ORIGINAL RESEARCH

Multidrug-resistant *Pseudomonas aeruginosa* from sputum of patients with cystic fibrosis demonstrates a high rate of susceptibility to ceftazidime–avibactam

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Purpose: Ceftazidime–avibactam is a novel antimicrobial combining a third-generation cephalosporin with a non-β-lactam β-lactamase inhibitor that was recently approved to treat Gram-negative hospital- and ventilator-acquired pneumonia. The use of ceftazidime–avibactam to treat *Pseudomonas aeruginosa* respiratory infections in patients with cystic fibrosis (CF) has not been evaluated. In this study, we assessed the ceftazidime–avibactam susceptibility of multidrug-resistant (MDR) *P. aeruginosa* sputum isolates from adults with CF.

Methods: Sputum was collected from individuals with CF, aged ≥18 years, known to be colonized with MDR *P. aeruginosa*, and tested for susceptibility to 11 different antipseudomonal antimicrobial agents. Isolates were included in the analysis if they were resistant to both ceftazidime and at least one agent in ≥3 different antimicrobial categories routinely used to treat *P. aeruginosa*. Subject demographics and clinical characteristics were collected. Ceftazidime–avibactam-resistant isolates were screened for the presence of β-lactam-resistant mechanisms.

Results: Thirty-two *P. aeruginosa* isolates were analyzed, of which 23 isolates were sensitive to ceftazidime–avibactam (71.9%). Ten of the isolates were mucoid and 22 isolates were non-mucoid, both demonstrating >70% susceptibility to ceftazidime–avibactam. The most notable difference in the subjects with resistant strains was an older age and lower body mass index (BMI). Ceftazidime–avibactam-resistant strains showed elevated AmpC expression in >60% of the strains and loss of OprD detection in >70% of the strains.

Conclusion: Ceftazidime–avibactam demonstrated a significant in vitro activity against highly resistant *P. aeruginosa* sputum isolates from individuals with CF. Further evaluation of the cause of resistance and clinical impact of ceftazidime–avibactam in CF patients with MDR *P. aeruginosa* is warranted.

Keywords: ceftazidime, avibactam, cystic fibrosis, *Pseudomonas aeruginosa*, multidrug-resistant

Introduction

Cystic fibrosis (CF) is a multisystem autosomal recessive genetic disorder caused by mutations in the CF transmembrane conductance regulator (CFTR) ion channel. The disease manifests predominantly with gastrointestinal and respiratory complications, including chronic bacterial infections. Airway obstruction from chronic infection and inflammation is the leading cause of premature morbidity and mortality in patients with CF. More than 50% of CF individuals aged 18 years and older in the USA are infected with *Pseudomonas aeruginosa*, of whom approximately one-third is multidrug-resistant (MDR).[1] MDR *P. aeruginosa* may be treated with an array of

available antibiotics, but the effectiveness of these antibiotics in practice has been quite variable. Clinicians and researchers have, therefore, been seeking newer antibiotics to treat infections in CF. Ceftazidime–avibactam is a novel antimicrobial that combines a third-generation cephalosporin, ceftazidime, with a non-β-lactam β-lactamase inhibitor.[2,3]

Ceftazidime–avibactam has shown a significant in vitro activity against a number of Gram-negative bacteria including *Proteus*, *Citrobacter*, *Escherichia coli*, *Klebsiella* species, extended spectrum beta lactamase (ESBL)-producing organisms, and *Burkholderia cepacia*.[4–7] In fact, *B. cepacia*, an organism well described to be resistant to antibiotics in patients with CF, showed 90% susceptibility to ceftazidime–avibactam.[8] Ceftazidime–avibactam was first approved for use in USA in 2015 for complicated intra-abdominal and urinary tract infections caused by MDR Gram-negative organisms.[8] More recently, ceftazidime–avibactam was approved and showed beneficial outcomes for use in patients with severe respiratory infections, including hospital- and ventilator-associated pneumonia, suggesting appropriate lung penetration and utility in respiratory disease.[9–11] Ceftazidime–avibactam has not been thoroughly evaluated in patients with CF. However, a pharmacokinetic–pharmacodynamic study of ceftazidime–avibactam use in patients with CF exacerbations did show similar total body clearance and total volume of distribution as published in healthy adults along with notable concentrations in sputum.[12]

P. aeruginosa is a common pathogen in the lungs of those with CF and is associated with frequent pulmonary exacerbations and high morbidity and mortality.[13] The lungs of patients with CF can harbor this organism for decades. With increasing levels of *P. aeruginosa* drug resistance, treatment of pulmonary exacerbations can be increasingly difficult over time. *P. aeruginosa* has several mechanisms of resistance that lead to eradication failure and chronic infections, including porin loss and overexpression of efflux pumps as well as production of inactivating enzymes, such as β-lactamases.[14,15] Another key mechanism of resistance is the generation of alginate polysaccharide biofilms; these are complex structures, which provide resistance by barrier protection and diffusion limitations.[15] Although difficult to eradicate, certain organisms leading to chronic infection in CF mandate antimicrobial therapy during acute pulmonary exacerbations in patients with CF.[16]

There are limited studies on the use of ceftazidime–avibactam against MDR *P. aeruginosa* in sputum specimens from CF patients. The purpose of this study is to evaluate the in vitro activity of ceftazidime–avibactam against MDR *P. aeruginosa* isolates from sputum samples of adult CF patients with highly drug-resistant chronic *P. aeruginosa* infection and to understand the mechanisms involved in β-lactamase resistance.

