



Serum-Associated Antibiotic Tolerance in Pediatric Clinical Isolates of *Pseudomonas aeruginosa*

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Background. When grown in human serum, laboratory isolates of *Pseudomonas aeruginosa* exhibit tolerance to antibiotics at inhibitory concentrations. This phenomenon, known as serum-associated antibiotic tolerance (SAT), could lead to clinical treatment failure of pseudomonal infections. Our purpose in this study was to determine the prevalence and clinical impact of SAT in *Pseudomonas* isolates in hospitalized children.

Methods. The SAT phenotype was assessed in patients aged <18 years admitted with respiratory or blood cultures positive for *P. aeruginosa*. The SAT phenotype was a priori defined as a ≥ 2 -log increase in colony-forming units when grown in human serum compared with Luria-Bertani medium in the presence of minocycline or tobramycin.

Results. SAT was detected in 29 (64%) patients. Fourteen patients each (34%) had cystic fibrosis (CF) and tracheostomies. Patient demographics and comorbidities did not differ by SAT status. Among CF patients, SAT was associated with longer duration of intravenous antibiotics (10 days vs 5 days; $P < .01$).

Conclusions. This study establishes that SAT exists in *P. aeruginosa* from human serum and may be a novel factor that contributes to differences in clinical outcomes. Future research should investigate the mechanisms that contribute to SAT in order to identify novel targets for adjunctive antimicrobial therapies.

Keywords: antibiotic resistance; antibiotic tolerance; cystic fibrosis; drug efflux; tracheitis.

Morbidity and mortality from infectious diseases continue to rise in both adult and pediatric hospitals throughout the United States [1]. Complicating the treatment of bacterial infectious diseases is clinical failure of antimicrobial therapy against strains shown to be susceptible in clinical laboratory testing (antibiotic treatment failure [ATF]). Observations in multiple clinical settings have shown that ATF leads to prolonged hospital lengths of stay, use of multiple antibiotics, and increased morbidity and mortality [2, 3]. The chronic persistence of bacteria (colonization) that can become pathogenic in susceptible hosts is an additional cause of morbidity related to infectious diseases. Individuals with chronic respiratory disease such as cystic fibrosis (CF) and chronic respiratory failure that requires a tracheostomy are particularly susceptible to colonization by the gram-negative bacterial pathogen *Pseudomonas*

aeruginosa. In CF, the acquisition of *P. aeruginosa* is associated with more frequent pulmonary exacerbations and hospitalizations as well as decreased survival [4–7]. Similarly, nearly 90% of children with tracheostomies will have respiratory cultures positive for *P. aeruginosa* [8], and acquisition of *P. aeruginosa* post-tracheostomy is independently associated with increased odds of bacterial tracheostomy-associated respiratory tract infections [9]. Both populations, although heterogeneous in underlying pathophysiology, are frequently treated with anti-pseudomonal antibiotics [9, 10]. Nevertheless, a substantial subpopulation of these children, for example, 13% of those with CF, develop chronic pseudomonal infections despite early and aggressive antibiotic therapy [11].

The pathophysiology and molecular basis of ATF in *P. aeruginosa* infection remain mostly undefined. Prior work has shown that ATF in patients with *P. aeruginosa* ventilator-associated pneumonia is not associated with antibiotic class or the resistance profile of the organisms, suggesting that additional factors of the pathogen itself may contribute to ATF [12]. *Pseudomonas aeruginosa* has been demonstrated to exhibit adaptive tolerance to antibiotics when grown in biologically relevant media such as human serum, a phenotype known as serum-associated antibiotic tolerance (SAT) [13].

Received 25 June 2019; editorial decision 4 December 2019; accepted 5 December 2019; Published online 30 December, 2019.

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Journal of the Pediatric Infectious Diseases Society 2020;9(6):671–9

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DOI: 10.1093/jpids/piz094

In contrast to static determinants of resistance where drug-binding sites are altered or enzymes degrade the antibiotic, SAT does not confer a change in the clinical minimum inhibitory concentration (MIC). Laboratory strains of *P. aeruginosa* are susceptible to ciprofloxacin and tobramycin in broth cultures but become tolerant to the antibiotics' MICs when exposed to human serum [13]. The SAT phenotype has been hypothesized to allow *P. aeruginosa* to persist in the host environment despite exposure to otherwise appropriate antimicrobial treatment. Furthermore, susceptibility of *P. aeruginosa* to ciprofloxacin but not tobramycin can be restored with treatment with the known efflux pump inhibitor phenylalanine- β -naphthylamide (PA β N), suggesting efflux- and nonefflux-mediated mechanisms for this tolerance [13].

