







Pseudomonas aeruginosa Type III Secretion System Virulotypes and Their Association with Clinical Features of Cystic Fibrosis Patients

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Edilene do Socorro Nascimento Falcão Sarges ^{1,2}
Yan Corrêa Rodrigues ¹
Ismari Perini Furlaneto ³
Marcos Vinícios Hino de Melo ¹
Giulia Leão da Cunha Brabo ⁴
Kátia Cilene Machado Lopes ²
Ana Judith Pires Garcia Quaresma ⁴
Luana Nepomuceno Godim Costa Lima ^{1,4}
Karla Valéria Batista Lima ^{1,4}

¹Programa de Pós-graduação em Biologia Parasitária na Amazônia, Universidade do Estado do Pará, Belém, Pará, Brazil; ²Hospital Universitário João de Barros Barreto, Universidade Federal do Pará, Belém, Pará, Brazil; ³Mestrado Profissional em Ensino em Saúde – Educação Médica, Centro Universitário do Pará, Belém, Pará, Brazil; ⁴Instituto Evandro Chagas, Seção de Bacteriologia e Micologia, Ananindeua, Pará, Brazil

Correspondence: Edilene do Socorro Nascimento Falcão Sarges
Ambulatório de Fibrose Cística, Hospital Universitário João de Barros Barreto, Universidade Federal do Pará, Rua dos Mundurucus, 4487, Bairro Guamá, CEP 66075-000, Belém, Pará, Brazil
Tel +5591 993484649
Email edifalcao@ufpa.br

Karla Valéria Batista Lima
Evandro Chagas Institute, Br 316, km 7, s/n., Ananindeua, Pará, Brazil
Tel +5591 32142116
Email karlalima@iec.pa.gov.br

Purpose: *Pseudomonas aeruginosa* appears as the main pathogen in cystic fibrosis (CF) involved in recurrent pneumonia and pulmonary exacerbations. The type III secretion system (T3SS) is one of its main determinants of virulence and is associated with poor clinical progression and increased mortality. This study determined the relationship of clinical features of patients with CF and *P. aeruginosa* T3SS virulotypes.

Materials and Methods: From January 2018 to March 2019, *P. aeruginosa* were isolated from sputum and/or oropharyngeal swabs. T3SS markers (*exoS*, *exoU*, *exoT* and *exoY*) were detected by PCR. Clinical severity according to Shwachman-Kulczycki score and spirometry data were associated with T3SS virulotypes.

Results: A total of 49 patients had positive cultures for *P. aeruginosa*. T3SS virulence-related markers were detected as follows: *exoS* 97.9% (n=48), *exoU* 63.2% (n=31), *exoT* 95.9% (n=47) and *exoY* 97.9% (n=48). The prevalence of *exoS*⁺/*exoU*⁺ virulotype was higher than previously reported in CF settings, being detected in 61.2% of the evaluated isolates, present in 70% of intermittent infections and with a significantly higher frequency in cases of exacerbations. The presence of *exoU* in chronic infection was not associated with poor clinical results. In chronic infections, the *exoS*⁺/*exoU*⁻ virulotype prevailed (77.8%) and was associated to worse clinical results according to the Shwachman-Kulczycki score and spirometric.

Conclusion: Our findings revealed a high prevalence of the atypical *exoS*⁺/*exoU*⁺ virulotype among *P. aeruginosa* isolates from patients with CF, which was associated with intermittent infection and early clinical alterations, while the *exoS*⁺/*exoU*⁻ virulotype was associated with chronic infection and worse clinical results. Finally, the presented data highlight the relevance of T3SS virulence markers in the clinical progression and disease severity in CF patients.

Keywords: virulence genes, type III secretory system, *exoS*, *exoU*, *exoT*, *exoY*

Introduction

Cystic fibrosis (CF) is an autosomal recessive disorder, which mutation on the cystic fibrosis transmembrane conductance regulator (CFTR) gene negatively affects the encoding of a protein that regulates chlorine and sodium transmembrane transport to intracellular medium, causing extracellular dehydration, electrolyte changes in sweat, production of thick mucus, and consequent obstruction of the airways, pancreatic, biliary and efferent pathways. In Brazil, it is estimated that the incidence of CF is 1:7.576 live births; however, due to its great population miscegenation, it presents regional differences, with higher values observed in Brazilian southern states.¹⁻³

The clinical presentation is variable, mostly occurring progressive respiratory disease and malnutrition, acute pulmonary exacerbations, increased cough, changes in the appearance of sputum secretions, fever, decrease in spirometric data, radiological changes and weight loss, leading to progressive respiratory and functional impairment and, later, respiratory failure. Thus, chronic lung disease remains the main responsible for the high morbidity and mortality among CF patients.^{3–5}