Methods
Study design and population
The University of Texas Southwestern adult CF clinic population was queried using the electronic medical record and local Cystic Fibrosis Foundation patient registry database to generate a list of eligible subjects for the study. The study was approved by the Institutional Review Board at the University of Texas Southwestern Medical Center (STU 052011-020). Inclusion criteria were subjects with a confirmed diagnosis of CF by sweat or genetic testing, the age of ≥18 years, and ≥2 sputum cultures positive for *P. aeruginosa* prior to January 1, 2015.[17] Exclusion criteria included subjects who had undergone lung transplantation. With informed and written consent, sputum was collected from eligible subjects. Isolates were included in the analysis if they were resistant to ceftazidime and to at least one agent in ≥3 different antimicrobial categories routinely used to treat *P. aeruginosa* including fluoroquinolones, aminoglycosides, β-lactams, carbapenems, and polymyxins.

Demographic information acquisition
Age, sex, race, and CFTR genetic information were collected from the University of Texas Southwestern electronic medical record. Body mass index (BMI) was calculated based on height and weight taken at the time of sputum sample collection using standard formulae. Percent predicted forced expiratory volume in 1 second (ppFEV$_1$) was calculated using the NHANES methodology from spirometry measurements taken at the time of sputum sample collection. Inpatient and outpatient oral and intravenous antibiotic exposures for each subject were collected for 2 years prior to sample collection.

Antibiotic susceptibility testing
Isolation of *P. aeruginosa* from sputum samples was performed in the University of Texas Southwestern microbiology laboratory. Sputum samples were inoculated onto MacConkey agar, sheep blood agar, chocolate agar, *B. cepacia* selective media, mannitol salt agar, and inhibitory mold agar. *P. aeruginosa* was identified as oxidase-positive, nonlactose-fermenting colonies on MacConkey agar and reported as mucoid vs nonmucoid. The isolates were identified definitively as *P. aeruginosa* by MicroScan Neg Urine Combo Panel Type 61 (Beckman Coulter, Inc., Brea, CA, USA). *P. aeruginosa* isolates were subsequently sent to JMI Laboratories (North Liberty, IA, USA) for susceptibility testing to ceftazidime–avibactam along with other standard antipseudomonal antibiotics including ceftazidime, cefepime, aztreonam, meropenem, piperacillin–tazobactam, amikacin, gentamicin, colistin, levofloxacin, and ciprofloxacin. JMI Laboratories was blinded to any patient data. All isolates were

tested for susceptibility using the reference broth microdilution method as described by the Clinical and Laboratory Standards Institute (CLSI).[18,19] Ceftazidime was combined with avibactam at a fixed concentration of 4 mg/L. Ceftazidime–avibactam breakpoints approved by the US-Food and Drug Administration (FDA) (≤8/4 mg/L for susceptible and ≥16/4 mg/L for resistant) when testing *P. aeruginosa* were applied. Susceptibility interpretations for comparator agents were those found in CLSI document M100-S26[19] and/or US-FDA package insert.[20] Quality control was performed using *E. coli* ATCC 25922 and 35218, *Klebsiella pneumoniae* ATCC 700603 and BAA-1705, and *P. aeruginosa* ATCC 27853. MIC_{50} and MIC_{90} calculations were made as previously described.[21] Drug-resistant categories were defined as follows: multidrug-resistant (MDR) strains were defined as isolates resistant to at least one agent in ≥3 different antimicrobial categories;[22] extensive drug-resistant (XDR) strains were defined as those resistant to at least one agent in all but two or fewer antimicrobial categories; and pan drug-resistant (PDR) strains were defined as those resistant to all classes except colistin.[22,23]

Biofilm assay

Biofilm breakdown assays were performed in 96-well minimum biofilm eradication concentration (MBEC) plates (Innovotech, Inc., Edmonton, AB, Canada) as described previously.[24] Briefly, *P. aeruginosa* strains were inoculated at 5×10^5 CFU/mL in Mueller Hinton II broth followed by exposure to ceftazidime–avibactam at concentrations ranging from 1 to 256 µg/mL for 24 hours. Biofilm was quantified using a crystal violet assay.[24] The OD of the eluted stain was then measured at 570 nm to quantify the biofilm.

Detection of acquired β-lactamases

Isolates displaying resistance to ceftazidime–avibactam with MIC values (≥16 µg/mL) were screened by PCR for the presence of bla_{IMP}, bla_{VIM}, bla_{KPC}, bla_{NDM}, bla_{OXA-48}, bla_{GES} (bla_{GES-2}, bla_{GES-4}, bla_{GES-5}, bla_{GES-6}, and bla_{GES-8}), bla_{NMC-A}, bla_{SME}, bla_{IMI}, bla_{FRI-1}, bla_{BKC-1}, $bla_{GIM-1/-2}$, bla_{SIM-1}, bla_{SPM-1}, bla_{KHM-1}, bla_{AIM-1}, bla_{BIC-1}, and bla_{DIM-1} genes.

Expression analysis of the chromosomally encoded AmpC and efflux pumps

The expression of *ampC*, *mexA* (MexAB-OprM), *mexC* (MexCD-OprJ), *mexE* (MexEF-OprN), and *mexX* (MexXY-OprM) was determined by quantitative real-time PCR (qRT-PCR) using DNA-free RNA preparations. Total RNA was extracted from mid-log-phase bacterial cultures (cell density at OD_{600} 0.3–0.5) using RNA Protect Reagent and RNeasy Mini Kit (Qiagen NV, Venlo, the Netherlands) in the QIAcube workstation (Qiagen NV), and residual DNA was eliminated with RNase-free DNase (Promega Corporation, Fitchburg, WI, USA). Quantification and quality of mRNA were done using the RNA 6000 Pico kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) according to manufacturer's instructions. Only preparations with RNA integrity number (RIN) >8 that showed no visual degradation were used for experiments.[25] Relative quantification of target genes was performed in triplicate by normalization to an endogenous reference gene (*rpsL*) on the StepOnePlus instrument (Thermo Fisher Scientific, Waltham, MA, USA) using Power SYBR® Green RNA-to-CT™ kit (Thermo Fisher Scientific) and custom-designed primers showing efficiency >96.0% previously validated.[26,27] Transcription levels were considered significantly different if at least 5- or 10-fold differences were noted compared with *P. aeruginosa* PAO1 for efflux pumps and AmpC expression, respectively.[26,28,29]