Our purpose in this study was to determine the prevalence of SAT among *P. aeruginosa* isolates obtained from pediatric patients, characterize the population with *P. aeruginosa* isolates that exhibit SAT, and investigate the association between SAT and relevant clinical outcomes.

METHODS

Study Design and Population

Children aged <18 years hospitalized between 1 July 2016 and 28 February 2017 at Johns Hopkins All Children's Hospital with cultures positive for *P. aeruginosa* obtained from blood, endotracheal tube aspirate, tracheostomy aspirate, or expectorated sputum in the emergency department (during the encounter that immediately preceded admission), general pediatric inpatient ward, or pediatric/cardiovascular intensive care units were included in the study. An individual patient could contribute multiple specimens during the study period. Specimens were excluded if they could not be processed within 5 days of collection or were resistant to minocycline or tobramycin according to MICs in broth medium. Specimens from patients hospitalized in the neonatal intensive care unit were also excluded in order to reduce the potential influence of longer neonatal hospitalizations on clinically relevant outcomes. Patient data were obtained from the electronic health information system by 2 investigators and were further reviewed by a pediatric infectious diseases specialist (D. B.). The Johns Hopkins All Children's Hospital Institutional Review Board approved the study, with waiver of informed consent granted.

Bacterial Strains and Growth Conditions

Pseudomonas aeruginosa laboratory strain PAO1 was provided by Barbara Iglewski (University of Rochester, Rochester, NY). All clinical specimens were collected according to institutional standards of care and grown on sheep's blood agar plates. A single colony of *P. aeruginosa* was subcultured onto agar slants and transferred to the laboratory at the University of Rochester. All strains were grown in either Luria-Bertani (LB) medium

(VWR, Radnor, PA) or 100% human serum (Corning, Corning, NY). Where indicated, LB or serum was supplemented with the indicated concentration of tobramycin (Abraxis Pharmaceutical Products, Wilmington, DE) or minocycline (MP Biomedicals, Solon, OH). Minocycline was chosen as a previously studied surrogate for efflux-mediated SAT in our laboratory [13, 14]. Tobramycin was chosen as a clinically relevant antibiotic for the treatment of pseudomonal infections.

Antibiotic Susceptibility Assays

Pseudomonas aeruginosa was grown overnight from the agar slants in LB medium, diluted into fresh medium (1:100), and grown to the mid-exponential phase (optical density at 600 nm [OD₆₀₀], 0.4) at 37°C with aeration. A total of 1×10^4 colony-forming units (CFUs) were transferred to individual wells of a 96-well round bottom plate containing 100 μ L of LB supplemented with 2-fold increasing concentrations of tobramycin or minocycline (0.25 μ g/mL to 256 μ g/mL). The MIC was considered to be the lowest concentration of antibiotic in which no bacterial growth could be detected by eye. Once the MIC was determined, a fresh colony from the same frozen stock of the indicated bacterial strain was grown overnight in LB medium, diluted into fresh medium (1:100), and grown to OD₆₀₀ = 0.4 at 37°C with aeration. The susceptibility assays were performed in triplicate by transferring 1×10^4 CFU to individual wells of a 96-well round bottom plate containing 100 μ L of LB or 100% human serum. LB medium was utilized for the SAT assays due to the decreased variability in MIC with differing cation concentrations in comparison to that seen with Mueller-Hinton broth [15]. Cells were incubated in the plates for 2 hours at 37°C without shaking and then supplemented with the indicated MIC concentrations of tobramycin or minocycline and then incubated at 37°C for 48 hours. The contents of each well were serially diluted in sterile 0.8% sodium chloride and plated on LB agar to quantitate CFU/mL. SAT was defined a priori as a ≥ 2 -log increase in CFU/mL grown in serum + antibiotic compared to LB + antibiotic. This definition was based on prior observations that *P. aeruginosa* laboratory strain PAO1 exhibited this level of growth in serum + antibiotic [13]. Growth controls for both LB and serum were performed without the addition of antibiotic; isolates that exhibited a difference in growth potential at the 48-hour time point were excluded. The increase in growth noted in the presence of serum + antibiotic was further assessed as resulting from inducible tolerance vs a conferred genetic mutation in PAO1 and a subset of clinical isolates. Single colonies obtained after exposure to serum + antibiotic (at the maximum concentration with observable visible growth) were subsequently subjected to MIC determination in LB + antibiotic as described.

