibiotic synergy testing or the multiple combination bactericidal antibiotic testing (MCBT) technique [11]. However, a randomized, double-blind, controlled trial of MCBT versus standard culture and susceptibility techniques did not show improved clinical outcomes in CF exacerbations when the bacterial isolates were grown in planktonic culture [12].

Given that bacteria in the CF lung may grow as biofilms and that the biofilm-grown bacteria are susceptible to different antibiotic combinations than those grown by conventional means, we hypothesized that growing the bacteria as biofilms and testing them against combinations of antibiotics may yield information to support better antibiotic choices against CF lung infections and result in improved clinical outcomes. The objective of this study was to retrospectively analyze whether CF patients treated with antibiotic combinations that inhibited growth of biofilm-grown bacteria retrieved from their sputa experienced improved clinical outcomes following a pulmonary exacerbation compared to those who were treated with antibiotics that did not inhibit biofilm-growth of their sputum bacteria.

2. Methods

A retrospective analysis was done using bacterial isolates and clinical data from a randomised, controlled trial assessing outcomes following CF exacerbations in patients treated according to combination antibiotic susceptibility testing versus conventional culture and susceptibility testing [12].

2.1. Study subjects

Patients 12 years and older were included in the trial if they had a confirmed diagnosis of cystic fibrosis and known chronic infection with multiresistant *Burkholderia cepacia*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* or *Achromobacter xylosoxidans* bacteria (at least two sputum cultures within the past 12 months that had grown these organisms). Patients were excluded if they were pregnant, unable to produce sputum, had a history of lung transplantation or had previously participated in the trial. All enrolled patients signed informed consent and the ethics committee at each participating hospital approved the study.

2.2. Study design

Patients were enrolled from 10 sites. Sputum samples were collected at exacerbation before intravenous antibiotics and following 14 days of therapy. Bacterial isolates from the sputum were kept frozen from the time of the original trial.

Patients were randomized when they experienced an exacerbation of CF pulmonary symptoms that, in the opinion of the patient's physician, required intravenous antibiotics. A pulmonary exacerbation was defined according to the 1994 Cystic Fibrosis Foundation Microbiology and Infectious Disease Consensus Conference [13] as the presence of at least three of the following 11 new findings or changes in clinical status compared to the most recent baseline visit: cough; increased sputum production, change in appearance of expectorated sputum, or both; fever (≥ 38 ° C for at least 4 h in a 24 hour period) on more than one occasion in the previous week; weight loss ≥ 1 kg or 5% of bodyweight associated with anorexia and decreased dietary intake; school or work absenteeism (due to illness) in the previous week; increased respiratory rate, increased work of breathing, or both; new finding on chest examination (e.g. rales, wheezing, crackles); decreased exercise tolerance; decrease in FEV_1 of $\geq 10\%$ from previous baseline study within the past 3 months; decrease in hemoglobin saturation (as measured by oximetry) of $\geq 10\%$ from baseline value within the past 3 months; new finding on chest radiograph.

At exacerbation, the antibiotic combination was selected based on either conventional antibiotic susceptibility tests of plankontically-grown bacteria or MCBT susceptibility results of planktonically-grown bacteria, depending on which group the patient was randomized into [12]. Patients received any two intravenous antibiotics ± inhaled tobramycin.

All bacterial isolates from the sputum collected at exacerbation were later grown as biofilms. The biofilm-grown bacterial isolates were then tested against combinations of antibiotics using the MCBT technique, including the combination of antibiotics that the patient had received at the time of exacerbation.

Clinical outcomes for patients in whom at least one biofilm-grown isolate was susceptible to the prescribed antibiotic combination were compared to those in whom none of the isolates were susceptible. Also, patients in whom all biofilm-grown isolates were susceptible were compared to those in whom at least one isolate was not susceptible.

