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Use of *Pseudomonas* biofilm susceptibilities to assign simulated antibiotic regimens for cystic fibrosis airway infection

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***Objectives*: Increasing evidence indicates that *Pseudomonas aeruginosa* grows as a biofilm in the lungs of cystic fibrosis (CF) patients. In contrast, the bacterial inoculum used in conventional susceptibility testing is composed of planktonic cells. As a prelude to a clinical trial of biofilm susceptibility testing in CF, simulated antibiotic regimens based on either biofilm or conventional susceptibility testing of CF patient isolates were compared.**

***Patients and methods*: Biofilm and conventional susceptibilities were determined for *P. aeruginosa* isolate sets from 40 CF patients. An algorithm was used to assign simulated regimens of two anti-pseudomonal antibiotics for each patient/susceptibility method dataset. For agents with equivalent activity, the algorithm included a drug selection hierarchy, the rationale for which was suppression of chronic infection. Substitution of an alternative hierarchy, based on treatment of acute exacerbation, was used to evaluate the robustness of the regimen assignments.**

***Results*: For both drug-ranking schemes, all 40 simulated regimens based on conventional susceptibilities included a β-lactam antibiotic. In contrast, based on biofilm testing, only 43% of chronic regimens and 65% of acute regimens included a β-lactam. Moreover, the conventional and biofilm regimens assigned to individual patients were discordant, with only 20% and 40% of chronic and acute regimens, respectively, consisting of drugs in the same two mechanistic classes by both methods.**

***Conclusions*: Biofilm susceptibility testing of CF *P. aeruginosa* isolate sets leads to different antibiotic assignments than conventional testing, with no single two-drug regimen predicted to provide optimal anti-biofilm activity against the majority of isolate sets.**

Keywords: susceptibility testing, *P. aeruginosa*, inhibitory quotients, biofilms

Introduction

Chronic lung infection with *Pseudomonas aeruginosa* is a common and serious complication of cystic fibrosis (CF), a lethal genetic disorder. In CF, *P. aeruginosa* primarily infects mucus plaques retained at airway surfaces,[1,2] and thus depends on markedly different adaptations than other well-known bacterial pathogens such as *Salmonella* or *Mycobacterium tuberculosis* that cause persistent infection by taking up residence within host cells.[3] Adaptations of chronically infecting *P. aeruginosa* strains include changes in lipopolysaccharide,[4] conversion into a mucoid phenotype,[5] shift to anaerobic metabolism,[1,6] and development of antibiotic resistance.[7–10] An additional and probably related adaptation is the formation of biofilms.[6,11–13] Classic *in vitro* biofilms display complex structures such as stalks and caps.[14] In contrast, published reports of biofilms within CF sputum have been limited to descriptions of microcolonies.[11,15]

Acute exacerbations of *P. aeruginosa* lung infection in CF patients are often treated with potentially synergic two-drug combinations of anti-pseudomonal antibiotics,[16,17] based on

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susceptibility data derived from conventional testing methods such as agar diffusion or broth microdilution.[18,19] Although CF patients may improve clinically in response to such treatment, eradication of *P. aeruginosa* in this context is rare.[8,16,17,20,21] Progressive lung destruction associated with persistent infection remains the primary cause of CF morbidity and mortality.

Biofilm-grown bacteria have different antimicrobial susceptibility patterns than bacteria grown planktonically to logarithmic phase.[22–24] Conventional methods for susceptibility testing use planktonic cultures growing in enriched media, rather than stationary phase organisms growing in biofilms. The use of such methods may partly explain why therapy based on conventional susceptibility results almost always fails to eradicate chronic airway infection.

We recently reported a clinically feasible antibiotic susceptibility assay for CF isolates of *P. aeruginosa* growing in biofilms.[25] This assay, which was developed in an effort to adapt a method for growing bacteria on plastic pegs[26] to the clinical laboratory, gave markedly different antimicrobial susceptibility results than conventional susceptibility testing. However, it was not clear that biofilm susceptibility testing would actually result in sufficiently different treatment regimens to justify further evaluation of this method in a prospective clinical trial.