Clinical Data Collection

Patient demographic information including age, sex, chronic diagnoses, and primary admitting diagnosis was collected.

Chronic diagnoses were categorized into the following organ systems: respiratory, neurologic, gastrointestinal, cardiac, renal, and hepatic disease and immunosuppression status. Patient home respiratory support including tracheostomy status, non-invasive positive-pressure ventilation requirement, and/or ventilator settings were recorded. The number of hospitalizations, the admitting diagnosis for these hospitalizations, and the number of respiratory and blood cultures in the 12 months preceding enrollment were collected. Additionally, we examined clinical outcomes including total length of stay (LOS), duration of intravenous (IV) antibiotic use, duration of fever (>38°C), number of critical care days, duration of intensity of respiratory support exceeding baseline requirements, and mortality during the study period. For patients with positive cultures from repeated hospitalizations within the study period, data from the first (“index”) hospitalization were used in the analysis of clinical outcomes described above.

Statistical Analyses

Continuous variables were reported as either medians (with interquartile range [IQR]) or means (standard deviation) dependent on whether the continuous variable was normally distributed based on visual and statistical assessment. Categorical variables were reported as frequency counts (with percentages). Differences in nonnormally distributed continuous variables between a priori specified groups were assessed using the Wilcoxon rank sum test, whereas the independent sample *t* test was used to determine whether differences in normally distributed continuous variables existed. The χ^2 test was used to determine whether categorical variables differed between a priori specified groups. Due to the small group sizes, exact methods were used for all calculations. Statistical significance was defined as a *P* value < .05. Statistical analysis was performed using SAS software (v. 9.4).

RESULTS

During the study period, 338 cultures were positive for *P. aeruginosa*, 32.5% (110/338) of which were obtained on hospitalized patients (Figure 1). SAT assays were performed on 72 cultures from 44 unique patients during 65 unique hospital admissions. Individual MIC values for each strain tested are described in Supplementary Table 1.

Patient Population

Comparisons of clinical characteristics and outcomes by SAT status were performed in 44 patients. Thirty-eight (86%) patients had at least 1 medical comorbidity identified (Table 1). The most common comorbidities were attributed to the respiratory, neurologic, and gastrointestinal systems. Equal proportions of patients had either CF (32%; *n* = 14) or were tracheostomy-dependent (32%; *n* = 14). The majority of patients (84%;

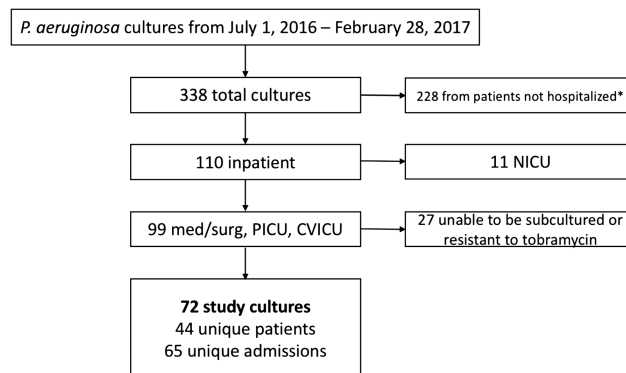


Figure 1. Determination of eligible cultures for inclusion in the serum-associated antibiotic tolerance assay. Cultures obtained from patients in the emergency department who were not admitted were considered outpatient cultures and not included in the study (*). Abbreviations: CVICU, cardiovascular intensive care unit; NICU, neonatal intensive care unit; med, general pediatric medicine unit; PICU, pediatric intensive care unit; surg, pediatric surgery unit.

n = 37) were hospitalized for respiratory-related conditions. The most common admitting diagnoses were respiratory distress (*n* = 14), exacerbation of CF disease (*n* = 11), and tracheitis (*n* = 6). Patients were previously admitted a median (IQR) of 1.5 (1.0–5.0) times in the 12 months prior to enrollment and had a median (IQR) of 4 (1.0–5.0) respiratory cultures obtained during the same time frame. Thirty-two (73%) of the patients had a respiratory culture positive for *P. aeruginosa* within 12 months prior to the study period.

