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Genomic Comparative of *Pseudomonas aeruginosa* Small Colony Variant, Mucoid and Non-mucoid Phenotypes Obtained from a Patient with Cystic Fibrosis During Respiratory Exacerbations

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Abstract

Pseudomonas aeruginosa, the most prevalent opportunistic pathogen in chronic obstructive pulmonary disease, associated with high morbidity and mortality in patients with cystic fibrosis (CF), is practically impossible to be eradicated from the airways in chronicity. Its extraordinary genomic plasticity is possibly associated with high antimicrobial resistance, virulence factors, and its phenotypic diversity. The occurrence of *P. aeruginosa* isolates promoting airway infection, showing mucoid, non-mucoid, and small colony variant (SCV) phenotypes, was observed simultaneously, in the present study, in sputum cultures obtained from a male CF young patient with chronic pulmonary infection for over a decade. The isolates belonged to a new ST (2744) were obtained in two moments of exacerbation of the respiratory disease, in which he was hospitalized. Genetic background and phenotypic analysis indicated that the isolates exhibited multi- and pan-antimicrobial resistant profiles, as well as non-susceptible to polymyxin and predominantly hypermutable (HPM) phenotypes. Whole genome sequencing showed variations in genome sizes, coding sequences and their determinants of resistance and virulence. The annotated genomes were compared for antimicrobial resistance, hypermutability, and SCV characteristics. We highlight the lack of reported genetic determinants of SCV emergence and HPM phenotypes, which can be explained in part due to the very short time between collections of isolates. To the best of our knowledge, this is the first report of genome sequencing of *P. aeruginosa* SCV from a CF patient in Brazil.

Introduction

Pseudomonas aeruginosa is the most prevalent microorganism and it is associated to chronic obstructive pulmonary disease, considered to a progressive and chronic respiratory condition characterized by airflow limitation and persistent breathing difficulties, often promoting morbimortality increasing rates in cystic fibrosis (CF) patients [1, 2]. CF is a multisystem autosomal recessive disease caused by a deficiency in the expression of the cystic fibrosis transmembrane conductance regulator (CFTR) protein, a chloride and bicarbonate ion channel in the cell membranes of the respiratory, digestive, reproductive epithelium, and sweat glands. Absence, decrease or defects in CFTR promote an increase in mucus viscosity in several organs, and the respiratory tract is greatly affected, affected by intermittent or chronic infections, important causes of death in these individuals [2]. Intermittent lung infection is susceptible to treatment with aggressive antibiotic therapy and eradication is still possible. After the establishment of chronic infection, the eradication of airway pathogen is practically impossible [3].

This opportunistic pathogen, with an extraordinary ability to build antimicrobials resistance by selected genomic mutations and by exchange of transferable resistance elements, present elevated plasticity of genome which increases its

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metabolic versatility, in addition to being highly adaptable to environmental variations [4, 5]. CF lungs, a unique environment rich in stressors, may support the appearance of persistent variant phenotypic, such as mucoid (MUC), and small colony variants (SCV) and hypermutable (HPM), associated to chronicity infection. Interestingly, occasional presence of these *P. aeruginosa* phenotypes, living with wild-type strains, confers heterogeneity to population in airways, and directly affects the therapy objectives [6–8].

Alginate exopolysaccharide production by *P. aeruginosa* is associated to MUC phenotype and related to chronic airways infections, which are correlated with poorer lung function [9]. The occurrence of mucoid isolates usually results from the mutation of the mucA gene [10]. HPM phenomenon displays spontaneous mutation increased rates, owing defects in mismatch repair system (MRS), with mutS being the most frequently affected gene, and it has been observed in *P. aeruginosa* lung chronic infection in CF patients [8, 11, 12]. The occurrence of *P. aeruginosa* HPM has been implicated in antipseudomonal drugs resistance and increasing microbial virulence, contributing as additive factor to infection severity [8, 13, 14].