The goal of this study was to test the need for and feasibility of individualized biofilm susceptibility testing in the clinical setting, by retrospectively simulating antibiotic regimen assignments for a group of CF subjects with chronic *P. aeruginosa* airway infection. Two questions were of particular interest: (i) were biofilm susceptibilities sufficiently variable between sets of isolates from different subjects as to preclude the adoption of a standardized anti-biofilm regimen? and (ii) were biofilm and conventional susceptibilities of isolate sets sufficiently different from each other as to result in selection of different antibiotic regimens for individual patients? Our hypothesis, based on the latter question, was that for most CF patients, the antibiotic regimen assigned on the basis of individual biofilm testing would differ from that based on conventional testing by at least one drug class.

Materials and methods

Subjects

Subjects (n = 43) were patients of the Cystic Fibrosis Center at the Children's Hospital and Regional Medical Center (CHRMC) in Seattle during the period from January 1998 through May 2002, who met the following criteria: diagnosis of CF;[8] age 5 years or older; maximum forced expiratory volume in 1 s (FEV_1) not more than 90% predicted[27] for previous 12 months; history of at least one course of intravenous antibiotics; and history of sputum culture positive for *P. aeruginosa* but not *Burkholderia cepacia* complex. These criteria were intended to select a study population similar to that of a planned prospective clinical trial of biofilm susceptibility testing. The actual study group consisted of 23 females and 20 males, with a median age of 15 years (range: 5–22) and a median FEV_1% predicted of 65 (range: 30–90). The CHRMC Institutional Review Board reviewed and approved the study (Protocol E-02-0039-04), including a waiver of informed consent.

Bacterial isolates

For each subject, a set of *P. aeruginosa* isolates representing distinct colony morphotypes from a single sputum sample obtained during a period of clinical stability was retrieved from storage at –80°C. Sputum samples had originally been cultured quantitatively by standard microbiological methods[18,19] as part of clinical care. Each set satisfied the requirement that the sputum density of the most abundant *P. aeruginosa* morphotype was at least 10^5 cfu/g; additional isolates were included in the set only if their sputum density was ≥1% that of the most abundant morphotype.

Conventional and biofilm antibiotic susceptibility testing

Conventional and biofilm antibiotic susceptibilities were evaluated for each set of *P. aeruginosa* isolates. Antibiotics from the following mechanistic classes were tested: β-lactam (aztreonam, ceftazidime, meropenem, piperacillin/tazobactam and ticarcillin/clavulanate), aminoglycoside (amikacin, gentamicin and tobramycin), macrolide (azithromycin and clarithromycin—biofilm only), fluoroquinolone (ciprofloxacin) and tetracycline (doxycycline). Minimum inhibitory concentration (MIC) values were determined by broth microdilution[19] at the time of initial bacterial isolation, as part of routine clinical care. Biofilm inhibitory concentration (BIC) values were determined by broth microdilution as part of a study to develop clinically feasible methods of biofilm susceptibility testing.[25] For these determinations, biofilms were formed overnight on the pegs of a special microtitre lid (catalogue no. 445497, Nunc TSP system) submerged in cation-adjusted Mueller–Hinton broth supplemented with 0.4% arginine, to promote biofilm growth under microaerophilic conditions. The medium used in the antibiotic challenge and biofilm recovery plates was cation-adjusted Mueller–Hinton broth without added arginine. In the antibiotic challenge step, peg-adherent biofilms were incubated in a range of antibiotic concentrations; pegs carrying control biofilms were submerged in antibiotic-free medium. The biofilms were then transferred to the recovery plate by centrifugation, and residual biofilm viability was measured as the mean OD_{650} difference before and after incubation for 6 h (OD_{650} at 6 h minus OD_{650} at 0 h). Adequate biofilm formation was defined as a mean OD_{650} difference ≥0.05 for the control biofilms, without detailed verification of biofilm structure by scanning electron microscopy or other methods. *P. aeruginosa* isolates that failed to satisfy this definition were excluded from the analyses.

Antibiotic selection algorithm

In order to assign appropriate antibiotic regimens, a standard set of rules was developed for drug selection. Based on current best practice for treatment of *P. aeruginosa*, the selection of a pair of antibiotics with different mechanisms of action (so-called 'double coverage') was thought to be desirable to decrease the likelihood of emergence of antibiotic resistance and to offer the potential for synergic activity. In addition, the availability of inhibitory concentration (MIC and BIC) data for the isolates, as opposed to categorization as susceptible, intermediate and resistant, permitted identification of the most active agents as defined by inhibitory quotient (IQ).[28,29] Because the maximum achievable sputum concentration is not known for many of the agents tested, calculations of IQ were based on the maximum achievable serum concentrations.