Prevalence of SAT in *P. aeruginosa* Isolates

Specimen sources were as follows: sputum (*n* = 19; 43%), tracheal aspirate (*n* = 15; 34%), endotracheal tube aspirate (*n* = 8; 18%), and blood (*n* = 2; 5%). Forty-five (63%) cultures exhibited the SAT phenotype that involved at least minocycline or tobramycin (Table 2). Tobramycin tolerance alone was observed in 11 (15%) of the isolates, whereas tolerance to minocycline alone was observed in 22 (31%). Tolerance to both antibiotics was observed in 12 (17%) of the isolates. When the analysis was limited to cultures from the index hospitalization (ie, each patient represented once), the frequency of SAT was similar to that for all isolates at 66% (*n* = 29). Six (14%) patients at the index hospitalization exhibited tolerance to tobramycin alone and 14 (34%) exhibited tolerance to minocycline alone. Overall, 9 (20%) exhibited tolerance to both minocycline and tobramycin.

Determination of SAT as an Adaptive Phenotype

The SAT phenotypes observed in laboratory strain PAO1 and a subset of clinical isolates (JHACHPA15, JHACHPA37, and JHACHPA39) were assessed for an inducible response (hypothesized mechanism) vs static resistance phenotypes conferred from a genetic mutation resulting from antibiotic exposure. For

Table 1. Baseline Characteristics of the Study Cohort (n = 44)

Baseline Characteristic	Total
Patient age at admission, median (IQR), y	12.2 (4.9, 16.5)
Sex, n (%)	
Male	20 (45.4)
Female	24 (54.6)
Admitting diagnoses, n (%) ^a	
Cough	8 (18.2)
Fever	6 (13.6)
Hypoxia	10 (22.7)
Pneumonia	11 (25.0)
Respiratory distress	14 (31.8)
Tachypnea	1 (2.3)
Cystic fibrosis/exacerbation	14 (31.8)
Tracheitis	6 (13.6)
Other	30 (68.2)
Chronic diagnoses, n (%) ^a	
Respiratory diagnoses	
Other respiratory ^b	17 (38.6)
Cystic fibrosis	14 (31.8)
Tracheostomy dependence	14 (31.8)
Neurologic diagnoses ^c	11 (75.0)
Cardiac diagnoses ^d	4 (9.1)
Gastrointestinal diagnoses ^e	38 (86.4)
Immunosuppressed ^f	3 (6.8)
Renal diagnoses	2 (4.6)
Hepatic diagnoses	3 (6.8)

^a Patients could have had multiple admitting or chronic diagnoses.

^b Asthma, bronchopulmonary dysplasia (BPD), chronic lung disease (CLD), home respiratory support.

^c Cerebral palsy, developmental delay, hydrocephalus, intellectual disability, nonambulatory, and/or ventriculoperitoneal shunt.

^d Congenital heart disease, pulmonary hypertension.

^e Aspiration, feeding-tube dependency, gastric fundoplication, supplemental nutrition requirement.

^f Immunosuppressed due to medication, genetic, or both medication and genetic.

all isolates tested, the measured MIC for cells subcultured after exposure to serum + antibiotic was equivalent to that for cells not previously exposed to serum or antibiotic (Table 3).

Relationship Between SAT and Patient Characteristics

Medical comorbidities did not differ by SAT status (Table 4). Among patients with SAT-negative *P. aeruginosa* isolates, 4 (27%) had a diagnosis of CF and 4 (27%) were tracheostomy-dependent. Similarly, 11 (38%) and 10 (35%) of SAT-positive patients had a diagnosis of CF or were tracheostomy-dependent, respectively. The majority of patients, regardless of SAT group,

Table 2. Prevalence of Serum-Associated Antibiotic Tolerance Phenotypes Among Total Study Cultures (n = 72) and Index Hospitalizations (n = 44)

Antibiotic Serum-Associated Antibiotic Tolerance Phenotype	Total Cultures, n (%)	Index Hospitalization, n (%)
No	27 (37)	14 (32)
Yes	45 (63)	29 (66)
Tobramycin alone	11 (15)	6 (14)
Minocycline alone	22 (31)	14 (24)
Tobramycin + minocycline	12 (17)	9 (20)

were hospitalized for respiratory conditions. The median (IQR) number of prior hospitalizations for SAT-negative patients (1.0 [1.0–5.0]) and SAT-positive patients (2.0 [0.0–3.0]) was not significantly different ($P = .93$).