SCV phenotype is a colonial variation type demonstrating in vitro, self-aggregative appearance, slow growth, and very small size. In *P. aeruginosa* isolated from chronic stage of CF respiratory infection, this phenotypic behavior associated to biofilm increased formation, multidrug resistance and persistence, being strongly related to genetic alterations in mutational targets as yfiN, yfiR, rsmA, wspF, mutS, fleQ e accBC [15, 16].

In the present study, we report a case of a male cystic fibrosis (CF) patient (F508del/Y913X), with chronic pulmonary infection for twelve years, hospitalized in April 2017, with pulmonary exacerbation. In this context, sputum microbiological analysis detected *P. aeruginosa* isolates that exhibited in the same culture the mucoid, non-mucoid, and small colony variant (SCV) phenotypes. Two months later, the patient had to undergo a new hospitalization due to worsening lung condition, when it was again identified in a microbiological culture the presence of *P. aeruginosa* isolates with the same previously found three phenotypes. The objective of this study was to compare, in the different phenotypes of *P. aeruginosa*, in two scenarios of pulmonary exacerbation in a CF young patient, their profiles of resistance, virulence and to evidence genotypic alterations associated with HPM and emergence of SCV.

Materials and Methods

Bacterial Identification

The isolates included in this retrospective study were stored and cataloged in a bacteriological collection at the State University of Rio de Janeiro. *P. aeruginosa* strains were isolated from microbiological cultures of respiratory secretions from CF patients, in two moments of pulmonary disease exacerbation, with an interval of two months, according to the methodology proposed by Miller and colls.[17].

P. aeruginosa isolates were identified by phenotypic tests, including oxidative metabolism of glucose (non-fermentative), detection of the production of oxidase (positive), arginine decarboxylation (positive), and physiological analyze, such as growth at 42 °C (positive) and motility test [17].

The non-mucoid (NM), mucoid (MUC), and small colony variant (SCV) morphotypes were established by visual verification of the colonial morphology. Regarding the consistency of the bacterial colonies, according to the growth in agar medium, those visually moist and sticky were considered mucoid. The macroscopic characteristics of colonies in relation to size were analyzed and visually classified, presenting in vitro a self-aggregative appearance, slow growth, and very small size [18].

Antimicrobial Susceptibility

Antimicrobial susceptibility were performed by disk-diffusion assay (DDT), and interpreted according to the Clinical & Laboratory Standards Institute (CLSI) for the following antimicrobials: piperacillin tazobactam (PTZ), ceftazidime (CAZ), cefepime (FEP), imipenem (IPM), aztreonam (ATM), gentamicin (GEN), amikacin (AMI), meropenem (MEM), ciprofloxacin (CIP), tobramycin (TOB), and doripenem (DOR) (Oxoid Ltd., Hampshire, England), and minimum inhibitory concentration (MIC), for polymyxin (POL) (Sigma, St. Louis, USA) [19–21] were performed for six isolates, named 21113 MUC, 21114 NM, and 21107 SCV morphotypes (first exacerbation), and 21168 MUC, 21167 NM, and 21169 SCV (second exacerbation). *P. aeruginosa* ATCC® 27853 and Escherichia coli ATCC® 25922 were used as quality controls. The isolates were classified as multi-resistant (MDR) and pan-resistant (PDR) based on their susceptibility profiles. The criteria used to determine the profiles were: MDR: non-susceptible to ≥ 1 agent in ≥ 3 antimicrobial categories, XDR: non-susceptible to ≥ 1 agent in all but ≤ 2 categories, and PDR: non-susceptible

to all antimicrobial agents tested, according to Magiorakos criteria [22].