Pseudomonas aeruginosa is one of the most frequent and mortality-related pathogens in CF patients, causing recurrent lower respiratory tract infections and/or chronic lung colonization, which accelerates the deterioration of lung function and nutritional status. The pathogen eradication and disease severity are associated with its impressive genetic and metabolic plasticity, antimicrobial resistance and secretion of virulence exoproducts.^{6–10}

Pathogenicity in *P. aeruginosa* is related to virulence factors that contribute to host persistence and invasion. Shared by several Gram-negative bacteria species, the type III secretion system (T3SS) is one of the most clinically relevant virulence determinants in *P. aeruginosa*, which acts through the translocation of up to four exotoxins (ExoS, ExoU, ExoT and ExoY) to the host cell by a needle-like appendix. The high virulence potential conferred by secretion ExoS and ExoU contributes to the deterioration of lung tissue and higher mortality probability. ExoT and ExoY play a minor role in virulence, as in vivo and in vitro assays have demonstrated little cytotoxicity.^{11,12} These effector proteins are encoded by *exoS*, *exoU*, *exoT* and *exoY* genes, respectively, and are variably detected in *P. aeruginosa*, where *exoS*, *exoT* and *exoY* are found in approximately all strains, while *exoU* is detected less frequently for being associated with a pathogenicity island (PAPI-2) not belonging to the core genome of *P. aeruginosa*. In addition, *exoS* and *exoU* genes are usually mutually exclusive, being the *exoS*⁺/*exoU*⁺ virulotype rarely detected.^{10–12}

Previously, several studies have associated the *exoS*⁺/*exoU*[−] virulotype with *P. aeruginosa* causing chronic infection in CF patients and most of *exoS*⁺/*exoU*⁺ virulotype have been reported on non-CF strains.^{13–19} In addition, studies evaluating the association between clinical features of patients and T3SS virulotype were performed including *P. aeruginosa* clinical isolates causing different types of acute infections.^{20–22} Therefore, the objective of this study was to determine the prevalence of T3SS genes in *P. aeruginosa* isolates from CF patients at a referral

center in the Brazilian Amazon, as well as its association with patients' clinical features.

Materials and Methods

Study Design and Inclusion Criteria

In this cross-sectional, descriptive and analytical study, 112 CF patients treated at a CF referral center in Pará state, Brazilian Amazon, which had positive cultures for nonspecific germs from oropharynx swab and/or sputum from January 2018 to March 2019, were included. Patients were grouped according to *P. aeruginosa* infection status as chronic, intermittent or infection-free. Chronic infection was defined as a patient with >50% of cultures positive for *P. aeruginosa* in the last 12 months or with the presence of 3 or more positive cultures in a period of 6 months, according to the routine adopted by the referral center.⁴

Bacterial Isolates

All isolates were plated on MacConkey Agar media and incubated for 24h/35°C for observation of colony morphology, presence/or absence of mucoid phenotype and Gram staining. Identification of isolates was performed on an automated VITEK-2 system (bioMérieux, Marcy l'Etoile, France) and complemented by phenotypical and biochemical assays such as oxidase test, absence of carbohydrate fermentation on triple sugar iron agar, oxidative behavior on Hugh Leifson medium and the cytochrome oxidase reaction.

Determination of Severity Degree and Spirometry Data

The variables of the Shwachman-Kulczycki score assessed the degree of clinical severity from CF patients, considering general activity assessment (0–25), radiological findings (0–25), physical examination (0–25) and nutritional status (0–25). A total score ranging from 86 to 100 points expressed an “excellent” evolution, 71–85 “good”, 56–70 “mild”, 41–55 “moderate” and <40 “severe”.²³ Spirometric data were collected from medical records, being evaluated the Forced Expiratory Volume in the 1st second (FEV₁), Forced Vital Capacity (FVC), Tiffenau Index (FEV₁/FVC) and Intermediate Forced Expiratory Flow (FEF_{25–75%}) for analysis of pulmonary function.²⁴

Molecular Detection of T3SS Genes

Genomic DNA was obtained from isolates of the last positive cultures of patients using the PureLink™ Genomic

DNA Mini Kit (Invitrogen™, Carlsbad, USA) following the manufacturer's recommendations. T3SS-related genes – *exoS*, *exoU*, *exoT* and *exoY* – were amplified by PCR as previously described by Finnan et al¹⁹ and Zhu et al,²⁵ with modifications (Table 1). Each PCR mixture was prepared to a final volume of 25 µL, 1x Master mix GoTaq® DNA Polymerase (Promega, Wisconsin, USA), 1x Q-solution (Qiagen, Hilden, Germany), 0.5 µM of each primer and DNA template, followed by amplification in Veriti Thermocycler (Applied Biosystem, USA) under the following conditions: 95°C for 2 minutes; followed by 35 cycles at 95°C for 1 minute, 60°C for 45 seconds, 72°C for 1 minute and a final extension step at 72°C for 7 minutes. The observation of PCR products under ultraviolet light was performed after electrophoresis on 1% agarose gel stained with Syber®Safe stain (Invitrogen™, Carlsbad, USA) at 100 V. *P. aeruginosa* ATCC 27,853 and PA14 strains were used as positive controls.