Relationship Between SAT and Clinical Outcomes

Clinical outcomes are summarized by SAT status in Table 5. Overall, median (IQR) total hospital LOS was 9 days (5.5–16). There was no significant difference in median LOS between SAT-positive and SAT-negative patients in the overall study population ($P = .53$). Similarly, mean duration of antibiotic administration did not differ by SAT status ($P = .23$). However, in the study subpopulation of CF patients (Table 6), those with SAT-positive isolates at the index hospitalization had a significantly longer median duration of IV antibiotic administration compared with SAT-negative isolates (10 vs 5 days, respectively; $P < .01$). Median LOS was also longer in SAT-positive CF patients whose isolates at the index hospitalization were SAT-positive when compared with those whose isolates were SAT-negative (12 vs 5 days; $P = .07$), although this difference did not meet the threshold for statistical significance. By contrast, in the tracheostomy subpopulation, there was a lack of information to detect a difference in duration of IV antibiotic use ($P = .56$) or LOS ($P = .73$) between patients whose isolates were SAT-positive and SAT-negative at the index hospitalization (Table 7). Patients with tracheostomy with the tobramycin SAT-positive phenotype had a longer median duration of IV antibiotic administration and LOS, although these results did not reach the threshold for statistical significance ($P = .72$ and $P = .90$, respectively; Table 7).

DISCUSSION

In this study, we are the first to report the existence of SAT among clinical isolates of *P. aeruginosa* obtained from pediatric patients. We demonstrated the SAT phenotype in more than two-thirds of all clinical isolates collected in this cohort, the majority of which was comprised of children with CF or tracheostomy who had 2 or more comorbidities. Furthermore, although there were no statistically significant differences in outcomes of the index hospitalization between SAT-positive and SAT-negative groups for the overall study population, we found a statistically significant increase in duration of IV antibiotics and a clinically significant (albeit not statistically significant) increase in hospital LOS in the CF subpopulation.

The aforementioned findings are important for better understanding the mechanisms by which *P. aeruginosa* evades eradication in its host, particularly those individuals affected by chronic and recurrent pseudomonal infections. The colonization of patients with CF and patients with tracheostomy by *P. aeruginosa* has been associated with poorer clinical outcomes [5, 9]. Treatment approaches aimed at eradicating *P. aeruginosa*

Table 3. Determination of Minimum Inhibitory Concentrations for Laboratory Strain PAO1 and Clinical Isolates Exhibiting the Serum-Associated Antibiotic Tolerance Phenotype Before and After Exposure to Serum + Antibiotic

Strain	Antibiotic ^a	MIC LB-pre Serum (µg/mL)	MIC Serum (µg/mL)	MIC LB-post Serum (µg/mL)
PAO1	Minocycline	8	>32	8
JHACHPA15	Minocycline	8	>32	8
	Tobramycin	2	8	2
JHACHPA37	Minocycline	16	>32	16
	Tobramycin	1	>1	1
JHACHPA39	Minocycline	16	>32	16

Abbreviation: LB, Luria-Bertani; MIC, minimum inhibitory concentration.

^a Minimum inhibitory concentrations determined only for antibiotics to which the strain exhibited serum-associated antibiotic tolerance.

from the airways of patients with CF have limited long-term success [4, 11, 16]. Similarly, children with tracheostomy have high readmission rates of approximately 30% for respiratory illnesses despite frequent administration of systemic anti-pseudomonal antibiotics [17]. The reasons for this microbial persistence are characterized best in patients with CF and are multifactorial [18–20]. Antibiotic extrusion through multidrug efflux pumps is one mechanism by which *P. aeruginosa* may persist in its host, conferring resistance to multiple antibiotics [21–28] and fitness

advantages for the organism [29, 30]. Further elucidation of the mechanisms that underlie SAT could therefore have important implications for enhanced therapeutic strategies in patients at risk for recurrent pseudomonal infection and/or colonization.

Within our clinical cohort, we determined that the SAT phenotype was present in 63% of total isolates and 68% of unique patients. Because this has not been previously established, it is difficult to determine whether a similar prevalence would be found in populations external to our institution.

Table 4. Baseline Patient Characteristics, Admitting Diagnoses, and Chronic Diagnoses by Serum-Associated Antibiotic Tolerance Status