Mutation Frequencies

Isolates with mutation frequencies whose rate was equal to or greater than 20 times the mutation frequency of the PAO1 strain were considered HPM. The classification HPM was performed according to the mutation frequency rates (f) of each isolate: strongly increased spontaneous frequency of mutation (SISf) when f ≥ 2x10−7; weakly increased spontaneous frequency of mutation (WISf), when < 2x10−7 f ≥ 2x10−8; and non-increased spontaneous frequency of mutation (NISf), when f < 2 10−8. [11, 23, 24].

Genome Sequencing and Bioinformatics Analysis

The whole genome sequencing (WGS) of all isolates, were performed using Illumina (Illumina Inc, USA) technology in a MiSeq System equipment. Sequence reads were assembled de novo with Spades 3.5 genome assembler [25]. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession codes WUTK00000000, WUTL00000000, WUTM00000000, WUTN00000000, WUTO00000000, and WUTP00000000.

Contigs were uploaded to the Rapid Annotation using Subsystem Technology (RAST) v.2.0 server (http://rast.nmpdr.org) for annotation. Additional databases and search engines used for more detailed genome annotation comprise Basic Local Alignment Search Tool (BLAST) (blast.ncbi.nlm.nih.gov), and BLASTp and BLASTn, ResFinder (cge.cbs.dtu.dk/services/ResFinder), in the Center of Genomic Epidemiology (CGE) platform (genomicepidemiology.org/), Pathosystems Resource Integration Center (PATRIC) (patricbrc.org/), PubMLST (pubmlst.org/), Phaster (phaster.ca), Islandviewer (pathogenomics.sfu.ca/islandviewer), IS Finder (is.biotoul.fr), and Universal Protein Resource (UniProt) (uniprot.org). Average Nucleotide Identity (ANI) was calculated with PyANI using the ANIb method [26] and compared with *P. aeruginosa* genomes from isolates recovered from the lungs of patients with cystic fibrosis and from cystic fibrosis murine models that were downloaded from GenBank (Supplementary material 1–Table S1).

Results

All isolates were classified as MDR, except the 21169 SCV that was classified as PDR, including polymyxin resistance (MIC= 4 μg/mL). The antibiogram results, of each isolate are described in Table 1.

According to the phenotypic detection of hypermutability (HPM), four isolates (21114 NM, 21167 NM, 21107 SCV, and 21169 SCV) were classified as HPM. The mucoid isolates, 21113 MUC and 21168 MUC, were categorized as non-hypermutable (NHPM) and non-increased spontaneous frequency of mutation (NISf), when f < 2 × 10−8 (Table 2).

The genomes were assembled in 45 to 52 contigs, showing genome sizes ranging from 6,384,973 to 6,392,517 bp. Genes related to virulence factors varied from 228 to 230, and those associated with antibiotic resistance ranged from 122 to 125 (Table 3). The six isolates were also typified by multilocus sequence typing (MLST), and none showed any match with other STs in the database, and therefore were assigned as a new sequence type (ST2744).

Five types of acquired resistance genes were detected in all isolates, including two for beta lactams (blaPAO and blaOXA-50), and one each for aminoglycoside (aph (3')-IIb), for fosfomycin (fosA), for chloramphenicol (catB7), and for ciprofloxacin (crpP); no carbapenemase and mcr genes were detected.

RAST annotation findings, confirmed by BLAST analysis of the porin genes, and nucleotide sequence alignments revealed the presence of point mutations in the oprD and oprF genes. The same analysis was applied to genes of efflux systems, and genes described as regulator/repressors, such as nalC, nfxB, mexS, and mexZ, revealed substitutions in all isolates.