For each *P. aeruginosa* isolate, IQs were calculated for each antibiotic/test method combination. For each patient's isolate set, the composite IQ, conservatively defined as the lowest IQ in the set (i.e. that of the most resistant isolate) was then determined for each antibiotic/test method combination, as illustrated (Table 1). The activity of an antibiotic against a given isolate set was assumed to correlate directly with its composite IQ. Therefore, in order to select the most active agents, antibiotic regimens for each set consisted of the two agents from different antibiotic classes with the highest composite IQs (e.g. for

Table 1. Illustration of composite inhibitory quotient (IQ) calculation

Antibiotic	Maximum achievable serum concentration (mg/L)	Inhibitory conc. (mg/L) for isolate			Inhibitory quotient[a] for isolate			Composite IQ[b]
		1A	1B	1C	1A	1B	1C	
β-Lactams								
ceftazidime	8	4	4	2	2	2	4	2
meropenem	4	1	1	1	4	4	4	4
piperacillin/tazobactam	64	32	16	16	2	4	4	2
ticarcillin/clavulanate	64	16	16	64	4	4	1	1
Aminoglycosides								
amikacin	16	16	8	32	1	2	0.5	0.5
tobramycin	4	2	1	2	2	4	2	2
Fluoroquinolone								
ciprofloxacin	1	0.5	1	0.25	2	1	4	1
Macrolide								
azithromycin	2	1	2	1	2	1	2	1

[a]Inhibitory quotient = $\frac{\text{maximum achievable serum concentration}}{\text{inhibitory concentration}}$
[b]Composite IQ = lowest IQ in the isolate set, i.e. IQ of the most resistant isolate(s).

Table 2. Hierarchies of antibiotic agents used when equivalent composite inhibitory quotients were identified

Chronic hierarchy	Acute hierarchy
1. azithromycin	1. tobramycin
2. ciprofloxacin	2. amikacin
3. ceftazidime	3. ceftazidime
4. meropenem	4. meropenem
5. ticarcillin/clavulanate	5. ticarcillin/clavulanate
6. piperacillin/tazobactam	6. piperacillin/tazobactam
7. tobramycin	7. ciprofloxacin
8. amikacin	8. azithromycin

the isolate set in Table 1, meropenem and tobramycin would be assigned). To simplify the analysis, the following antibiotics were excluded from further consideration at this step: doxycycline (due to overall lack of conventional and anti-biofilm activity), and aztreonam, gentamicin and clarithromycin (due to conventional and anti-biofilm activities generally less than or the same as those of ceftazidime, tobramycin and azithromycin, respectively).

When two or more agents had the same composite IQ, drug selection was based on antibiotic hierarchies. These prioritization schemes were based on two distinct rationales: suppression of chronic infection and treatment of acute exacerbation (Table 2). In the initial analysis, the data were interpreted by applying a set of rules that might reasonably be used to assign antibiotics for suppression of chronic *P. aeruginosa* airway infection (Table 2, chronic hierarchy). These rules assumed that for the purpose of chronic suppression, drugs administered by the oral route would be favoured over those administered intravenously, drugs administered less frequently would be favoured over those administered more frequently, and drugs with a better safety profile would be favoured over those with more severe side effects or toxicities requiring serum concentration monitoring. To evaluate the sensitivity of the results to these rules for ranking drugs, the dataset was also re-interpreted by applying alternative rules that might reasonably be used to assign antibiotics for treatment of acute CF pulmonary exacerbations, favouring intravenously administered drugs commonly regarded as most effective for *P. aeruginosa* infection (Table 2, acute hierarchy).

To evaluate the effect of including subjects with multiresistant *P. aeruginosa*, some analyses included only subjects with non-multiresistant isolate sets (n = 30). For this purpose, non-multiresistance was defined as composite IQ ≥ 1 (equivalent to categorization of all isolates as 'susceptible' in conventional MIC interpretation) for at least one agent and composite IQ ≥ 0.5 (equivalent to 'susceptible' or 'intermediate') for at least one additional agent from a different antibiotic class.