Porin detection

Outer membrane proteins were purified by using the Fast-Prep®-24 instrument (MP Biomedicals, Solon, OH, USA), according to the manufacturer's instructions. Normalized concentrations of purified outer membrane proteins were electrophoretically separated and transferred onto PVDF membranes. Western blots were probed with an affinity-purified polyclonal antibody raised in rabbits using the synthetic OprD peptide N″-SDKTGTGNLPVMNDGKPPD-C″ (Thermo Fisher Scientific) and revealed with the WesternBreeze Chromogenic Kit (Thermo Fisher Scientific).[30] *P. aeruginosa* PAO1 and two OprD downregulated laboratory constructs were used as positive and negative controls for comparative analysis.

Statistical analysis

Differences in ceftazidime–avibactam-sensitive vs -resistant strains were compared using a chi-squared test and Fisher's exact test for categorical variables and unpaired *t*-test for continuous variables. A *P*-value of <0.05 was considered statistically significant. Statistical analysis was conducted with GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA).

Results
Subjects

From January 1, 2015, to December 31, 2015, a total of 263 patients with CF who had not undergone lung transplant were seen in the University of Texas Southwestern adult CF

clinic, of whom 142 patients were excluded due to the lack of *P. aeruginosa* in ≥2 previous cultures and a further 73 patients were excluded due to the lack of ≥2 ceftazidime-resistant *P. aeruginosa* strains (Figure 1). Forty-eight subjects were eligible for the study. Forty of these patients were stable at the time of their visit, produced sputum, and consented to participate in the study. A total of 32 isolates were MDR, and ceftazidime-resistant *P. aeruginosa* isolates were tested, of which 23 (71.9%) isolates were ceftazidime–avibactam sensitive while nine (28.1%) isolates were ceftazidime–avibactam resistant.

Patients' characteristics

We compared demographic data of subjects who had *P. aeruginosa* isolates found to be ceftazidime–avibactam sensitive vs those who had resistant strains (Table 1). Given that four subjects had two *P. aeruginosa* isolates each, some of which had differing susceptibility profiles to ceftazidime–avibactam, a subject's demographics was included twice if they had two isolates. Individuals with ceftazidime–avibactam-sensitive *P. aeruginosa* were significantly younger than those with resistant strains (27.8±8.3 vs 36.9±8.1 years, respectively, $P=0.017$). There was no significant difference in sex or racial
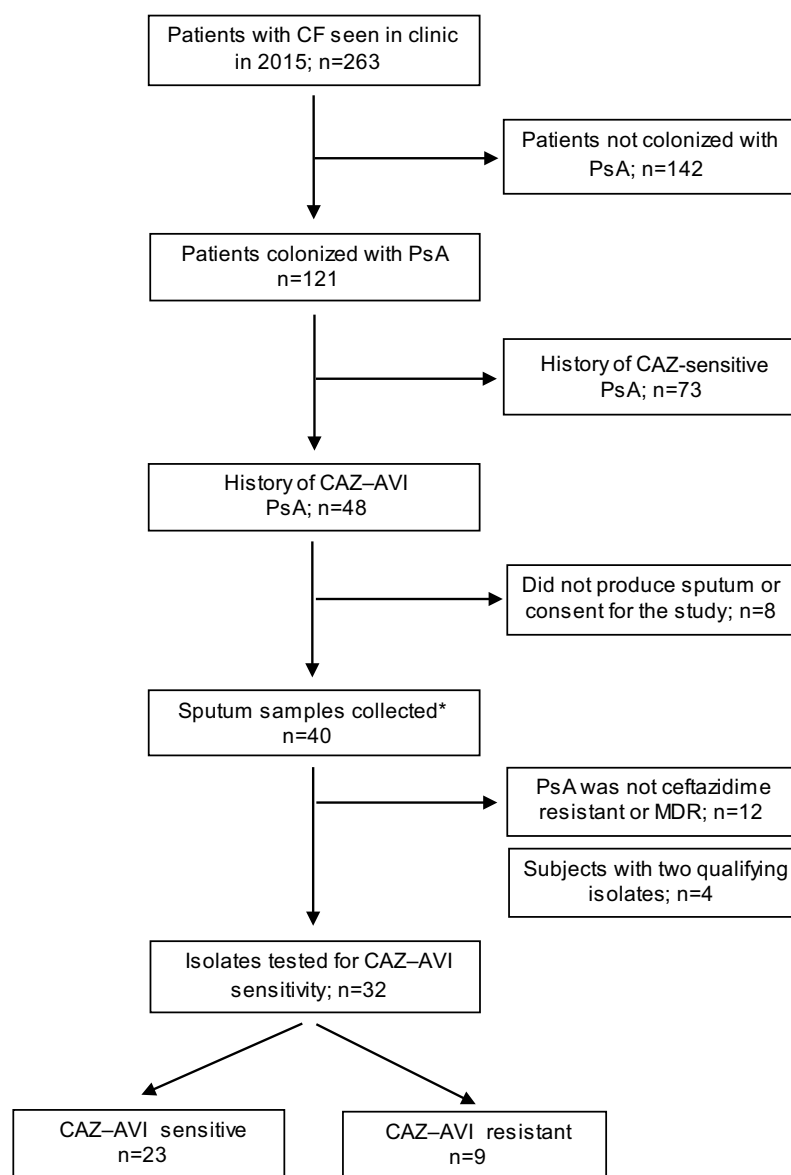
Figure 1 Flowchart of subjects and isolate collection.
Note: A flowchart of CF individuals seen at the University of Texas Southwestern adult clinic from January 1, 2015, to December 31, 2015.
Abbreviations: AVI, avibactam; CAZ, ceftazidime; CF, cystic fibrosis; PsA, *Pseudomonas aeruginosa*; MDR, multidrug-resistant.