Statistical Analysis

The results were treated using descriptive statistics and expressed using central tendency and dispersion measures appropriate to each distribution. Data normality was tested by the Shapiro–Wilk test. The independence between categories of qualitative variables and groups was tested using the G-test of independence, followed by the Chi-square residue analysis in case of statistical significance. The comparison of ordinal or numerical variables with asymmetric distribution between groups was performed with Kruskal-Wallis variance analysis, complemented by Dunn's post-test in case of significance. Values of $P \leq 0.05$ were considered statistically significant. All analyses were performed using the statistical software BioEstat® 5.4.²⁶

Ethics Statement

Samples included in this study were obtained during the routine laboratory procedure at the CF referral center. This study was approved by the Ethics Committee of Hospital Universitário João de Barros Barreto (Nº 1.910.716) and registered in the National System of Management of Genetic Heritage and Associated Traditional Knowledge (SisGen; Nº AF44CCB).

Results

The referral center had 136 CF patients active in the treatment program. One hundred and twelve patients with oropharyngeal and/or sputum swab cultures were included, of which 49 (43.7%) had positive cultures for *P. aeruginosa* and classified as chronic infection (n =23), intermittent infection (n =26) and infection-free (n =63). The number of annual exacerbations was significantly higher in chronically infected patients when compared to infection-free patients (Table 2/ $p =0.0087$). Patients with chronic infection and infection-free were similar in age and both were older than those with intermittent infection (Table 2/ $p =0.0023$). Regarding age at diagnosis, chronically infected patients tended to be diagnosed later when compared to patients with intermittent infection (Table 2/ $p =0.0025$).

Comparing the distribution of cases according to the severity category, a significantly higher frequency of “mild” and “severe” cases was observed among patients with chronic infection. In addition, it was observed a significantly lower frequency of cases with “excellent” classification among patients with chronic infection and of cases classified as “mild”, “moderate” and “severe” among infection-free patients (Table 3/ $p <0.0001$).

Table 1 Primers Sequences Used in the Molecular Detection of T3SS Related-Genes

Gene	Primers Sequence	Amplicons Size (bp)	Reference
<i>exoS</i>	F: 5'-CGTCGTGTTCAAGCAGATGGTGCTG-3' R: 5'-CCGAACCGCTTACCAGGC-3'	444	Finnan et al ¹⁹
<i>exoT</i>	F: 5'-CAATCATCTCAGCAGAACCC-3' R: 5'-TGTCGTAGAGGATCTCCTG-3'	1159	
<i>exoY</i>	F: 5'-TATCGACGGTCATCGTCAGGT-3' R: 5'-TTGATGCACTCGACCAGCAAG-3'	1035	
<i>exoU</i>	F: 5'-GGGAATACTTTCCGGGAAGTT-3' R: 5'-CGATCTCGCTGCTAATGTGT-3'	428	Zhu et al ²⁵

Abbreviation: bp, base pairs.

Table 2 *P. aeruginosa* Infection Profile According to the Number of Exacerbations/Year, Age and Age at Diagnosis

Variable	Groups			p-value*
	Chronic Infection n=23	Intermittent Infection n=26	Infection-Free n=63	
Number of exacerbations/year Min. – Max. Median (DIQ) P25/P75 CV%	0–4,0 0 (2,0) ^a 0/2,0 127,5	0–2,0 0 (1,0) 0/1,0 141,2	0–4,0 0 (0) ^a 0/0 241,9	0,0087 [†]
Age (years) Min. – Max. Median (DIQ) P25/P75 CV%	2,0–63,0 21,0 (17,0) ^a 9,0/26,0 73,5	1,0–38,0 9,0 (8,3) ^{a,b} 7,0–15,3 66,1	0,2–70,0 15,0 (12,0) ^b 11,0/23,0 73,3	0,0023 [†]
Age at diagnosis (years) Min. – Max. Median (DIQ) P25/P75 CV%	0,3–62,0 12,0 (13,0) ^a 6,0/19,0 101,6	0,1–30,0 3,0 (5,5) ^a 0,8–6,3 135,4	0,1–64,0 6,0 (9,0) 2,0/11,0 133,0	0,0025 [†]

Notes: *Kruskal-Wallis/Dunn test. DIQ, interquartile deviation; P25/P75, 25th and 75th percentiles; CV, coefficient of variation; [†]Statistically significant. Equal superscript letters indicate the groups that showed statistically significant differences from each other.