Because calculating composite IQs and assigning antibiotics for each patient according to these logical rules and numerical definitions required multiple steps, spreadsheet software (Excel X for Mac, Microsoft Corporation) was used to create a workbook ('Inhibitory Value Interpreter', or IVI) that automated the selection algorithm described above. (A sample IVI workbook containing simulated data that corresponds to the example in Table 1 is available as online supplementary data; its first worksheet is a guide to the program entitled, 'About IVI.') Individual modules within this program are structured to: (i) review the sputum bacterial density data; (ii) enter the MIC and biofilm densitometry data; (iii) determine the adequacy of biofilm growth, assess the quality of the biofilm inhibitory patterns, and calculate the BICs from these patterns; (iv) calculate the IQs and composite IQs for each test method; (v) evaluate the isolate set for multiresistance and data completeness; and (vi) determine the antibiotics from different classes with the highest composite IQs, applying the drug hierarchies as needed. Given the volume of data to be entered, error-checking functions have been incorporated into the program to screen for data entry errors and other data consistency problems. Compared with hand processing of data by the investigators, automated processing using the IVI workbook decreased the incidence of quality control failures and mistaken antibiotic assignments.

Statistical analysis

Data analysis consisted of descriptive characterization of the proportion of patients assigned to a given antibiotic regimen. χ^2 analysis was used to assess the relationship between bacterial phenotype and adequacy of biofilm formation. The sample size was determined by

feasibility considerations for a pilot study, with the goal of studying a sufficient number of subjects such that potential discordances between regimens assigned by each test method were likely to be observed.

Results

Adequacy of biofilm formation by P. aeruginosa CF isolate sets

Overall, 117 isolates from 43 subjects were tested (one isolate set per subject; mean isolates per set = 2.7), with 93 isolates (80%) forming biofilms that satisfied the minimum growth criterion. χ^2 analysis indicated that the frequency of biofilm formation among mucoid isolates ($n = 73$; 75%) was not significantly different than that among non-mucoid isolates ($n = 44$; 86%) ($P = 0.15$). For 28 subjects (65%), all isolates formed biofilms that satisfied the minimum growth criterion for the interpretation of antibiotic susceptibilities. For the other 15 subjects, one or more isolates either never formed adequate biofilms or did so too inconsistently to provide reliable susceptibility results; these isolates were excluded from the analyses of their respective isolate sets. Only three of 43 subjects (7%) were excluded from the analyses because all isolates displayed inadequate or inconsistent biofilm growth.

Chronic regimens for P. aeruginosa CF airway infection based on conventional and biofilm antibiotic susceptibilities

To examine the effect of susceptibility test method on simulated antibiotic regimen assignments for suppression of chronic *P. aeruginosa* airway infection, an antibiotic assignment algorithm was applied to biofilm (BIC) and conventional (MIC) susceptibility data (Tables S1 and S2, online supplementary data) for sets of CF clinical isolates ($n = 40$). All chronic regimens assigned on the basis of conventional susceptibilities included a β-lactam agent (Table 3). In contrast, only 42.5% of biofilm-based chronic regimens included a β-lactam, whilst 57.5% included a macrolide (azithromycin). In addition, inclusion of a fluoroquinolone (ciprofloxacin) was slightly more prevalent in biofilm-based regimens than in conventional regimens (75% versus 60%). The most common conventional regimen consisted of a fluoroquinolone and a β-lactam, whereas the most common biofilm-based regimen consisted of a fluoroquinolone and a macrolide. Exclusion of isolates with a density <10% of the most abundant isolate, or exclusion of multiresistant isolate sets, resulted in only minor variation in these distinct patterns of chronic regimen assignment (data not shown).

Effect of drug selection rules on antibiotic regimen assignments for P. aeruginosa CF airway infection

The chronic hierarchy was adopted to emulate clinical reasoning in the selection of antibiotic regimens for suppression of CF airway infection. Because these rules favour the use of azithromycin, a once-daily oral agent with fairly good anti-biofilm activity but little or no conventional antibacterial activity against *P. aeruginosa*,[25] their use might have biased the interpretation of biofilm susceptibility data toward assignment of antibiotic regimens different from those assigned on the basis of conventional susceptibilities. The fact that the chronic hierarchy was invoked to break ties in the assignment of 72.5% of conventional regimens and 67.5% of biofilm-based regimens underscores the possibility that the ranking scheme might have represented an unintended source of bias.