Table 1 Demographic information of patients for each *P. aeruginosa* isolate collected categorized by CAZ–AVI susceptibility

	CAZ–AVI sensitive (n=23)[a]	CAZ–AVI resistant (n=9)[a]	P-value
Age (years), mean ± SD	27.8±8.3	36.9±8.1	0.017[b]
Sex			0.433[c]
Male, n (%)	13 (52.6)	3 (33.3)	
Female, n (%)	10 (47.4)	6 (66.7)	
Race			0.509[c]
Caucasian, n (%)	17 (73.6)	8 (88.9)	
African American, n (%)	4 (15.7)	1 (11.1)	
Hispanic, n (%)	2 (10.5)	0	
Others	0	0	
BMI, mean ± SD	21.6±2.7	19.1±5.4	0.049[b]
ppFEV$_1$, mean ± SD	39.2±15.0	35.6±22.1	0.208[b]
Genotype			0.056[c]
F508del homozygous	12 (52.6)	1 (11.1)	
F508del heterozygous	8 (47.3)	4 (44.4)	
No F508del mutations	3 (15.7)	4 (44.4)	

Notes: [a]Because four subjects had 2 *P. aeruginosa* isolates some of which had differing sensitivity to CAZ–AVI, the subject's demographics with two isolates was included twice. [b]Unpaired *t*-test. [c]chi-square test. [d]Fisher's exact test.
Abbreviations: AVI, avibactam; BMI, body mass index; CAZ, ceftazidime; *P. aeruginosa*, *Pseudomonas aeruginosa*; ppFEV$_1$, percent predicted forced expiratory volume in 1 second.

characteristics in ceftazidime–avibactam-sensitive vs -resistant samples. Approximately 55.6% of ceftazidime–avibactam-resistant isolates came from patients with a BMI of <19, which correlates clinically with more severe disease burden in patients with CF.[31] There was no significant genotypic difference or mean ppFEV$_1$ difference in the two groups (39.2±15.0 vs 35.6±22.1; *P*=0.208). Overall, these data demonstrate a higher association between individual's colonization with β-lactamase inhibitor-resistant *P. aeruginosa* strains who are older and less well nourished. Antibiotic history demonstrated no notable differences in the average number of antibiotic exposure days between subjects with ceftazidime–avibactam-resistant vs -sensitive strains in all tested antibiotics other than meropenem (*S*=16.4±5.5 days; *R*=53.1±16.7 days, *P*=0.018) (Figure 2). Average antibiotic days for other antibiotics are as follows: tobramycin (*S*=29.6±6.8 days; *R*=51.3±19.8 days, *P*=0.224), ceftazidime (*S*=14.7±4.8 days; *R*=15.9±7.8 days, *P*=0.886), piperacillin–tazobactam (*S*=21.2±8.2 days; *R*=25.0±14.4 days, *P*=0.805), levofloxacin (*S*=38.0±12.2 days; *R*=51.0±16.6 days, *P*=0.528), colistin (*S*=12.4±6.67 days; *R*=12.7±4.9 days, *P*=0.976), ciprofloxacin (*S*=74.2±18.2 days; *R*=44.9±13.1 days, *P*=0.268), aztreonam (*S*=2.12±1.18 days; *R*=3.5±2.39 days, *P*=0.567), and cefepime (*S*=5.24±3.97 days; *R*=3.40±2.31 days, *P*=0.741).