Table 3 Severity Category According to the Shwachman-Kulckycki Score Among the Studied Groups

Gravity	Groups			p-value*
	Chronic Infection n=23 (%)	Intermittent Infection n=26 (%)	Infection-Free n=63 (%)	
Excellent	0 (0,0) [‡]	12 (46,2)	30 (47,6) [†]	<0,0001 [†]
Good	05 (21,8)	09 (34,6)	23 (36,5)	
Mild	12 (52,2) [†]	03 (11,5)	09 (12,3) [‡]	
Moderate	03 (13,0)	02 (7,7)	01 (1,6) [‡]	
Severe	03 (13,0) [†]	0 (0,0)	0 (0,0) [‡]	

Notes: *G-test of independence/Chi-Square Residue Analysis. [†]Statistically significant. [‡]Frequency lower than expected at random. [†]Frequency higher than expected at random.

Of the 49 patients with a positive culture for *P. aeruginosa*, 39 had data on spirometry and 57 infection-free patients performed the test. Table 4 presents the comparison of the spirometric profile among the investigated groups. It was observed that patients with chronic infection had FEV₁, FVC and FEF_{25–75%} values significantly lower than those with intermittent infection and control group ($p < 0.0001$, $p < 0.0001$ and $p = 0.0003$, respectively), with no difference in the spirometric profile among patients from other groups. The Tiffenau Index (FEV₁/FVC) was similar between the three groups ($p = 0.0660$), while FEF_{25–75%} parameter was the most variable among all groups.

The prevalence of *exoS*, *exoU*, *exoT* and *exoY* genes among the 49 isolates of *P. aeruginosa* were 97.9%

($n = 48$), 63.2% ($n = 31$), 95.9% ($n = 47$) and 97.9% ($n = 48$), respectively. The *exoS*⁺/*exoU*⁺ virulotype was detected in 61.2% ($n = 30$) of isolates. There was a significantly higher frequency of cases of intermittent infection in the presence of *exoS*⁺/*exoU*⁺ virulotype and cases of chronic infection in the presence of *exoS*⁺/*exoU*[–] (Table 5/ $p = 0.0087$). No significant association between T3SS virulotypes and colonies morphology was observed (Table 5/ $p = 0.8163$).

The individual analysis of the Shwachman-Kulckycki score categories revealed that patients infected with *P. aeruginosa* harboring *exoS*⁺/*exoU*[–] virulotype obtained lower scores in “general activity” ($p = 0.0232$), “physical examination” ($p < 0.0001$) and “radiological findings” (p

Table 4 Spirometric Data of CF Patients According to Infection Status

Variable	Groups			p-value*
	Chronic Infection n=18	Intermittent Infection n=21	Infection-Free n=57	
FEV ₁ , % Min. – Max. Median (DIQ) P25/P75 CV%	18,0–124,0 43,0 (51,0) ^{a,b} 28,0/79,0 57,4	30,0–121,0 86,0 (32,0) ^a 70,0/102,0 30,4	31,0–136,0 92,0 (24,3) ^b 79,7/104,0 22,0	<0,0001 [†]
FVC, % Min. – Max. Median (DIQ) P25/P75 CV%	22,0–132,0 54,0 (37,0) ^{a,b} 44,0/81,0 46,4	42,0–133,0 102,0 (29,5) ^a 80,5/110,0 28,4	45,0–140,0 102,0 (25,5) ^b 87,0/112,5 20,1	<0,0001 [†]
FEV ₁ /FVC, % Min. – Max. Median (DIQ) P25/P75 CV%	55,0–97,0 82,0 (25,0) 72,0/97,0 16,3	72,0–108,0 89,0 (25,0) 83,0/108,0 9,1	65,0–109,0 89,0 (11,0) 84,0/95,0 10,4	0,0660
FEF _{25–75} , % Min. – Max. Median (DIQ) P25/P75 CV%	10,0–120,0 30,0 (59,0) ^{a,b} 16,0/75,0 81,4	13,0–155,0 86,0 (47,0) ^a 63,0/110,0 45,8	11,0–156,0 86,0 (33,5) ^b 67,0/100,5 34,7	0,0003 [†]

Notes: *Kruskal-Wallis/Dunn test. FEV₁: forced expiratory volume in the first second. FVC: forced vital capacity. FEF 25–75: forced expiratory flow. DIQ, interquartile deviation. P25/P75: 25th and 75th percentiles. CV, coefficient of variation. [†]Statistically significant. Equal superscript letters indicate the groups that showed statistically significant differences from each other.