To examine this possibility, an alternative hierarchy was developed to emulate clinical reasoning in the selection of antibiotic regimens for treatment of acute CF pulmonary exacerbations (acute hierarchy). Using the acute hierarchy, all conventional regimens still included a β-lactam agent (Table 3). In comparison, 65% of biofilm-based acute regimens included a β-lactam, whilst only 15% included a macrolide. Inclusion of a fluoroquinolone remained more prevalent in biofilm-based regimens than conventional regimens (55% versus 30%). The most common acute regimen based on either testing method consisted of a β-lactam and an aminoglycoside, but to different extents (70% of conventional regimens versus 35% of biofilm-based regimens). In addition, the combination of an aminoglycoside and a fluoroquinolone comprised 27.5% of biofilm-based acute regimens, but none of the conventional acute regimens. As with the chronic regimens,

Table 3. Effect of susceptibility test method and drug selection hierarchy on antibiotic assignments ($n = 40$)

	Assignment to antibiotic class or combination (% of regimens)			
	chronic hierarchy		acute hierarchy	
Antibiotic class or combination	MIC	BIC	MIC	BIC
β-Lactam	100.0	42.5	100.0	65.0
Aminoglycoside	40.0	25.0	70.0	65.0
Fluoroquinolone	60.0	75.0	30.0	55.0
Macrolide	ND[a]	57.5	ND[a]	15.0
β-Lactam + aminoglycoside	40.0	10.0	70.0	35.0
β-Lactam + fluoroquinolone	60.0	22.5	30.0	22.5
Aminoglycoside + fluoroquinolone	0	10.0	0	27.5
β-Lactam + macrolide	ND[a]	10.0	ND[a]	7.5
Aminoglycoside + macrolide	ND[a]	5.0	ND[a]	2.5
Fluoroquinolone + macrolide	ND[a]	42.5	ND[a]	5.0

[a]Because of historical high-level resistance, azithromycin MICs were not determined, thus macrolides were not considered for conventional regimens.

exclusion of isolates with a density <10% of the most abundant isolate and exclusion of multiresistant isolate sets resulted in only minor variation in patterns of acute regimen assignment (data not shown).

Discordance of conventional and biofilm-based antibiotic regimens for P. aeruginosa CF airway infection

Aggregate rates of antibiotic regimen assignments might have underestimated the discordance of the individual assignments. The antibiotic regimens assigned by each test method were therefore compared head-to-head for each subject. An analysis using the chronic hierarchy revealed that only 20% of subjects were assigned to conventional and biofilm-based regimens consisting of agents from the same two antibiotic classes (15% β-lactam and fluoroquinolone by both methods; 5% β-lactam and aminoglycoside by both methods). Using the chronic hierarchy, assignment to the most common biofilm-based assignment (a macrolide and a fluoroquinolone) corresponded to conventional assignment to a β-lactam and a fluoroquinolone for 25% of subjects and to a β-lactam and an aminoglycoside for 17.5% of subjects. A similar analysis using the acute hierarchy showed that 40% of subjects were assigned to agents in the same two antibiotic classes by both methods (27.5% β-lactam and aminoglycoside by both methods; 12.5% β-lactam and fluoroquinolone by both methods).

Discussion

In this pilot study, a numerical algorithm was developed and used to simulate antibiotic regimen assignments for CF patients based on conventional and biofilm-based susceptibility testing of *P. aeruginosa* isolate sets. Within the study group, 40 of 43 isolate sets (93%) included at least one isolate with biofilm formation sufficient for susceptibility testing and interpretation. Biofilm susceptibility testing led to substantially different simulated regimens compared with conventional testing. Moreover, for a majority of CF patients, biofilm and conventional regimens were discordant (i.e. differed by at least one drug class) when compared head-to-head. These results were largely independent of the procedure used to break ties between drugs with equivalent activity, although applying different selection hierarchies to a given set of susceptibility test results did alter the distribution of simulated regimens and pattern of discordance.