Ceftazidime–avibactam susceptibility testing results

P. aeruginosa isolates from sputum samples were tested for susceptibility to ceftazidime–avibactam and to a panel of 10 commonly used antipseudomonal antibiotics (Table 2). US-FDA clinical breakpoints were applied for ceftazidime–avibactam, ceftazidime, and cefepime while CLSI breakpoints were applied for the remaining antimicrobials. In our cohort of sputum isolates, 71.9% were susceptible to ceftazidime–avibactam, 3.1% were susceptible to aztreonam, 34.3% were susceptible to meropenem, and 3.1% were susceptible to piperacillin/tazobactam. Susceptibility to fluoroquinolones such as levofloxacin and ciprofloxacin was 21.9 and 25.0%, respectively, and susceptibility to aminoglycosides such as amikacin and gentamicin was 25.0 and 12.5%, respectively. Analysis of MIC data across all isolates demonstrated that ceftazidime–avibactam had the highest rate of susceptibility of all antimicrobial classes tested outside of colistin, which demonstrated 93.7% susceptibility to the ceftazidime-resistant *P. aeruginosa* respiratory isolates. Full antimicrobial susceptibility results are shown in Table S1. The MIC$_{50}$ for ceftazidime–avibactam was 4 µg/mL, which was also the lowest of all antimicrobials tested other than colistin, which was found to have an MIC$_{50}$ of 1 µg/mL. We evaluated the percentage of ceftazidime–avibactam-sensitive and -resistant isolates that were MDR, XDR, and PDR and found that isolates resistant to ceftazidime–avibactam had a significantly higher rate of XDR and PDR phenotypes than the ceftazidime–avibactam-sensitive strains (*P*=0.0054) (Figure 3A). This suggests that ceftazidime–avibactam-resistant isolates show an extensive antimicrobial resistance to multiple drug classes.
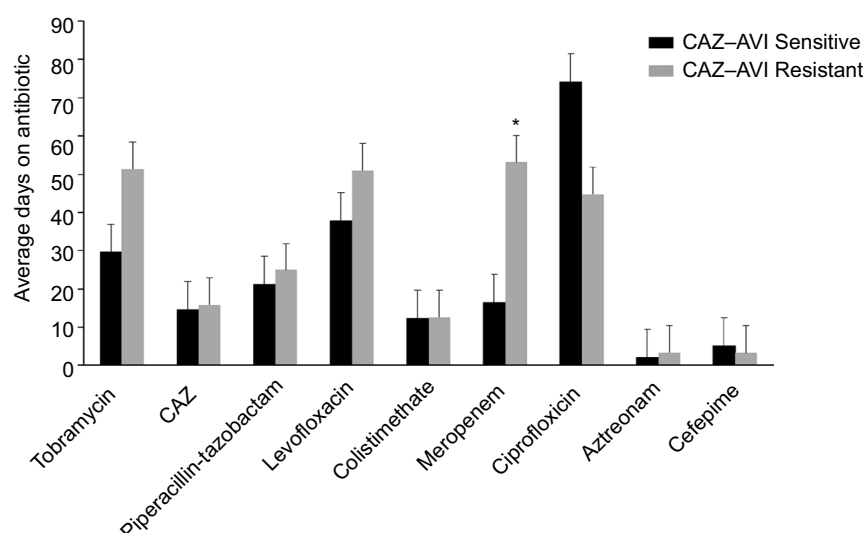
Figure 2 Antibiotic exposure of subjects prior to sputum collection.
Notes: Average number of days of subject exposure to listed antibiotic with standard error bars displayed in the 2 years prior to sputum sample collection. *$P<0.05$.
Abbreviations: AVI, avibactam; CAZ, ceftazidime.

Table 2 Antimicrobial susceptibility of 32 *P. aeruginosa* isolates from 28 patients with cystic fibrosis

Antimicrobial agent	MIC$_{50}$ (µg/mL)	MIC$_{90}$ (µg/mL)	MIC range (µg/mL)	%S[a] (CLSI/US-FDA)	%I or R[a] (CLSI/US-FDA)
Ceftazidime–avibactam	4	64	0.5–>128	71.9	28.1
Ceftazidime	64	>128	16–>128	0	100
Aztreonam	64	>128	≤1–>128	3.1	96.9
Cefepime	>16	>16	8–>16	0	100
Meropenem	8	>16	≤0.12–>16	34.3	65.7
Piperacillin/tazobactam	>128	>128	16–>128	3.1	96.9
Amikacin	32	>32	4–>32	25.0	75.0
Gentamicin	>8	>8	2–>8	12.5	87.5
Colistin	1	2	0.25–8	93.7	6.3
Levofloxacin	>4	>4	0.12–>4	21.9	78.1
Ciprofloxacin	>4	>4	0.06–>4	25.0	75.0

Note: [a]US-FDA breakpoints were applied for ceftazidime–avibactam, ceftazidime, and cefepime (≥8/≤16 µg/mL for *S/R* for all three drugs), and CLSI breakpoints were applied for the remaining drugs.
**Abbreviations:** FDA, Food and Drug Administration; CLSI, Clinical and Laboratory Standards Institute; R, resistant; I, intermediate; S, sensitive.

Relationship to mucoid phenotype

P. aeruginosa in CF sputum has the unique ability to secrete alginate and acquire a mucoid phenotype, which can demonstrate high drug resistance in some cases.[15] Ceftazidime–avibactam-sensitive vs -resistant isolates in our study were stratified by mucoid and nonmucoid phenotypes (Figure 3B). There was no significant difference in the number of mucoid and nonmucoid isolates in these groups (seven of the 23 ceftazidime–avibactam-sensitive isolates were mucoid [30.4%], while three of the nine ceftazidime–avibactam-resistant isolates were mucoid [33.3%], $P=0.874$), suggesting that mucoid phenotype may not play a role in resistance mechanisms to ceftazidime–avibactam.