Baseline Characteristic	SAT-Negative (n = 15)	SAT-Positive (n = 29)	P Value
Patient age at admission, median (IQR), y	9.8 (2.6,16.7)	12.6 (8.0,16.4)	.34
Sex, n (%)			.34
Male	5 (33.3)	15 (51.7)	...
Female	10 (66.7)	14 (48.3)	...
Admitting diagnoses, n (%) ^a			
Cough	1 (6.7)	7 (24.1)	.23
Fever	2 (13.3)	4 (13.8)	1.0
Hypoxia	4 (26.7)	6 (20.7)	.71
Pneumonia	3 (20.0)	8 (27.6)	.72
Respiratory distress	5 (33.3)	9 (31.0)	1.0
Tachypnea	0 (0.0)	1 (3.5)	1.0
Cystic fibrosis/exacerbation	4 (26.7)	10 (34.5)	.74
Tracheitis	1 (6.7)	5 (17.2)	.65
Other	8 (53.3)	22 (75.9)	.18
Chronic diagnoses, n (%)			
Respiratory diagnoses			
Other respiratory ^b	13 (86.7)	21 (72.4)	.45
Cystic fibrosis	4 (26.7)	10 (34.5)	.74
Tracheostomy dependence	4 (26.7)	10 (34.5)	.74
Neurologic diagnoses ^c	11 (73.3)	22 (75.9)	1.0
Cardiac diagnoses ^d	2 (13.3)	2 (6.9)	.60
Gastrointestinal diagnoses ^e	13 (86.7)	25 (86.2)	1.0
Immunosuppressed ^f	0 (0.0)	3 (10.3)	.54
Renal diagnoses	0 (0.0)	2 (6.9)	.54
Hepatic diagnoses	1 (6.7)	2 (6.9)	1.0

Abbreviation: SAT, serum-associated antibiotic tolerance.

^a Patients could have had multiple admitting or chronic diagnoses.

^b Asthma, bronchopulmonary dysplasia (BPD), chronic lung disease (CLD), home respiratory support.

^c Cerebral palsy, developmental delay, hydrocephalus, intellectual disability, nonambulatory, and/or ventriculoperitoneal shunt.

^d Congenital heart disease, pulmonary hypertension.

^e Aspiration, feeding-tube dependency, gastric fundoplication, supplemental nutrition requirement.

^f Immunosuppressed due to medication, genetic, or both medication and genetic.

Table 5. Clinical Outcomes for Entire Study Cohort by Overall Serum-Associated Antibiotic Tolerance (SAT) Status and SAT Pharmacologic Phenotype

Outcome	SAT-Negative (n = 15)		P Value	Minocycline SAT-Positive (n = 23)		Tobramycin SAT-Positive (n = 13)	
Duration with fever, median (IQR), d	1.0 (0.0, 1.0)	0.0 (0.0, 1.0)	.16	0.0 (0.0, 1.0)	.22	0.0 (0.0, 1.0)	.45
Duration in pediatric intensive care unit, median (IQR), d	1.0 (0.0, 5.0)	0.0 (0.0, 7.0)	.87	0.0 (0.0, 10.0)	.90	0.0 (0.0, 11.0)	.87
Hospital length of stay, median (IQR), d	7.0 (5.0, 14.0)	9.0 (6.0, 18.0)	.53	9.0 (6.0, 18.0)	.52	11.0 (7.0, 20.0)	.28
Duration of antibiotics, median (IQR), d	5.0 (5.0, 8.0)	8.0 (5.0, 10.0)	.23	8.0 (3.0, 11.0)	.25	8.0 (6.0, 10.0)	.14
Duration of oxygen therapy (>home requirement), median (IQR), d	5.0 (0.0, 11.0)	4.0 (0.0, 8.0)	.66	5.0 (0.0, 9.0)	.80	3.0 (0.0, 8.0)	.72
Mortality, n (%)	0 (0.0)	2 (6.9)	.54	2 (8.7)	.51	2 (15.4)	.21

Abbreviation: IQR, interquartile range; SAT, serum-associated antibiotic tolerance.

Multidrug-resistant *P. aeruginosa*, which may share common molecular mechanisms to SAT, has been estimated to affect up to 45% of patients with CF [31–33]. The prevalence of multidrug-resistance in non-CF patients is not as well characterized. The influence of SAT on clinical outcomes warrants an understanding of the drug-targetable mechanisms that could inhibit this phenomenon. Multiple mechanisms for antibiotic resistance in *P. aeruginosa* have been previously described [19]. While intrinsic and acquired mechanisms for resistance have been well characterized in *P. aeruginosa*, adaptive mechanisms by which the pathogen responds to an environmental stimulus to alter its tolerance to antibiotics are a more recent discovery [13, 34–38]. Previously, we showed that the laboratory strain of *P. aeruginosa*, PAO1, becomes tolerant to ciprofloxacin and tobramycin when exposed to human serum and does so in both an efflux-dependent and -independent manner [13]. The present work confirms this in vitro observation from a laboratory strain via ex vivo findings from patient-derived isolates in a cohort of children with CF or tracheostomy.