Table 5 Association of T3SS Virulotypes with Infection Profile and Colony Morphology

Profile	Virulotype			p-value*
	<i>exoS</i> ⁺ / <i>exoU</i> ⁺ n=30 (%)	<i>exoS</i> ⁺ / <i>exoU</i> ⁻ n=18 (%)	<i>exoS</i> ⁻ / <i>exoU</i> ⁺ n=1 (%)	
Groups				
Chronic infection	9 (30,0) [‡]	14 (77,8) [†]	0 (0,0)	0,0087 [†]
Intermittent infection	21 (70,0) [‡]	4 (22,2) [‡]	1 (100)	
Morphology				
Mucoid	7 (23,4)	8 (44,4)	0 (0,0)	0,8163
Non-mucoid	22 (73,3)	9 (50)	1 (100)	
Both	1 (3,3)	1 (5,6)	0 (0,0)	

Notes: *G-test of independence/Chi-Square Residue Analysis. [†]Statistically significant. [‡]Frequency lower than expected at random. [†]Frequency higher than expected at random.

<0.0001) when compared to infection-free patients. Similarly, the scores for “physical examination” ($p < 0.0001$) and “radiological findings” ($p < 0.0001$) were lower in patients with *P. aeruginosa* harboring *exoS*⁺/*exoU*⁺ virulotype when compared to infection-free patients (Table 6).

Regarding the spirometric data, the FEV₁, FVC and FEF_{25–75%} values were significantly lower in the presence of the *exoS*⁺/*exoU*⁻ virulotype when compared to infection-free patients ($p = 0.0005$, $p = 0.0007$ and $p = 0.0067$, respectively). The Tiffenau Index (FEV₁/FVC) was similar between the three groups ($p = 0.1473$), while FEF_{25–75%}

Table 6 Association of Shwachman-Kulkycki Score Categories with T3SS Genotypes and Infection-Free Patients

Variable	<i>exoS</i> ⁺ / <i>exoU</i> ⁺ n=30	<i>exoS</i> ⁺ / <i>exoU</i> ⁻ n=18	Infection-Free n=63	p-value*
General activity				
Min. – Max.	5,0–25,0	5,0–25,0	15,0–25,0	0,0232 [†]
Median (DIQ)	20 (5,0)	20,0 (7,7) ^a	25,0 (5,0) ^a	
P25/P75	20,0/25,0	17,3/25,0	20,0/25,0	
CV%	22,8	28,0	13,1	
Physical examination				
Min. – Max.	5,0–25,0	5,0–25,0	10,0–25,0	<0,0001 [†]
Median (DIQ)	20,0 (10,0) ^a	15,0 (3,5) ^b	20,0 (5,0) ^{a,b}	
P25/P75	15,0/25,0	15,0/18,5	20,0/25,0	
CV%	30,2	28,5	18,0	
Nutritional status				
Min. – Max.	10,0–25,0	10,0–25,0	10,0–25,0	0,2394
Median (DIQ)	22,5 (6,2)	20,0 (10,0)	25,0 (5,0)	
P25/P75	18,8/25,0	15,0/25,0	20,0/25,0	
CV%	27,4	25,3	20,7	
Radiological findings				
Min. – Max.	5,0–20,0	5,0–20,0	10,0–25,0	<0,0001 [†]
Median (DIQ)	15,0 (10,0) ^a	15,0 (8,0) ^b	20,0 (5,0) ^{a,b}	
P25/P75	10,0/20,0	10,0/18,0	15,0/20,0	
CV%	32,8	32,5	21,3	

Notes: *Kruskal-Wallis/Dunn test. DIQ, interquartile deviation. P25/P75: 25th and 75th percentiles. CV, coefficient of variation. [†]Statistically significant. Equal superscript letters indicate the groups that showed statistically significant differences from each other.

parameter was the most variable among the compared groups (Table 7).

Considering only patients with *P. aeruginosa* chronic infection, significantly lower FEV₁ ($p < 0.0001$) and FEF_{25–75%} ($p = 0.0004$) values were observed associated with *exoS*⁺/*exoU*⁻ virulotype when compared to infection-free patients. In addition, FVC ($p < 0.0001$) and overall Shwachman-Kulky severity score ($p < 0.0001$) were significantly lower in patients with *exoS*⁺/*exoU*⁻ virulotype when compared with *exoS*⁺/*exoU*⁺ virulotype and infection-free groups. The Tiffenau Index was similar among the three groups tested ($p = 0.0669$) (Table 8).