## Ceftazidime–avibactam can eliminate *P. aeruginosa* biofilm

We studied whether ceftazidime–avibactam would have activity in the biofilm setting. We tested four sensitive strains with an MIC of 1 or 2 µg/mL and four resistant strains with an MIC of 16 or 64 µg/mL. At a concentration of 1 µg/mL, there was a 69% decrease in biofilm biomass and this effect was dose dependent with a >50% reduction at 16 µg/mL (Figure 4). The resistant strains did not show antibiofilm activity even at doses of ceftazidime–avibactam as high as 256 µg/mL. These results overall demonstrate potential benefit of ceftazidime–avibactam in eradicating MDR *P. aeruginosa* even in a biofilm setting.
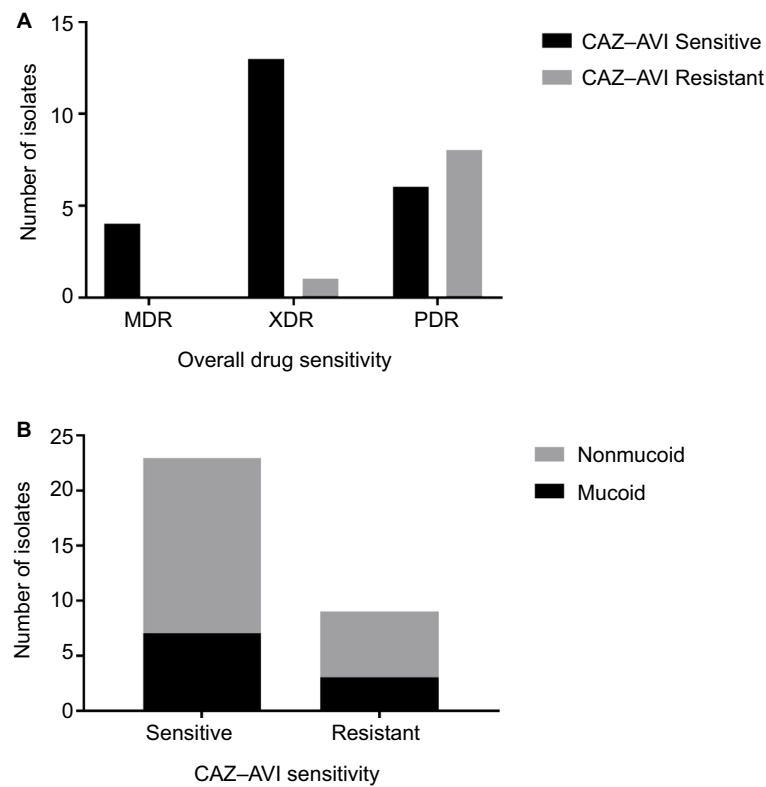
Figure 3 Characteristics of *P. aeruginosa* strain.
Notes: (**A**) Distribution of MDR, XDR, and PDR isolates found to be ceftazidime-avibactam sensitive versus resistant, *P*=0.0054. (**B**) CAZ–AVI susceptibility of *P. aeruginosa* isolates by morphotype, *P*=0.874.
Abbreviations: AVI, avibactam; CAZ, ceftazidime; MDR, multidrug-resistant; *P. aeruginosa*, *Pseudomonas aeruginosa*; PDR, pan-drug resistant; XDR, extensive drug resistant.
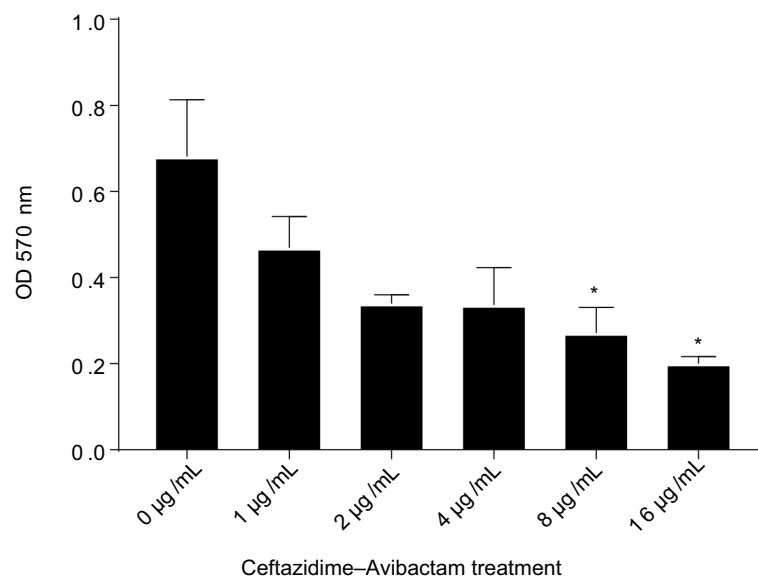
Figure 4 Ceftazidime–avibactam MBEC.
Notes: Dose-dependent response of ceftazidime–avibactam on eradication of a *Pseudomonas aeruginosa* biofilm generated from a representative strain with an MIC of 1 μg/mL to ceftazidime–avibactam. **P*<0.05.
Abbreviation: MBEC, minimum biofilm eradication concentration.

Mechanisms of resistance to β-lactams among ceftazidime–avibactam-resistant strains

The nine ceftazidime–avibactam-resistant isolates were evaluated further for the presence of acquired carbapenemases, AmpC gene expression, Mex-family efflux pumps, and detection of OprD (Table 3). Acquired β-lactamase, including the most common metallo-β-lactamases, was not detected among ceftazidime–avibactam-resistant isolates. OprD protein loss was noted in 77.78% of strains (Figure S1), elevated AmpC gene expression was noted in 66.67% of strains, elevated MexC expression was noted in 22.22% of strains, moderate expression of MexX and MexA was noted in 22.2% of strains, and no expression of MexE was noted.

Discussion

This study demonstrates for the first time that MDR *P. aeruginosa* respiratory isolates from adults with CF have a high rate of in vitro susceptibility to ceftazidime–avibactam (71.9%). Ceftazidime–avibactam demonstrated an MIC_{50} of 4 and an MIC_{90} of 64 (MIC range 0.5–128 μg/mL) against these highly antibiotic-resistant strains. There was no notable difference in susceptibility patterns to ceftazidime–avibactam when assessing mucoid vs nonmucoid strains. Ceftazidime–avibactam use also inhibited biofilm formation in a dose-dependent manner in strains noted to be ceftazidime–avibactam sensitive. Analysis of MIC data across the isolates collected demonstrated that ceftazidime–avibactam had the highest rate of susceptibility of all antimicrobial classes tested outside of colistin. The ceftazidime–avibactam-resistant *P. aeruginosa* strains also showed a high expression of AmpC and the protein detection of OprD and interestingly no expression of tested β-lactamases.