Table 1 Antimicrobial susceptibility profile of *Pseudomonas aeruginosa* strains recovered from cystic fibrosis patients during two episodes of pulmonary exacerbation

Isolate	Date	AMI	TOB	CIP	DOR	PTZ	CAZ	FEP	ATM	IPM	MEN	GEN	POL*
21114 NM	4/6/17	R	R	I	R	R	R	R	R	R	R	R	I
21113 MUC	4/6/17	S	S	S	S	S	S	S	S	S	S	S	I
21107 SCV	4/6/17	R	R	R	R	R	R	R	R	R	R	R	I
21167 NM	6/5/17	R	S	I	R	S	R	R	R	R	S	R	I
21168 MUC	6/5/17	S	S	I	S	S	R	S	S	S	S	S	I
21169 SCV	6/5/17	R	R	R	R	R	R	R	R	R	R	R	R

AMI amikacin, *TOB* tobramycin, *CIP* ciprofloxacin, *DOR* doripenem, *PTZ* piperacillin + tazobactam, *CAZ* ceftazidime, *FEP* cefepime, *ATM* aztreonam, *IPM* imipenem, *MEN* meropenem, *e, GEN* gentamicin, *POL**polymyxin B (tested by minimal inhibitory concentration (MIC), according to CLSI 2020 recommendations and breakpoints), *MUC* mucoid, *NM* non-mucoid, *SCV* small colony variant, *S* susceptible, *I* intermediate, *R* resistant

Table 2 Nucleotide sequence comparison of small colony variant and hypermutable phenotype related genes of six *Pseudomonas aeruginosa* isolates and PAO1 and spontaneous mutation frequency (*f*) rates

	Gene	Primary accession number	Length (bp)	21114 NM HPM/SISf ($f = 0.33 \times 10{-}6$)	21113 MUC NHPM/NISf ($f = 1.0 \times 10{-}10$)	21107 SCV HPM/SISf ($f = 2.4 \times 10{-}6$)	21167 NM HPM/SISf ($f = 27 \times 10{-}5$)	21168 MUC NHPM/NISf ($f = 0$)	21169 SCV HPM/SISf ($f = 2.5$ x $10{-}6$)
Small Colony Variant	rsmA	PA0592	807	subs: 7 n	subs: 7 n	subs: 7 n	subs: 7 n	subs: 7 n	subs: 7 n
	yfiN	PA1120	1308	subs: 3 n	subs: 3 n	subs: 3 n	subs: 3 n	subs: 3 n	subs: 3 n
	yfiR	PA1121	573	–	–	–	–	–	–
	fleQ	PA1097	1473	subs: 13 n	subs: 13 n	subs: 13 n	subs: 13 n	subs: 13 n	subs: 13 n
	wspF (cheB)	PA0173	1050	–	–	–	Δ21 n subs: 6 n	Δ6 n subs: 3 n	–
	accBC	PA4847	1350	subs: 4 n	subs: 4 n	subs: 4 n	subs: 4 n	subs: 4 n	subs: 4 n
SCV/ HPM	mutS	PA3620	2568	subs: 10 n	subs: 10 n	Δ1 n subs: 10 n ins:10 n	Δ1 n subs: 10 n ins:10 n	subs: 10 n	Δ1 n subs: 10 n ins:10 n
Hypermutability	mutL	PA4946	1902	subs: 22 n	subs: 22 n	subs: 22 n	subs: 22 n	subs: 22 n	subs: 22 n
	mutT	PA4400	mutT1. 555 mutT2. 438	subs: 3 n subs: 4 n	subs: 3 n subs: 4 n	subs: 3 n subs: 4 n	subs: 3 n subs: 4 n	subs: 3 n subs: 4 n	subs: 3 n subs: 4 n
	mutM	PA0357	813	–	–	–	–	–	–
	uvrD	PA5443	2187	subs: 6 n	subs: 6 n	subs: 6 n	subs: 6 n	subs: 6 n	subs: 6 n

n nucleotide, Δ deletion, *subs* substitution, *ins* insertion

MUC mucoid, *NM* non-mucoid, *SCV* small colony variant, *HPM* hypermutability/hypermutable, *NHPM* non-hypermutable, *bp*:base pairs

f = mutation frequency rates; SISf (strongly increased spontaneous frequency of mutation) when $f \geq 2 \times 10{-}7$; WISf (weakly increased spontaneous frequency of mutation), when $< 2 \times 10{-}7$ $f \geq 2 \times 10{-}8$; and NISf (non-increased spontaneous frequency of mutation), when $f < 2$ $10{-}8$