The mechanisms for the SAT identified here are not entirely clear. Previous studies in the gram-negative bacterium *Acinetobacter baumannii* have shown that SAT is the result of the overexpression of a wide repertoire of efflux pumps when exposed to human serum [14] and physiological concentrations of sodium chloride [39]. Treatment with the known efflux pump inhibitor PAβN restored the susceptibility of *A. baumannii* to antibiotic exposure. Similar to *A. baumannii*, *P. aeruginosa* harbors numerous known and putative efflux pumps [40]. Most notable

are the resistance-nodulation-division (RND) efflux systems that are capable of extruding antibiotic and nonantibiotic substrates [41]. Specifically, the MexXY and MexAB-OprM efflux pumps are capable of extruding aminoglycosides such as tobramycin [42]. While constitutive overexpression of these pumps is known to contribute to multidrug-resistance in *P. aeruginosa* [43], adaptive mechanisms for SAT are possible in response to bacterial exposure to human plasma in vivo and serum ex vivo. Induction of RND-type efflux pumps has been described in *P. aeruginosa* after exposure to electrophilic stressors [44] and nitrosative stresses [45]; human serum as a growth medium may represent conditions that elicit a similar physiologic response. Albumin, a prominent component of human serum, has also been linked to regulation of quorum-sensing apparatus [46], the function of which is affected by drug efflux pump expression [47–50]. Although not directly evaluated in our study, it is possible that serum-specific components increase the expression of drug efflux pumps and thereby result in the SAT phenotype. The relevance of the SAT phenotype within the lung environment and whether exposure to alveolar epithelial lining fluid (aELF) induces a similar tolerance to antibiotic challenge were not definitively addressed in this study. While it was determined in previous studies that the composition of aELF protein [51–54] and elemental compositions in healthy volunteers [55, 56] are similar to those in serum, further investigation to elucidate the host factors responsible for SAT and whether these factors are specific to serum or ubiquitous among biologically relevant growth media is warranted.

Table 6. Clinical Outcomes for Cystic Fibrosis Patient Subpopulation by Overall Serum-Associated Antibiotic Tolerance (SAT) Status and SAT Pharmacologic Phenotype

Outcome	SAT-Negative (n = 4)		P Value	Minocycline SAT-Positive (n = 7)		Tobramycin SAT-Positive (n = 5)	
Duration with fever, median (IQR), d	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	1.0	0.0 (0.0, 0.0)	1.0	0.0 (0.0, 0.0)	1.0
Duration in pediatric intensive care unit, median (IQR), d	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	1.0	0.0 (0.0, 0.0)	1.0	0.0 (0.0, 0.0)	1.0
Hospital length of stay, median (IQR), d	5.0 (4.0, 10.0)	12.0 (9.0, 18.0)	.07	11.0 (9.0, 18.0)	.09	14.0 (11.0, 21.0)	.08
Duration of antibiotics, median (IQR), d	5.0 (4.0, 5.5)	10.0 (8.0, 15.0)	<.01	10.0 (9.0, 17.0)	<.01	10.0 (8.0, 15.0)	.02
Duration of oxygen therapy (>home requirement), median (IQR), d	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	1.0	0.0 (0.0, 0.0)	1.0	0.0 (0.0, 0.0)	1.0
Mortality, n (%)	0 (0.0)	0 (0.0)	1.0	0 (0.0)	1.0	0 (0)	1.0

Abbreviation: IQR, interquartile range; SAT, serum-associated antibiotic tolerance.

Table 7. Clinical Outcomes for Tracheostomy Patient Subpopulation by Overall Serum-Associated Antibiotic Tolerance (SAT) Status and SAT Pharmacologic Phenotype