Ceftazidime is a β-lactam cephalosporin with a robust history in treating *P. aeruginosa* and other Gram-negative lung infections, including in individuals with CF. Avibactam is a member of a novel class of non-β lactam-β lactamase inhibitors, the diazabicyclooctanes (DBOs).[32] Compared with the inhibitors currently available for clinical use, DBOs are more potent and have a broader spectrum and different mechanisms of action. While co-administration of a β-lactamase inhibitor can help restore antibacterial activity to cephalosporins, previously approved β-lactamase inhibitors such as tazobactam and clavulanic acid do not inhibit certain important β-lactamases.[33–35] Avibactam effectively inactivates class A (including *K. pneumoniae* carbapenemase [KPC]), class C (AmpC), and some class D (OXA) lactamases, with a 50% inhibitory concentration (IC_{50}) value and low turnover numbers, but is not active against New Delhi metallo-*b*-lactamase 1 (NDM-1).[36,37] Thus, avibactam extends the antibacterial activity of ceftazidime against many ceftazidime-resistant organisms that produce the above-cited enzymes, including *P. aeruginosa.*

The study design for this work has some inherent limitations. We stringently limited our analysis to *P. aeruginosa* isolates from individuals with CF with a known history of resistance to ceftazidime and MDR *P. aeruginosa.* As a result, our sample size was small. Our study samples were also collected from a single-center study, so practice patterns on antibiotic use in the CF population may impact susceptibility and gene expression results seen. However, our data are consistent with the evaluation of ceftazidime–avibactam against another fastidious respiratory pathogen in CF, *Burkholderia* species, in which >90% of the isolates were sensitive to avibactam.[8] Finally, we acknowledge that results of susceptibility testing in vitro do not always correlate with treatment efficacy in individuals with CF[38] and, thus,

Table 3 Expression analysis of OprD, AmpC, and efflux pumps from nine resistant isolates

	MIC	OprD loss[a]	Fold change in gene expression compared to control:				
			AmpC[b]	MexCD-OprJ[b]	MexXY-OprM[b]	MexAB-OprM[b]	MexEF-OprD[b]
1	64	+	3702.7[++]	29.8[++]	0.9	1.9	0.03
2	64	+	2141[++]	1.9	1.7	1.5	0.06
3	64	+	1.9	2.8	0.64	0.87	0.05
4	16	+	25.4[++]	0.047	1.2	3.0	0.01
5	128	+	18.1[++]	0.28	1.1	0.88	0.12
6	>128	+	677.8[++]	14.9[++]	0.39	2.0	0.04
7	16	−	0.9	0.03	0.67	1.8	0.02
8	128	+	28.0[++]	0.46	8.8[+]	5.3[+]	0.01
9	64	−	1.7	1.7	5.3[+]	6.5[+]	0.02

Notes: [a]OprD loss: +, protein not detected; -, protein was detected. [b]AmpC and efflux pumps' interpretation: [++], ≥10-fold change = elevated expression; [+], >5-fold change but <10-fold change = moderate expression.

use of this data primarily serves as a foundation to support the evaluation of ceftazidime–avibactam for use in treating *P. aeruginosa* respiratory infections in CF.[39,40]

Despite limitations, this is the first evaluation of susceptibility testing of MDR *P. aeruginosa* strains from the sputum of patients with CF with an evaluation of potential resistance mechanisms. In our study, ceftazidime–avibactam-resistant *P. aeruginosa* isolates predominantly displayed the loss of OprD and elevated AmpC enzyme, suggesting the role of porins and β-lactamases in the resistance mechanisms involved. In support of these findings, others have similarly shown that *P. aeruginosa* resistance to ceftazidime–avibactam is through modification of the Ω loop region of AmpC, though these isolates were not from patients with CF.[41] Others report resistance mechanisms that include AmpC and efflux pump overexpression but not OprD involvement; these data were from *P. aeruginosa* isolates taken from blood stream infections again in patients without CF.[42] Finally, in samples taken from blood, urine, tracheal aspirates, and wounds from a variety of patients, investigators showed ceftazidime–avibactam resistance mechanism that predominantly involved the overexpression of efflux pumps.[43] Taken together, these data suggest that ceftazidime–avibactam resistance in *P. aeruginosa* isolates is likely multifactorial and may vary depending on the source of the specimen along with the underlying disease process of the patient. Our data provides initial mechanistic insight into the characteristics of ceftazidime–avibactam resistance in *P. aeruginosa* strains isolated from the sputum of patients with CF.

Conclusion

Ceftazidime–avibactam is a novel antimicrobial drug targeting difficult to treat Gram-negative organisms. This study demonstrates excellent susceptibility profiles for ceftazidime–avibactam against highly drug-resistant *P. aeruginosa* strains collected from the sputum of individuals with CF. Strains resistant to ceftazidime–avibactam demonstrated high rates of OprD loss and elevated AmpC expression. Future studies are needed to determine the efficacy of ceftazidime–avibactam in vivo in individuals with CF being actively treated for pulmonary exacerbations who harbor highly drug-resistant *P. aeruginosa*.

Acknowledgment

We would like to thank the patients with CF who participated in this study.

Author contributions

SA, SDA, MF, and AK contributed to subject identification, sample collection, and writing and editing the article. RH contributed to sputum processing and editing the article. DEG, MB, and JDF contributed to writing and editing the article. HS and MC contributed to the susceptibility studies and gene expression data along with editing the article. RJ contributed to oversight of the project along with sample collection, subject identification, manuscript writing, and editing. All authors contributed toward data analysis, drafting and revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