Table 3 Overview of genomic sequences annotated in Rapid Annotation Subsystem Technology (RAST) platform of *Pseudomonas aeruginosa* isolates

	21114 NM	21113 MUC	21107 SCV	21167 NM	21168 MUC	21169 SCV
Genomic features						
NCBI accession no	NA580450	NA580449	NA580445	NA580453	NA580455	NA580457
Genome size (bp)	6,390,267	6,391,355	6,384,973	6,386,325	6,392,517	6,386,368
G-C Content	66.4	66.4	66.4	66.4	66.4	66.4
Number of Contigs	46	52	51	55	45	50
Number of Subsystems	564	566	567	567	566	565
Number of Coding Sequences (CDS)	5986	5987	6010	6009	5992	6004
Number of RNAs	65	65	65	65	65	65
Resistance to antibiotics and toxic compounds	122	122	125	125	122	123
Number of virulence factors	228	229	229	230	228	230

Content G-C: percentage of Guanine-Cytosine nucleotides of region

bp base pairs, *MUC* mucoid, *NM* non-mucoid, *SCV* small colony variant

One intact prophage was found in all *P. aeruginosa* isolates, named YMC11/02/R656 (NC_028657). Five insertion sequences, ISPa2, ISPa6, ISPa32, ISPa57, and IS222, were observed in all isolates, with origins related to *P. aeruginosa*. Nine genomic islands (GIs) were predicted. Three of them were common among all isolates, and one harbored the exotoxin A and pyocin virulence factors. The virulence factors detected were associated with adherence, types III and VI secretion systems, serum resistance, membrane-damage, motility, antiphagocytosis, siderophore, biosurfactant, protease production, toxin regulation, and quorum sensing. Plasmids were not found using RAST, PATRIC, and BLASTn analysis.

Average Nucleotide Identity analysis was used to compare our six isolates with 24 *P. aeruginosa* genomes of strains isolated from murine models of cystic fibrosis or

from human cystic fibrosis patients who exhibited either wild-type, SCV or MUC phenotypes. The genomes that exhibited the highest identity with our isolates were SCVFeb SCVJan, Nhmuc, and DK1 (Fig. 1 and Supplementary material 2–Figure S1).

The comparison between the nucleotide sequences of all isolates with PAO1, aimed at detecting differences between the main genes associated with SCV and HPM phenotypes, is shown in Table 2. Mutations that appeared to be more relevant, detected in the mutS gene, given that their differences were more prominent in the SCV isolates, were not exclusive of these strains. mutS was also the gene that showed the most relevant changes when the HPM phenotype was considered.

Discussion

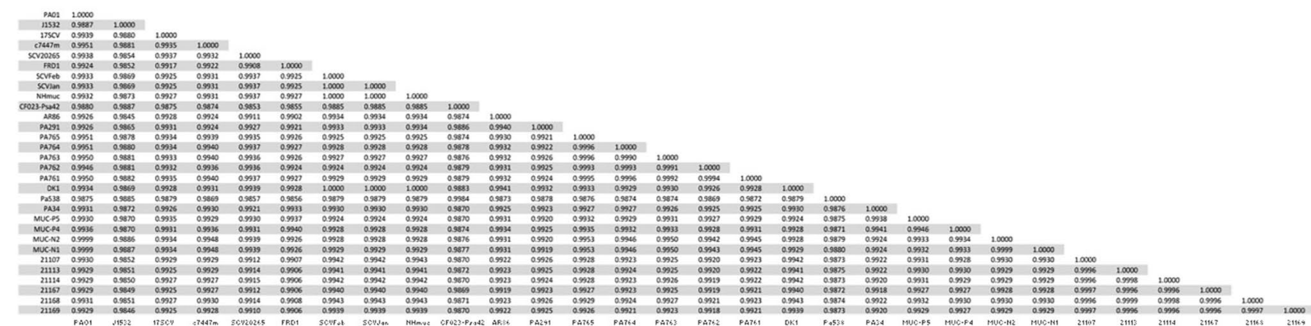
In contrast to other centers around the world [27, 28], in Brazil, *P. aeruginosa* isolates recovered from respiratory infections in CF patients [6, 29] have proven to be generally more susceptible to antimicrobials. However, in the present study, half of the six isolates recovered from a chronically infected CF patient over a decade ago, were classified as MDR and, one of them, PDR, following a trend pointed out by epidemiological studies in recent years, referring to a greater circulation of *P. aeruginosa* more resistant to antimicrobials. These bacteria cause healthcare-related infections, like pneumonia occurring in immunocompromised individuals, and in those with respiratory disease, such as CF [4, 30–32].