Outcome	SAT-Negative (n = 4)	SAT-Positive (n = 10)	P Value	Minocycline SAT-Positive (n = 7)	P Value	Tobramycin SAT-Positive (n = 5)	P Value
Duration with fever, median (IQR), d	1.0 (0.5, 4.0)	0.0 (0.0, 0.0)	.21	0.0 (0.0, 0.0)	.21	0.0 (0.0, 1.0)	.63
Duration in pediatric intensive care unit, median (IQR), d	1.0 (0.0, 2.0)	0.5 (0.0, 3.0)	.83	0.0 (0.0, 3.0)	.82	1.0 (0.0, 19.0)	.73
Hospital length of stay, median (IQR), d	6.0 (4.5, 39.0)	5.5 (3.0, 11.0)	.73	6.0 (5.0, 11.0)	.94	11.0 (2.0, 18.0)	.90
Duration of antibiotics, median (IQR), d	6.0 (4.0, 8.0)	3.0 (0.0, 9.0)	.56	3.0 (3.0, 9.0)	.74	9.0 (3.0, 10.0)	.72
Duration of oxygen therapy (>home requirement), median (IQR), d	6.5 (4.5, 10.0)	6.0 (4.0, 9.0)	1.0	6.0 (6.0, 9.0)	.85	9.0 (3.0, 11.0)	1.0
Mortality, n (%)	0 (0.0)	1 (10.0)	1.0	1 (11.1)	1.0	1 (20)	1.0

Abbreviation: IQR, interquartile range; SAT, serum-associated antibiotic tolerance.

In addition, nonefflux-mediated mechanisms may be contributing to the SAT phenotype. For instance, human serum may be a relatively nutrient-limited environment and thus induce the bacterium's stringent response with formation of persister cells known to be more tolerant to antibiotics [38]. One alternative explanation for the increased growth in human serum includes decreased effective antibiotic concentration due to significant protein-binding of the antibiotic itself. This has been shown to be negligible for aminoglycoside antibiotics such as tobramycin [57] but could be relevant for minocycline, which is known to exhibit significant binding [58]. However, this mechanism alone would not explain the variability of the minocycline SAT phenotype observed in this study, particularly for isolates with equivalent minocycline MICs. Furthermore, the restoration of susceptibility to minocycline by efflux pump inhibition suggests that efflux contributes, at least in strong part, to the SAT phenotype [13]. Additional studies into the mechanism(s) that underlies the SAT phenotype will provide more insight into the feasibility of this phenomenon as a druggable target for the treatment of pseudomonal infections.

There are several limitations to this study. First, as the inclusion criteria for the study was pathogen-based and not disease-based, the study population was heterogeneous with respect to baseline clinical characteristics and the clinical management that may have affected the results of our secondary clinical outcomes. The resultant limitation of the between-group comparisons for clinical outcomes may have led to an underestimation of the relationship between SAT and clinical outcomes. Second, the nonprospective design of our study engenders heterogeneity in patient management, which historically tends to regress putative associations toward the null of no association. A prospective study will help not only to validate our cross-sectional study findings but also to generate more reliable estimates of the strength of the association between SAT phenotype and clinical outcomes. Third, given its sample size, the study may have been underpowered to definitively perform our secondary objective of investigating the relationship between SAT and clinical outcomes. Nevertheless, we were able to demonstrate that duration of IV antibiotic use in the CF population was significantly increased for patients with SAT-positive isolates compared with those with SAT-negative isolates at the index hospitalization. In addition, our study did not collect aELF from patients in whom

our isolates were obtained. Thus, we cannot address whether aELF alone would induce the antibiotic tolerance observed in this study. Identification of additional media in which the SAT phenotype is observed is an important next step for future research efforts. Last, we were unable to accurately measure the influence of antibiotic exposure preceding inclusion in this cross-sectional study. Future studies are warranted to investigate the influence of a patient's longitudinal exposure to antibiotics on the prevalence of SAT and clinically relevant outcomes associated with SAT. Notwithstanding these limitations, our findings warrant further adequately powered research in expanded pediatric populations with infectious disease or heightened infectious disease risk.

In conclusion, this study establishes that clinical isolates of *P. aeruginosa* harbor the SAT phenotype and that this phenotype is found in multiple pediatric populations at risk for chronic and acute-on-chronic pulmonary infections. Our results further show that SAT is a novel factor associated with differences in clinical outcomes of patients affected by *P. aeruginosa*. Future research should investigate the mechanisms that contribute to SAT in pediatric populations, such as those with CF or tracheostomy dependence, toward the identification of novel targets for future adjunctive antimicrobial therapies.

Supplementary Data

Supplementary materials are available at *Journal of the Pediatric Infectious Diseases Society* online.

Notes

Acknowledgments. We are grateful for the efforts of the faculty and staff in the Johns Hopkins All Children's Hospital Clinical and Translational Research Track. We give special thanks to Ernest Amankwah, PhD, for assistance with study design.

Financial support. This work was supported by an institutional grant from the Johns Hopkins All Children's Foundation.

Potential conflicts of interest. D. B. is a clinical consultant to Teleflex Inc. All remaining authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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