Table S1 Antimicrobial susceptibility of the 32 isolates

	CAZ	FEP	ATM	TZP	MEM	LVX	CIP	AMK	GEN	CST	CAZ–AVI
1	64	>16	32	>128	≤0.12	1	0.5	32	>8	2	2
2	32	16	16	>128	0.5	1	0.5	16	8	2	1
3	128	>16	>128	>128	>16	>4	4	16	>8	1	8
4	32	>16	32	64	>16	4	2	16	4	0.5	2
5	128	>16	128	>128	4	>4	>4	>32	>8	2	8
6	16	16	16	128	2	>4	4	32	>8	2	1
7	64	>16	32	>128	0.5	4	2	16	4	2	2
8	16	16	16	64	0.25	>4	4	32	>8	2	2
9	16	8	16	128	8	>4	2	>32	>8	0.5	2
10	>128	>16	128	>128	4	>4	>4	>32	>8	1	4
11	>128	>16	128	128	0.25	4	1	32	>8	0.5	2
12	64	>16	64	>128	16	>4	>4	>32	>8	2	4
13	>128	>16	32	>128	≤0.12	1	1	16	8	1	8
14	64	>16	32	>128	1	2	1	>32	>8	0.5	2
15	128	>16	64	>128	16	>4	4	>32	>8	4	4
16	16	8	8	16	0.5	4	2	32	>8	8	1
17	>128	>16	>128	>128	>16	>4	2	>32	>8	0.5	8
18	16	8	16	64	1	0.25	0.06	8	2	2	2
19	64	>16	64	64	2	2	1	32	>8	0.25	4
20	64	>16	>128	>128	8	>4	>4	4	2	1	2
21	64	>16	>128	32	8	>4	<4	16	8	0.5	2
22	16	>16	128	32	16	>4	4	32	>8	2	8
23	>128	>16	16	>128	16	>4	2	>32	>8	0.5	8
24	>128	>16	64	>128	8	4	4	32	>8	0.25	64
25	>128	>16	128	>128	>16	>4	>4	>32	>8	0.5	64
26	>128	>16	>128	>128	0.5	0.12	0.12	>32	>8	1	16
27	64	>16	128	32	>16	>4	>4	32	>8	2	64
28	>128	>16	128	>128	>16	>4	>4	32	>8	0.5	64
29	>128	>16	64	>128	16	>4	>4	>32	>8	1	16
30	>128	>16	>128	>128	8	>4	>4	>32	>8	0.5	>128
31	>128	>16	128	>128	>16	>4	>4	>32	>8	0.5	128
32	64	>16	16	64	4	>4	4	32	>8	0.25	16

Abbreviations: AMK, amikacin; ATM, aztreonam; CAZ–AVI, ceftazidime–avibactam; CAZ, ceftazidime; CIP, ciprofloxacin; CST, colistin; FEP, cefepime; GEN, gentamicin; LVX, levofloxacin; MEM, meropenem; TZP, piperacillin–tazobactam.

| MW Ladder | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | PAO1 control |

← ~49 kDa
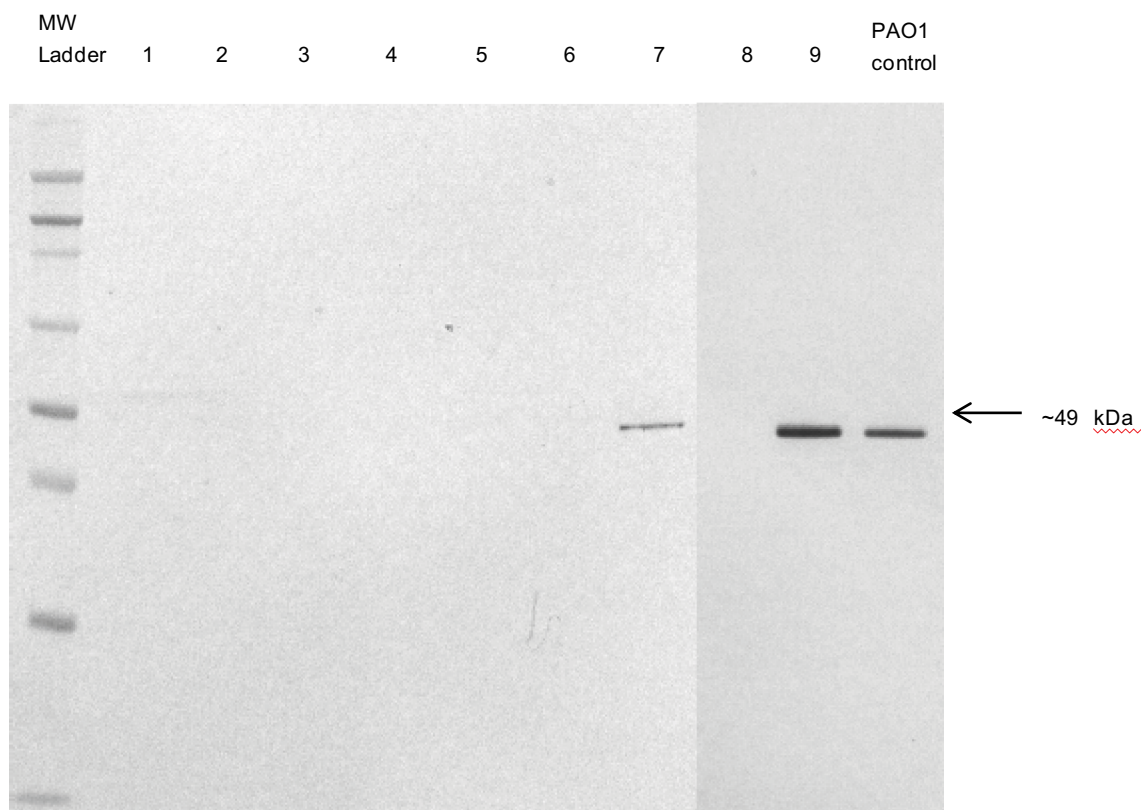
Figure S1 Outer membrane protein (OprD) expression.
Notes: Outer membrane protein (OprD) expression by Western blot of the nine ceftazidime–avibactam-resistant *Pseudomonas aeruginosa* strains. PAO1 was used as a positive control.