Several alternative methods for antibiotic susceptibility testing of CF isolates have been proposed in the past decade, including direct sputum susceptibility testing,[30,31] mixed morphotype susceptibility testing,[32–35] CF synergy testing,[36] multiple combination bactericidal testing (MCBT),[37–39] and biofilm susceptibility testing.[25] Non-conventional susceptibility testing methods are generally difficult and expensive to implement in a way that conforms with CLSI (formerly NCCLS) recommendations.[40] Alternative susceptibility testing of CF isolates is justified only if the following requirements can be met: (i) the results cannot be predicted on the basis of current microbiological characterization; and (ii) the results can be interpreted in a way that provides clinical benefit. Our results indicate that no single combination of agents predominates the conventional or biofilm susceptibility profiles of CF isolate sets. Saiman *et al*.[36] and Aaron and coworkers,[38,39] using CF synergy testing and MCBT, respectively, were similarly unable to identify a single most active antibiotic combination. This suggests that each of these alternative methods meets the first requirement. Meeting the second requirement requires prospective study in the CF patient population. Remarkably, a randomized controlled trial to test the clinical efficacy of conventional antibiotic susceptibility testing in CF has never been conducted, and only a single retrospective analysis has been published.[41] Randomized controlled trials to evaluate the effectiveness of assigning antibiotic regimens based on alternative methods such as MCBT[39] or biofilm susceptibility testing (S. Moskowitz, J. Emerson, S. McNamara, J. Foster, W. Benton, R. Gibson and J. Burns, unpublished results) represent some recent efforts to remedy this situation.

Susceptibility methods that can identify potentially synergic antibiotic combinations are sometimes used to test multiresistant CF isolates for clinical purposes. In the recently completed randomized controlled trial, MCBT-based regimens were not clinically superior to conventional regimens among CF patients with acute exacerbation of chronic multiresistant lung infection.[39] This study, which was conducted as a prelude to the biofilm randomized controlled trial, did not include testing of antibiotic combinations. Only a few of the isolates in this study had previously undergone CF synergy testing, a method in which the fractional inhibitory concentrations of antibiotic pairs are determined using a dilutional chequerboard.[36] Thus, whether synergy testing of the isolate sets would have changed the antibiotic regimens significantly is not known; moreover, the possibility that some of the assigned combinations might be antagonistic cannot be excluded. In the original description of the CF synergy testing method, combinations consisting of a β-lactam and either ciprofloxacin or tobramycin were active against 25–35% of multiresistant CF isolates.[36] (Ciprofloxacin and tobramycin were not tested in direct combination.) These investigators subsequently showed that combinations consisting of azithromycin and ciprofloxacin, tobramycin or a β-lactam were active against a smaller proportion of such isolates (4–12%).[42] The most common conventional regimens in this study were similar to the combinations identified as most active in CF synergy studies, whereas the most common biofilm-based regimens were less commonly synergic or were not tested in the earlier studies.[36,42] The development of hybrid testing methods, such as those proposed and piloted by Aaron *et al*.[38] that merge biofilm susceptibility testing with multiple combination testing, may help to resolve these discrepancies by more accurately simulating the interactions of antibiotic combinations with CF biofilms.

This study has additional limitations related to the criteria for antibiotic and bacterial isolate inclusion, as well as the method and interpretation of susceptibility testing. Because many CF clinicians in the USA would have objected to the first-line use of anti-pseudomonal antibiotics such as colistin and chloramphenicol in an interventional trial, these drugs were excluded. The bacterial isolates that this study utilized had been defined and quantified on the basis of phenotypic differences. To estimate the antibiotic susceptibility of the overall bacterial load in each patient, isolates that satisfied a minimum relative sputum density (≥1%) were tested. This isolate inclusion criterion was adopted to avoid pitfalls inherent in direct susceptibility testing of sputum[30,31] or mixed cultures,[32] but is somewhat arbitrary and may have resulted in sampling error. Moreover, the bacteria studied here had been isolated during a period of clinical stability. It has been shown that for the vast majority of patients, isolates obtained during acute CF pulmonary exacerbations are clonally related to those present during the preceding chronic period of clinical stability, with similar conventional susceptibilities.[43] However, it is not known whether the biofilm susceptibilities of serial CF isolates display similar

consistency. Thus, whether the biofilm susceptibility data generated in this study would be relevant to the assignment of acute exacerbation regimens, as simulated in this study, cannot be predicted.