Discussion

The findings in this study regarding the percentage of chronic infection were lower than those previously reported, since it is expected that 60–80% of adults become chronically infected.⁶ However, the presence of chronically infected children rises concern (one, with associated with *exoS*⁺/*exoU*⁺ virulotype, and two to *exoS*⁺/*exoU*⁻ virulotype), since in early childhood *P. aeruginosa* infection occurs intermittently and is eliminated with antimicrobial therapy.¹⁴ In addition, persistence in children

under 2 years of age increases morbidity and mortality in the first 10 years after diagnosis of infection chronicity when associated with *Staphylococcus aureus*. Thus, this chronic infection is the highest predictor of morbidity and mortality (2.6 times higher than those not infected).⁴ This fact demonstrates the importance of microbiological monitoring of patients' sputum and the early introduction of antibiotics when initial and intermittent infections are detected in the first months of life. The diagnosis at the center studied was late since many patients joined the CF program before the implementation of neonatal screening in Pará state. The delay in diagnosis must also have contributed to the onset of chronic infection, highlighting the importance of neonatal screening.¹

Regardless of the presented virulotype, the number of annual exacerbations was significantly higher among patients with chronic infection. In accordance with our findings, previous studies have also demonstrated decreasing in spirometric values after episodes of exacerbations (Table 2).²⁷ However, the previous association of the *exoU* with a higher risk of exacerbation in patients with non-cystic fibrosis bronchiectasis could not be established in our CF study population.²⁸

Table 7 Association of Spirometric Data with T3SS Genotypes and Infection-Free Patients

Spirometric Data	Virulotype		Infection-Free n=57	p-value*
	<i>exoS</i> ⁺ / <i>exoU</i> ⁺ n=24	<i>exoS</i> ⁺ / <i>exoU</i> ⁻ n=14		
FEV ₁ , % Min. – Max. Median (DIQ) P25/P75 CV%	18,0–124,0 82,5 (54,3) 44,8/99,00 39,2	18,0–121,0 49,0 (51,0) ^a 28,0–79,0 56,6	31,0–136,0 92,0 (24,3) ^a 79,7/104,0 22,0	0,0005 [†]
FVC, % Min. – Max. Median (DIQ) P25/P75 CV%	22,0–132,0 92,0 (51,8) 56,5/108,3 35,5	22,0–133,0 54,0 (43,0) ^a 47,0–90,0 46,6	45,0–140,0 102,0 (25,5) ^a 87,0/112,5 20,1	0,0007 [†]
FEV ₁ /FVC, % Min. – Max. Median (DIQ) P25/P75 CV%	55,0–108,0 87,0 (11,0) 82,0/93,0 12,0	57,0–99,0 83,0 (19,0) 72,0–91,0 15,6	65,0–109,0 89,0 (11,0) 84,0/95,0 10,4	0,1473
FEF _{25–75} , % Min. – Max. Median (DIQ) P25/P75 CV%	10,0–143,0 81,5 (65,3) 30,0–95,8 53,9	10,0–150,0 30,0 (66,0) ^a 21,0–87,0 83,1	11,0–156,0 86,0 (33,5) ^a 67,0/100,5 34,7	0,0067 [†]

Notes: *Kruskal-Wallis/Dunn test. FEV₁: forced expiratory volume in the first second. FVC: forced vital capacity. FEF 25–75: forced expiratory flow. DIQ, interquartile deviation. P25/P75: 25th and 75th percentiles. [†]Statistically significant. Equal superscript letters indicate the groups that showed statistically significant differences from each other.

P. aeruginosa clinical isolates exhibit variations in genotypic and phenotypic virulence profiles, which influence infection severity and clinical outcomes.^{13,29} Regarding T3SS-related genes, several authors have shown those variations analyzing isolates from distinct setting, including individual, environmental and clinical isolates, demonstrating the important role in the evolution of human disease and persistence in high-pressure settings.^{16,17,30,31} In CF *P. aeruginosa* isolates, the prevalence of these genes is also variable. In a study conducted at an Iranian CF Center evaluating 143 *P. aeruginosa* isolates, the *exoS* was the most prevalent (90.2%), followed by *exoU* (39.8%), in addition to low frequencies of *exoT* (28.6%) and *exoY* (25.2%).⁷ In a Bulgarian study, 42 CF *P. aeruginosa* isolates the presence of *exoS* genes was observed in 52.4% and *exoU* in 28.6%. The *exoS*⁺/*exoU*⁺ virulotype was present in 11.4% among the 202 isolates studied, including CF and non-CF isolates.³⁰ Overall, previously reported frequencies were lower than those found in the present study, in which the prevalence of virulence genes was *exoS* (97.9%), *exoU* (63.2%), *exoT* (95.9%) and *exoY* (97.9%).