Only recently, our center has observed an increase in the frequency of carbapenem-resistant *P. aeruginosa* obtained from CF patients [33]. On the other hand, *P. aeruginosa* non-sensitive to polymyxin in Brazil remain rare including isolates from hospital settings [34, 35]. The occurrence of isolates that were not susceptible to polymyxin in CF patients in our country was reported for the first time in this study. However, the mcr gene was not detected in our isolates.

HPM phenotype in *P. aeruginosa* has been related to chronic lung diseases in CF patients, including reports in Brazil [6, 11, 12]. In the present study, increased rates of mutation were observed in the non-mucoid and SCV isolates, in both periods of hospitalization (Table 2). Although HPM is not related exclusively to the increased resistance, these isolates were MDR or PDR [6, 13, 14]. The new ST (2744) that was observed in all isolates confirmed the presence of clones in patients with chronic infection. Extrinsic resistance genes were associated with the antibiotic resistance profile displayed by isolates. We highlight the detection of the crpP gene, related to ciprofloxacin resistance, initially described in clinical isolates of Enterobacteriaceae (non-CF) in Mexico [36], and here we report the first case in Brazil. This is worthy of attention because its circulation can contribute to therapeutic failure, worsening the patient's condition.

Mutations in the oprD gene, associated with resistance to imipenem and other drugs in the absence of genes for carbapenemases, are consistent with the antibiogram results. A single substitution in the oprF gene was observed in 21107SCV. Interestingly, in this same SVC isolate, the genomic island carrying exotoxin A and pyocin was not detected. It has already been described that mutations in this gene are associated to resistance to beta-lactams and disorganized quorum sensing, resulting in deficiency of virulence factors, such as pyocin and exotoxin A. The absence of genes in the pathogenicity island may be indicative of a less virulent isolate [37–40]. Substitutions occurring in the repressor/regulator genes from efflux systems are implicated in the MDR phenotype in *P. aeruginosa* [41, 42], corroborating the susceptibility profile of our isolates.

One intact phage was detected in all isolates, YMC11/02/R656, that was the same prophage described by our group in an Achromobacter ruhlandii isolate recovered from a CF patient [43]. This highlights the potential ability of these isolates to incorporate transmissible genetic