Outcomes measured over the 14 day treatment period included mean change in sputum bacterial density (measured as the difference in the sum of all the retrieved isolates from each sputum), absolute changes in FEV_1 and forced vital capacity (FVC), and changes in dyspnea as measured by the Transitional Dyspnea Index [14]. Other outcomes included time to next exacerbation, mean length of hospital stay and proportion of treatment failures. A treatment failure was defined as: requirement for patient transfer to an intensive care unit, or new requirement for assisted ventilation, or development of acute respiratory acidosis (arterial pH < 7.30, with arterial pCO_2 - 48 mm Hg, or unremitting fever >38 °C for 5 days while on study antibiotic therapy, or other evidence of clinical deterioration while taking study antibiotics which in the opinion of the local study physician required urgent administration of alternative open-label antibiotics.

3. Methods

Bacterial biofilms were grown from each of the bacterial isolates from the sputum collected at the time of exacerbation using a modified version of the Calgary biofilm technique [15]. The organism inoculum for the biofilm consisted of 100 uL of a 0.5 MacFarland turbidity standard added to each well of a 96 well, round-bottom microtitre plate with a peg lid (Nunc Inc., Roskilde, Denmark). The plates with peg lid were incubated, in the absence of antibiotics, at 35 °C on a rocking table (Bellco Glass Inc., Vineland, NJ) for 18–24 h overnight to allow for bacterial biofilm formation on the pegs.

Microtitre plates containing antibiotics in double (two intravenous antibiotics) or triple (two intravenous antibiotics and inhaled tobramycin 300 mg twice daily) combinations were prepared as previously described [12]. The plate included one well containing the combination of antibiotics that the patient received at the time of exacerbation, and wells that served as growth and sterility controls (no antibiotics and no organism in the well, respectively). The biofilm-laden peg lids were placed in the antibiotics and incubated at 35 °C on a rocking table. After 48 h of exposure to antibiotics, the peg lids were rinsed in sterile broth, placed in a fresh microtitre plate, and sonicated for 5 min to remove the biofilm. The peg lid was discarded and the plate with the sonicated bacteria was incubated overnight. The following day, the wells were examined for turbidity. If there was no growth in the well, the antibiotic combination was assumed to have eradicated the biofilm.

3.1. Analysis

Statistical analysis was done using SAS version 9. Continuous variables were analyzed by independent *t* test. The proportion of treatment failures was compared using an unadjusted Fisher exact test. Kaplan-Meier survival curves were used to describe time to next exacerbation.

In addition, linear regression procedures were employed to adjust continuous outcomes for imbalances in infection rates with *B. cepacia* or A *xylosoxidans* between the two groups. Hazard ratios for time to next exacerbation were also computed using Cox proportional hazards models with adjustment for *B. cepacia* and *A. xylosoxidans* infection.

4. Results

One hundred and thirty-two patients had been included in the original clinical trial however 22 of these patients were not included in the current study for the following reasons: their isolates were not frozen for storage (13 patients), their isolates were missing (3 patients), or their frozen isolates did not grow when thawed (6 patients). The remaining 110 patients had the original bacterial isolates from the time of their pulmonary exacerbation available for biofilm susceptibility testing, and these patients were included in the current study.

Two hundred thirty-four bacterial isolates from these 110 patients with acute CF pulmonary exacerbations were analyzed. Biofilm growth controls (tested in the absence of antibiotics) were positive for each of these isolates, suggesting that all of these isolates were able to successfully grow in-vitro as bacterial biofilms.

When the bacterial isolates were grown planktonically as part of the original trial, 66 of the 110 patients (60%) included in the current study were treated with antibiotic combinations that inhibited all of their planktonically-grown bacterial isolates. However, when the bacteria were grown as a biofilm, only 24 patients (22%) had all of their biofilm-grown isolates remaining susceptible to the antibiotics ($P=<0.001$). Sixty-one patients (55%) were treated with intravenous antibiotic combinations that inhibited at least one of the biofilm-grown bacterial isolates retrieved at exacerbation.