Evidence observed in previous studies indicates the mutual exclusion of *exoS/exoU* genes, being the *exoS*⁺/*exoU*⁺ virulotype rarely reported. However, in our study, a high prevalence of *exoS*⁺/*exoU*⁺ virulotype isolates was observed (61.2%), mainly, in the initial phase of infection and significantly less frequency among chronically infected patients. In a study by Hu et al,¹⁴ *exoU*⁺ strains were observed in the initial and subsequent infections of respiratory specimens in children up to 5 years old diagnosed with CF and followed up after the neonatal screening. Similarly, the obtained data demonstrate the *exoS*⁺/*exoU*⁺ virulotype in a higher frequency among intermittent infections and in patients significantly younger than those from other groups (Table 5).

According to the Shwachman-Kulcyski score, the high frequency of *exoS*⁺/*exoU*⁺ virulotype was associated with radiological changes and physical examination findings when compared to infection-free patients (Table 6). This fact could be better understood evaluating ExoU secretion since this is a marker of highly virulent strains associated with specific sites to immunocompromised

Table 8 Association of Spirometric Data and Shwachman-Kulczycki Score of Patients with *P. Aeruginosa* Chronic Infection with T3SS Genotypes and Infection-Free Patients

Variable	Virulotypes of Chronically Infected Patients		Infection-Free n=57	p-value*
	<i>exoS</i> ⁺ / <i>exoU</i> ⁺ n= 07	<i>exoS</i> ⁺ / <i>exoU</i> ⁻ n=12		
FEV ₁ , % Min. – Max. Median (DIQ) P25/P75 CV%	18,0–124,0 43,0 (54,0) 29,0/83,0 64,5	18,0–100,0 44,0 (40,2) ^a 27,3/67,5 51,9	31,0–136,0 92,0 (24,3) ^a 79,7/104,0 22,0	<0,0001 [†]
FVC, % Min. – Max. Median (DIQ) P25/P75 CV%	22,0–132,0 78,0 (48,0) ^a 38,0/86,0 53,3	22,0–104,0 52,0 (32,0) ^s 44,8/76,8 41,0	45,0–140,0 102,0 (25,5) ^{a,b} 87,0/112,5 20,1	<0,0001 [†]
FEV ₁ /FVC, % Min. – Max. Median (DIQ) P25/P75 CV%	55,0–97,0 82,0 (17,0) 77,0/94,0 17,3	57,0–96,0 82,5 (20,0) 70,5/90,5 16,4	65,0–109,0 89,0 (11,0) 84,0/95,0 10,4	0,0669
FEF _{25–75} , % Min. – Max. Median (DIQ) P25/P75 CV%	10,0–120,0 20,0 (80,0) 16,0/96,0 90,3	10,0–88,0 30,0 (48,2) ^a 12,8/61,0 73,5	11,0–156,0 86,0 (33,5) ^a 67,0/100,5 34,7	0,0004 [†]
Overall Severity Score	n=09	n=14	n=63	
Min. – Max. Median (DIQ) P25/P75 CV%	25,0–85,0 65,0 (25,0) ^a 47,5/72,5 29,7	25,0–85,0 65,0 (11,8) ^b 59,5/71,3 24,6	50,0–100,0 85,0 (15,0) ^{a,b} 75,0/90,0 13,5	<0,0001 [†]

Notes: *Kruskal-Wallis/Dunn test. DIQ, interquartile deviation. P25/P75: 25th and 75th percentiles. CV, coefficient of variation. [†]Statistically significant. Equal superscript letters indicate the groups that showed statistically significant differences from each other.

patients.¹³ Also, in chronically infected patients, the *exoS*⁺/*exoU*⁺ virulotype was not associated with worse clinical data when compared to the *exoS*⁺/*exoU*⁻ virulotype (Table 8); however, such analysis was limited given the reduced number of *exoS*⁺/*exoU*⁺ virulotype in chronic infection. Other studies demonstrate that ExoU-producing strains are poorly represented in persistent CF infection, which might be related to: (I) deletion over time, probably due to treatment with anti-pseudomonal drugs, which causes a selection against ExoU-producing *P. aeruginosa* with loss of *exoU* gene; (II) the deficiency in the secretion of ExoU contributes to the evasion of the immune system and promotes chronic infection; and finally, (III) the previous evidenced role played by ExoS in the pathogenesis of CF, selecting strains harboring *exoS* and allowing *P.*