To ensure consistent interpretation of susceptibility data, antibiotic regimens were assigned according to uniformly applied assumptions and rules, i.e. through the use of algorithms. These algorithms were based on complex sets of rules that, whilst non-arbitrary, were nonetheless chosen for practical reasons that limited the range of therapeutic options and might not have accurately simulated the actual reasoning of contemporary clinicians. For example, overall isolate set susceptibility was estimated using the composite IQ score (i.e. the minimum IQ). However, this approach might have been overly conservative, or might be functionally equivalent to a simpler approach (e.g. using the IQ values of the one or two most prevalent isolates). The use of serum concentrations to calculate the IQ values is an additional limitation. Available data indicate that intravenous antibiotic administration results in CF sputum concentrations that are proportional to, if more variable than, serum concentrations.[44–50] However, because sputum/serum ratios of the area under the concentration–time curve (AUC) following intravenous administration can be highly variable amongst different agents (e.g. mean AUC ratio of 0.46 for ciprofloxacin versus 0.09 for piperacillin)[51,52] and between individuals (e.g. range of AUC ratios from 0.01 to 0.61 for amikacin),[53] significant errors in the rank ordering of composite IQ scores may result from assuming fixed ratios of sputum/serum antibiotic concentration.

The assumption of intravenous administration is another potential oversimplification. CF patients are frequently prescribed inhaled antibiotics, and sometimes receive inhaled and intravenous forms of the same agent simultaneously. A number of interventional trials have assessed the clinical utility and microbiological efficacy of intravenous and inhaled antibiotics in CF patients.[16,17,54–56] Pharmacokinetic studies of tobramycin have shown that, depending on the nebulizer system used, a single inhaled dose can result in much higher peak sputum concentrations than a 3 week course of thrice-daily intravenous doses (e.g. 40–4000 mg/L versus 10–100 mg/L).[45,57] If peak sputum concentrations associated with antibiotic inhalation were used as the basis for IQ calculations, markedly higher values than those based on serum concentrations would result. However, antibiotic inhalation in the setting of focal airway obstruction may result in regionally diminished sputum deposition, and probably accounts for the high degree of sputum concentration variability seen with this route of administration. One potential consequence is exposure of bacterial subpopulations to lower, potentially subinhibitory concentrations. These potential complications illustrate why calculation of IQ values based on peak sputum concentrations achievable through antibiotic inhalation would probably provide an even less accurate approximation of CF lung pharmacodynamics than the approach taken in this study.

Many of these assumptions and limitations apply not only to the testing and interpretation methods used in this study, but also to conventional susceptibility testing currently in use for CF isolates of *P. aeruginosa*. These methods have generally been developed for reproducibility and ease of implementation. Thus, they rely on shortcuts and assumptions to simplify and standardize operations in the clinical laboratory that may diminish their relevance to susceptibility testing of CF isolates. Moreover, these methods were originally developed for single organism isolates from bloodstream and urinary tract infections, not for the sequestered polymicrobial infections that are associated with CF lung disease.[58] It has previously been demonstrated that *P. aeruginosa* isolates from CF patients display phenotypes that are quite distinct from those seen in most other chronic *P. aeruginosa* infection states, and require specific methodology to ensure reproducible results during susceptibility testing.[18,19,59]

The development of biofilm susceptibility testing methods was intended to promote the overall goal of CF bacterial susceptibility testing, namely, to simulate the effect of antibiotic combinations on the total bacterial biomass in CF airway secretions. This bacterial biomass is likely to consist of both biofilm and non-biofilm components. It has recently been suggested that acute CF pulmonary exacerbations represent a combination of two bacterial states, namely, a biofilm-like chronic infection of mucus plaques within CF airways, and an acute, inflammation-stimulating 'planktonic bloom' that emerges from the biofilm.[60] If so, optimal treatment of CF airway infection might require different approaches at different times. Periods of clinical stability might be maintained using an approach that chronically suppresses biofilm growth. In contrast, treatment of acute exacerbations might require targeting both biofilm and planktonic components of the infection using algorithms that assign multi-drug regimens based on results from multiple methods of antibiotic susceptibility testing. The creation of hybrid testing methods that combine biofilm susceptibility methods with other methods, such as conventional susceptibility testing[26] or multiple combination testing,[38] could streamline the testing process. Such approaches, supported by computer software like the IVI workbook to monitor and interpret the complex datasets that they would generate, could lead to more effective antimicrobial regimens for CF airway infection.

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Supplementary data

Supplementary data for this paper are available online at http://jac.oxfordjournals.org/.

Transparency declarations

The authors have no interests to declare.

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