Clinical outcomes for the patients in whom at least one biofilm-grown isolate was susceptible to the received antibiotic combination ($n=61$ patients, 154 isolates) were compared to patients in whom none were susceptible ($n=49$ patients, 80 isolates). The baseline characteristics for these patients are listed in Table 1. The antibiotic combinations most commonly received by the patients are listed in Table 2. There was a significant decrease in bacterial density after 2 weeks of antibiotic treatment ($P=0.02$) in the patients in whom at least one isolate was susceptible compared to those with no susceptible isolates (Table 3). These patients had a shorter mean hospital length of stay (13.3 days vs. 17.4 days, $P=0.04$). The treatment failure rate was 3.2% (2/61 patients) in those with at least one biofilm-grown susceptible isolate, compared to 12.2% (6/49 patients) in those with no susceptible isolates, although this difference did

Table 1
Baseline characteristics of included patients

	At least one biofilm-grown isolate susceptible ($n=61$)	No biofilm-grown isolates susceptible ($n=49$)
Age (SD)	27.2 (7.1)	27.9 (8.7)
Sex (male:female)	27:34	26:23
Body-mass index in kg/m^2 (SD)	20.3 (5.0)	22.1 (4.3)
Number of isolates	154	80
Infecting organism		
Pseudomonas aeruginosa	34 (56%)	16 (33%)
Burkholderia cepacia complex	13 (21%)	20 (41%)
Achromobacter xylosoxidans	0 (0%)	5(10%)
Stenotrophomonas maltophilia	2 (3%)	1(2%)
P. aeruginosa+B. cepacia	11 (18%)	4 (8%)
P. aeruginosa+A. xylosoxidans	0 (0%)	2 (4%)
P. aeruginosa+S. maltophilia	1 (2%)	0 (0%)
S. maltophilia+A. xylosoxidans	0 (0%)	1 (2%)
Baseline lung function		
FEV_1 in L (SD)	1.67 (0.69)	1.66 (0.69)
FEV_1 % predicted (SD)	48.7 (20.5)	46.6 (18.1)
FVC in L (SD)	2.79 (0.92)	2.78 (0.98)
FEV_1/FVC % (SD)	59.2 (11.4)	57.8 (13.8)
Lung function on day of exacerbation		
FEV_1 in L (SD)	1.50 (0.66)	1.46 (0.62)
FEV_1 % predicted (SD)	43.3 (18.1)	40.7 (17.0)
FVC in L (SD)	2.48 (0.91)	2.45 (0.94)
FEV_1/FVC % (SD)	58.5 (10.7)	60.5 (11.4)
Oxygen saturation % (SD)	94.4 (3.0)	93.2 (4.8)

SD=standard deviation.

Table 2
Antibiotic combinations most commonly received by patients in whom at least one biofilm-grown isolate was susceptible and patients in whom no biofilm-grown isolates were susceptible

Antibiotic combinations	At least one biofilm-grown isolate susceptible ($n=61$)	No biofilm-grown isolates susceptible ($n=49$)
Tobramycin+meropenem	17 (28%)	13 (27%)
Tobramycin+ceftazidime	4 (7%)	8 (16%)
Piperacillin-tazobactam+ceftazidime	4 (7%)	6 (12%)
Ciprofloxacin+ceftazidime	4 (7%)	5 (10%)
Ciprofloxacin+meropenem	7 (11%)	1 (2%)
Ceftazidime+meropenem	6 (10%)	1 (2%)
Trimethoprim-sulfamethoxazole+meropenem	4 (7%)	2 (4%)
Piperacillin-tazobactam+meropenem	5 (8%)	1 (2%)
Inhaled tobramycin as third antibiotic	51 (84%)	29 (59%)

not reach statistical significance ($P=0.14$). Changes in FEV1 and FVC, as well as the change in Transitional Dyspnea Index, were similar in the two groups (Table 3).