aeruginosa to colonize and persist in the lung of CF patients.^{14,16,32}

In general, *P. aeruginosa* isolates from adults and older children with CF and chronically infected presents the *exoS*⁺/*exoU*⁻ virulotype,¹³ in agreement with our findings (Tables 2 and 5). This virulotype was related to lower values in spirometric data (FEV₁, FVC and FEF_{25–75}%) and severity score in the categories that directly express pulmonary impairment such as physical examination, general activity and radiological findings (Tables 6 and 7). This is a worrying fact, since the presence of *P. aeruginosa* in patients with CF causes a decrease in the radiological score and Tiffenau Index (FEV₁/FVC),⁴ with the latter being similar among the studied groups (Table 7). In an Australian longitudinal study, 77 (83.7%) were *exoS*⁺, where strains from initial infections

secreted five times more ExoS than those from subsequent infections. However, there was a diminishing in ExoS secretion with increasing age, in line with the hypothesis that bacterial persistence in chronic infection leads to the deregulation of virulence factors; despite this, the formation of antibodies against T3SS still occurs, suggesting its role in the pathogenesis of lung injury.^{14,32} Among environmental samples from the same study, 60.7% presented *exoS*, and 39.1% were *exoU*.¹⁴ It is important to note that the strains of CF patients are mostly environmental, in which *exoU*⁺ is more prevalent.

ExoS is the major virulence factor associated with invasion, colonization and spread in chronic lung infections and burns.³¹ Regardless of the infection status, *exoS* had a higher prevalence, with only one isolate not harboring such a gene, in addition to a higher frequency than *exoU* in the evaluated isolates, indicating an important role in lung infection. Feltman et al¹⁶ reported an *exoS* prevalence of 85% (17/20) in respiratory isolates from CF patients; however, in isolates from other clinical sources, the prevalence varied with statistical significance in relation to isolates from bloodstream infection. In environmental isolates, 80% (16/20) were *exoS*⁺, suggesting that ExoS is not essential for *P. aeruginosa* survival in nature. *exoU* was detected in 10% (2/20) of CF isolates, less frequent than in the other studied and with a significant difference in relation to isolates from bloodstream infection and wound. *exoU* was detected in 20% (4/20) of the environmental samples. None of the isolates demonstrated *exoS*⁺/*exoU*⁺ virulotype. *exoT* was present in 100% of samples and *exoY* in 90% of CF samples, with a significant difference in relation to urine samples. In a larger study comprising CF and non-CF *P. aeruginosa* respiratory isolates, the prevalence of *exoS* was 90% (36/40) and 73% (29/40) while *exoU* was 8% (3/40) and 25% (10/40), respectively, with a significant difference.¹⁶ Lanotte et al³³ also observed the presence of *exoS* among 93.8% of 81 CF isolates, with a significant difference when compared to the other 81 samples from four different clinical origins.

Once colonized, the patient often remains with the same strain for years and it is conceivable that T3SS genes were deleted after years of growth within the CF lung or that it was possible for the ExoS and ExoU to antagonize, performing a selective pressure on *P. aeruginosa* strains to delete one of the encoding genes.³⁴ This fact is in agreement with a cohort with analysis by PFGE and MLST of clinical isolates of *P. aeruginosa* from CF patients, in which 87% had concordant fingerprints for up

to 25 years.³⁵ Also, indistinguishable strains were observed in 77% of patients with chronic infection in a longitudinal study for 32 months.³⁶

Furthermore, previous reports showed that 90% of ExoU-producing *P. aeruginosa* strains were associated with a severe infection in patients with pneumonia associated with mechanical ventilation.³⁷ Thus, the spread and emergence of multidrug-resistant *P. aeruginosa* strains capable of producing ExoU is a challenge in hospital settings and CF referral centers, given the large number of hospitalizations and cycles of antibiotic therapy that these patients may undergo throughout life limiting antimicrobial therapeutic options. Therefore, detection of the *exoU* gene can predict early clinical severity, aiding in the management, antimicrobial therapy and adoption of cross infection control measures.

Conclusions

In conclusion, compared to previous reports, the atypical *exoS*⁺/*exoU*⁺ virulotype was frequently found among our *P. aeruginosa* population, mainly associated with intermittent infections, changes in the physical, spirometric and radiological findings and related to a greater occurrence of pulmonary exacerbations, however without worse clinical results associated with chronic infection. The presence of this atypical virulotype did not determine a worse clinical picture, as reported in other infection sites, such as isolated from the bloodstream infections and in pneumonia associated with mechanical ventilation. The *exoS*⁺/*exoU*⁺ virulotype was associated with chronic infections and worse clinical results, including a significantly lower spirometric profile. Finally, the importance of *P. aeruginosa* virulotypes detection has been demonstrated, since virulence factors play a decisive role in pulmonary impairment, clinical progression and disease severity in CF patients.

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Disclosure

The authors report no conflicts of interest in this work.

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