	PA01	J1532	17SCV	c7447m	SCV20265	FRD1	SCVFeb	SCVJan	NHmuc	CF023-Psa42
PA01	1.0000									
J1532	0.9887	1.0000								
17SCV	0.9939	0.9880	1.0000							
c7447m	0.9951	0.9881	0.9935	1.0000						
SCV20265	0.9938	0.9854	0.9937	0.9932	1.0000					
FRD1	0.9924	0.9852	0.9917	0.9922	0.9908	1.0000				
SCVFeb	0.9933	0.9869	0.9925	0.9931	0.9937	0.9925	1.0000			
SCVJan	0.9933	0.9869	0.9925	0.9931	0.9937	0.9925	1.0000	1.0000		
NHmuc	0.9932	0.9873	0.9927	0.9931	0.9937	0.9927	1.0000	1.0000	1.0000	
CF023-Psa42	0.9880	0.9887	0.9875	0.9874	0.9853	0.9855	0.9885	0.9885	0.9885	1.0000
AR86	0.9926	0.9845	0.9928	0.9924	0.9911	0.9902	0.9934	0.9934	0.9934	0.9874
PA291	0.9926	0.9865	0.9931	0.9924	0.9927	0.9921	0.9933	0.9933	0.9934	0.9886
PA765	0.9951	0.9878	0.9934	0.9939	0.9935	0.9926	0.9925	0.9925	0.9925	0.9874
PA764	0.9951	0.9880	0.9934	0.9940	0.9937	0.9927	0.9928	0.9928	0.9928	0.9878
PA763	0.9950	0.9881	0.9933	0.9940	0.9936	0.9926	0.9927	0.9927	0.9927	0.9876
PA762	0.9946	0.9881	0.9932	0.9936	0.9936	0.9924	0.9924	0.9924	0.9924	0.9879
PA761	0.9950	0.9882	0.9935	0.9940	0.9937	0.9927	0.9929	0.9929	0.9929	0.9879
DK1	0.9934	0.9869	0.9928	0.9931	0.9939	0.9928	1.0000	1.0000	1.0000	0.9883
Pa538	0.9875	0.9885	0.9879	0.9869	0.9857	0.9856	0.9879	0.9879	0.9879	0.9984
PA34	0.9931	0.9872	0.9926	0.9930	0.9921	0.9933	0.9930	0.9930	0.9930	0.9870
MUC-P5	0.9930	0.9870	0.9935	0.9929	0.9930	0.9937	0.9924	0.9924	0.9924	0.9870
MUC-P4	0.9936	0.9870	0.9931	0.9936	0.9931	0.9940	0.9928	0.9928	0.9928	0.9874
MUC-N2	0.9999	0.9886	0.9934	0.9948	0.9939	0.9926	0.9928	0.9928	0.9928	0.9876
MUC-N1	0.9999	0.9887	0.9934	0.9948	0.9939	0.9926	0.9929	0.9929	0.9929	0.9877
21107	0.9930	0.9852	0.9929	0.9929	0.9912	0.9907	0.9942	0.9943	0.9943	0.9870
21113	0.9929	0.9851	0.9925	0.9929	0.9914	0.9906	0.9941	0.9941	0.9941	0.9872
21114	0.9929	0.9850	0.9927	0.9927	0.9915	0.9906	0.9942	0.9942	0.9942	0.9870
21167	0.9929	0.9849	0.9925	0.9927	0.9912	0.9906	0.9940	0.9940	0.9940	0.9869
21168	0.9931	0.9851	0.9927	0.9930	0.9914	0.9908	0.9943	0.9943	0.9943	0.9871
21169	0.9929	0.9846	0.9925	0.9928	0.9910	0.9906	0.9939	0.9939	0.9939	0.9870