When patients in whom all biofilm-grown isolates were susceptible to the received antibiotic combination ($n=24$ patients, 46 isolates) were compared to those in whom at least one of the isolates was not susceptible ($n=86$ patients, 188 isolates), the groups did not have statistically different mean changes in sputum bacterial density or length of stay (Table 4). However, patients in whom all biofilm-grown isolates were susceptible to their received antibiotic combination tended to have improved lung function (FEV_1 and FVC) and better dyspnea scores after 14 days of antibiotic therapy, although these results fell slightly short of statistical significance.

Statistical analyses which adjusted for baseline imbalances in infection with *B. cepacia* or *A xylosoxidans* did not appreciably affect the results for any of the continuous outcome variables. For instance, the mean unadjusted difference in length of hospital stay for patients in whom at least one biofilm-grown bacterial isolate was susceptible to the received antibiotics compared to those with no susceptible isolates, was 4.3 days, and the mean adjusted difference in length of stay was 4.1 days ($P=0.04$, for both the adjusted and the unadjusted comparisons). Similarly, the mean unadjusted difference in FEV_1 was 0.03 L, and the mean adjusted difference in FEV_1 was 0.01 L ($P=0.73$ and 0.93, respectively).

Survival analyses of the time to next exacerbation using Kaplan-Meier curves showed non-significant trends in both groups favoring patients treated with antibiotics that inhibited their biofilm-grown airway bacteria. The hazard ratio was 0.88 for at least one isolate susceptible compared to no isolates susceptible (95% CI 0.60–1.29, $P=0.51$) and the hazard ratio was 0.75 for all isolates susceptible compared to not all isolates susceptible (95% CI .47–1.20, $P=0.23$) (Fig. 1).When the survival analysis was adjusted for infection with *B. cepacia* or A *xylosoxidans,* the trend to prolonged time to next exacerbation was further enhanced for the groups treated with antibiotics that inhibited their biofilm-grown airway bacteria. The adjusted hazard ratios decreased from 0.88 to 0.76 (adjusted $P=0.21$) and from 0.75 to 0.69 (adjusted $P=0.13$), respectively.

Table 3
Changes in lung function, dyspnea, hospital length of stay, treatment failure rates and sputum bacterial density in patients in whom at least one biofilm-grown isolate was susceptible compared to patients in whom no biofilm-grown isolates were susceptible

	At least one biofilm-grown isolate susceptible ($n=61$)	No biofilm-grown isolates susceptible ($n=49$)	P-value
Mean change in sputum bacterial density from day 0 to 14 in CFU/ml	-6.26×10^7	-1.50×10^7	0.02
Changes in FEV1 in L, day 0 to 14	0.26 (0.38)	0.23 (0.35)	0.73
Changes in FVC in L, day 0 to 14	0.39 (0.46)	0.38 (0.45)	0.85
Transitional Dyspnea Index score, day 14	6.36 (3.49)	5.31 (4.03)	0.15
Treatment failure rates (%)	2/61 (3.2)	6/49 (12.2)	0.14
Patients infected with *B. cepacia* or A *xylosoxidans*	1/24	4/32	0.38
Patients not infected with *B. cepacia* and A *xylosoxidans*	1/37	2/17	0.23
Mean hospital length of stay in days	13.3 (6.9)	17.4 (11.3)	0.04

Table 4
Changes in lung function, dyspnea, hospital length of stay, treatment failure rates and sputum bacterial density in patients in whom all biofilm-grown isolates were susceptible compared to patients in whom at least one biofilm-grown isolate was not susceptible

	All biofilm-grown isolates susceptible ($n=24$)	At least one biofilm-grown isolate not susceptible ($n=86$)	P-Value
Mean change in sputum bacterial density from day 0 to 14 in CFU/ml	-3.82×10^7	-3.96×10^7	0.94
Changes in FEV1 in L, day 0 to 14 (SD)	0.35 (0.27)	0.22 (0.38)	0.18
Changes in FVC in L, day 0 to 14 (SD)	0.54 (0.39)	0.35 (0.46)	0.11
Transitional Dyspnea Index score, day 14 (SD)	6.95 (2.66)	5.61 (3.96)	0.06
Treatment failure rates (%)	2/24 (8.3)	6/86 (7.0)	1.00
Patients infected with *B. cepacia* or *A xylosoxidans*	1/10	4/46	1.00
Patients not infected with *B. cepacia* and *A. xylosoxidans*	1/14	2/40	1.00
Mean hospital length of stay in days (SD)	14.7 (9.5)	15.4 (9.4)	0.74

5. Discussion

This study is the first in the literature to examine the effect of antibiotic therapy directed against biofilm-grown bacteria on clinical outcomes in CF patients with pulmonary exacerbation. Perhaps the most important finding is that only a minority of CF patients treated for pulmonary exacerbation with antibiotics chosen based on susceptibility testing of planktonically-grown bacteria, actually received antibiotics that were effective against their same sputum bacteria grown as biofilms. This finding

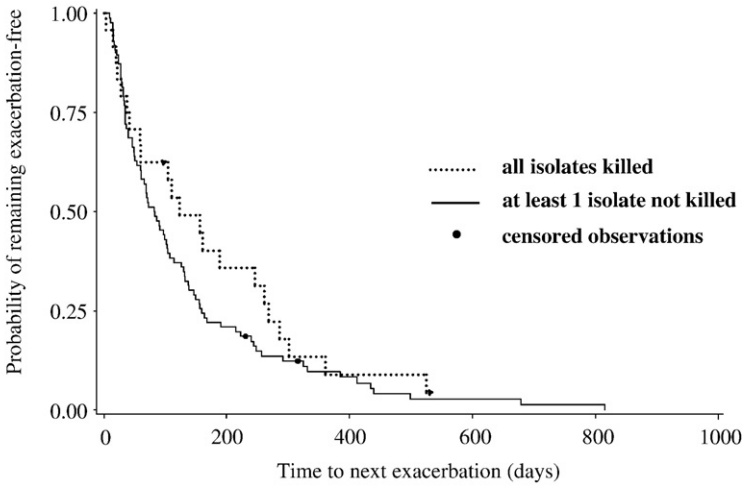

Fig. 1. Kaplan-Meier estimates of the probability of remaining exacerbation-free in patients in whom all biofilm-grown isolates were susceptible compared to not all isolates susceptible. Censored observations represent the four patients who did not have a next exacerbation during the study period .They were followed until the end of the study and were censored in the survival analysis at the point when the study follow-up ended.

supports our hypothesis that clinicians cannot reliably select antibiotics effective against biofilm-grown bacteria using conventional planktonic culturing techniques.

Why is it important to use biofilm-effective antibiotics in treating CF exacerbations? Increasing evidence supports the hypothesis that bacteria grow as biofilms within the lungs of CF patients. Using transmission electron microscopy, biofilm colonies of *Pseudomonas aeruginosa* have been observed in CF sputum [7]. Pathologic studies have also shown *Pseudomonas* bacteria existing in biofilm colonies within the small airways of CF lungs [16]. Furthermore, antibiotic resistance is increased when susceptibility testing is done on bacteria grown as biofilms. Other studies of CF patients have shown that biofilm-grown organisms are less susceptible to single or combinations of antibiotics than planktonically grown organisms from the sputum of CF patients infected with both *Pseudomonas aeruginosa* [8,9] and *Burkholderia cepacia* [17]. This was confirmed by our current study where 60% of the treated patients had all of their planktonically-grown bacterial isolates susceptible to the received antiobitics but only 22% had all of the same isolates susceptible when they were grown as biofilms. It has also been shown that the biofilm inhibitory concentrations are much higher than the corresponding conventionally determined minimum inhibitory concentrations (MIC) for many antibiotics used in CF [9]. Therefore, CF patients may require different combinations of antibiotics than suggested by conventional planktonic susceptibility testing to achieve inhibitory activity against the biofilm-forming bacteria in their airways.

Another question is whether treating CF patients with antibiotics that are effective against the bacterial biofilm will result in improvements in clinical outcomes from pulmonary exacerbations. The evidence suggests that there is little correlation between conventional *in-vitro* antibiotic susceptibility testing of planktonically-grown bacteria and clinical response to antibiotics in CF pulmonary exacerbations [18]. By growing the bacteria as biofilms *in vitro* prior to antibiotic susceptibility testing, we may more closely mimic *in vivo* bacterial growth and therefore select antibiotics with better activity against the bacteria growing within the airways of CF patients.

The biofilm culture technique used in our study has been previously validated by other groups [15]. In addition, we previously showed that combination antibiotic biofilm susceptibility testing is reproducible over time when biofilm-grown isolates were frozen, thawed, and then re-tested 8 weeks later [8]. In the current study, all growth controls were positive indicating that each isolate was able to be grown as a biofilm.

The patients in this study who were treated with antibiotics that inhibited biofilm growth of at least one sputum isolate showed significant improvement in sputum bacterial density and length of hospital stay (Table 1). The other clinical variables examined, although not achieving statistical significance, showed trends toward improved clinical outcomes in those treated with antibiotics effective against their biofilm-grown bacteria. One possible explanation for the trends toward improved clinical outcomes in patients treated with biofilm-effective therapy is that these patients were infected with less resistant organisms and therefore would have improved clinical outcomes regardless of antiobiotic choice. However, when the continuous outcomes were adjusted for infection with *Burkholderia cepacia* and/or *Achromobacter xylosoxidans,* bacteria which are typically more resistant to antibiotics, there were no significant changes to the results.

Obviously failure to show statistical significance for all of the evaluated clinical outcomes limits our ability to make definitive conclusions regarding efficacy of biofilm-directed

antibiotic treatment. However, the group of patients treated with antibiotics that inhibited all biofilm-grown isolates retrieved at exacerbation was relatively small ($n=24$). Therefore, comparisons using this small group of patients may be limited by lack of power, and this may explain why comparisons involving this group yield P values that fall short of statistical significance.

Other limitations of this study include its retrospective design. There may be confounding variables associated with biofilm-effective therapy that were not accounted for and may influence clinical response to therapy. For example, a higher percentage of patients who received triple antibiotic therapy in the form of inhaled tobramycin added to two intravenous antibiotics had at least one biofilm-grown isolate that was susceptible to the antibiotics (Table 2). If inhaled tobramycin has greater activity against biofilm-grown bacteria, and if use of inhaled tobramycin is associated with improved clinical outcomes in CF exacerbation independent of its anti-biofilm properties, then this could also potentially confound our data.

This was not a prospective randomized controlled trial comparing biofilm culturing techniques to conventional planktonic culturing techniques. Antibiotic combinations were prescribed according to results of either conventional or combination antibiotic susceptibility testing on planktonic bacterial cultures, not according to the results of biofilm susceptibility testing. The patients were then regrouped based on retrospective results from their biofilm cultures. Thus, this study is hypothesis-generating, and should be used to spur development of future prospective trials.

Although the retrospective study design limits definitive conclusions, patients with CF exacerbations who were treated with antibiotic combinations that were effective against their biofilm-grown bacteria seemed to have improved clinical outcomes. Future research is needed in the form of a prospective trial comparing clinical outcomes in CF patients randomized to conventional susceptibility testing compared to biofilm susceptibility testing, to determine if antibiotic treatment based on biofilm susceptibility testing leads to improved clinical outcomes for patients with CF pulmonary exacerbations.

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