	AR86	PA291	PA765	PA764	PA763	PA762	PA761	DK1	Pa538	PA34
AR86	1.0000									
PA291	0.9940	1.0000								
PA765	0.9930	0.9921	1.0000							
PA764	0.9932	0.9922	0.9996	1.0000						
PA763	0.9932	0.9926	0.9996	0.9990	1.0000					
PA762	0.9931	0.9925	0.9993	0.9993	0.9991	1.0000				
PA761	0.9934	0.9924	0.9995	0.9996	0.9992	0.9994	1.0000			
DK1	0.9941	0.9932	0.9933	0.9929	0.9930	0.9926	0.9928	1.0000		
Pa538	0.9873	0.9878	0.9876	0.9874	0.9874	0.9869	0.9872	0.9879	1.0000	
PA34	0.9925	0.9923	0.9927	0.9927	0.9926	0.9925	0.9925	0.9930	0.9876	1.0000
MUC-P5	0.9931	0.9920	0.9932	0.9929	0.9931	0.9927	0.9929	0.9924	0.9875	0.9938
MUC-P4	0.9934	0.9925	0.9935	0.9932	0.9933	0.9928	0.9931	0.9928	0.9871	0.9941
MUC-N2	0.9931	0.9920	0.9953	0.9946	0.9950	0.9942	0.9945	0.9928	0.9879	0.9924
MUC-N1	0.9931	0.9919	0.9953	0.9946	0.9950	0.9943	0.9945	0.9929	0.9880	0.9924
21107	0.9922	0.9926	0.9928	0.9923	0.9925	0.9920	0.9923	0.9942	0.9873	0.9922
21113	0.9923	0.9925	0.9928	0.9924	0.9925	0.9920	0.9922	0.9941	0.9875	0.9922
21114	0.9923	0.9924	0.9928	0.9923	0.9926	0.9919	0.9922	0.9942	0.9873	0.9920
21167	0.9919	0.9923	0.9927	0.9923	0.9925	0.9919	0.9921	0.9940	0.9872	0.9918
21168	0.9923	0.9926	0.9929	0.9924	0.9927	0.9921	0.9923	0.9943	0.9874	0.9922
21169	0.9922	0.9925	0.9926	0.9921	0.9923	0.9918	0.9921	0.9939	0.9873	0.9920

	MUC-P5	MUC-P4	MUC-N2	MUC-N1	21107	21113	21114	21167	21168	21169
MUC-P5	1.0000									
MUC-P4	0.9946	1.0000								
MUC-N2	0.9933	0.9934	1.0000							
MUC-N1	0.9932	0.9933	0.9999	1.0000						
21107	0.9931	0.9928	0.9930	0.9930	1.0000					
21113	0.9930	0.9930	0.9929	0.9929	0.9996	1.0000				
21114	0.9931	0.9929	0.9929	0.9929	0.9996	0.9998	1.0000			
21167	0.9927	0.9927	0.9928	0.9928	0.9997	0.9996	0.9996	1.0000		
21168	0.9932	0.9930	0.9930	0.9930	0.9996	0.9999	0.9998	0.9996	1.0000	
21169	0.9929	0.9927	0.9929	0.9929	0.9997	0.9996	0.9996	0.9996	0.9997	1.0000

Fig. 1 ANIb percentage identity figure. (Fig. 1): On the horizontal axis (x), contrast isolates are shown

elements that can promote the dissemination/acquisition of resistance determinants, even from other bacterial genera.

Besides porins and efflux pumps, the presence of ISs could partially contribute to the MDR and PDR observed in the isolates. Nine GIs were detected among six isolates, being absent only in 21107SCV (Supplementary material 3–Table S2), related to bacterial virulence and increased pathogenesis, containing both exotoxin A, with cytotoxic activity [44] and pyocin [45]. All isolates shared the same virulence genes, although the number of copies of the algB and phzA1 and phzB1genes presented variation. This may explain the respiratory damage caused in this patient during the chronic infection process. Virulence genes collaborate to an increased accessory genome, and avirulent strains seem to have less correlation with resistance, and greater, with microbial persistence in the lungs rich in stressors of CF patients [5, 46].

In fact, a genomic level comparison has been considered the gold standard in bacterial characterization and identification. Therefore, with the availability of existing in silico genomic analysis tools, such as ANI, it is possible to obtain high quality results with good reproducibility rates. The reference genomes used for ANI belong to 24 strains of *P. aeruginosa*, and the analysis showed that our six isolates were most similar to SCVFeb (PRJNA291145), SCVJan (PRJNA291144), Nhmuc (PRJNA291143), and DK1 (PRJEB9823). The isolates SCVFeb and SCVJan, obtained from a mouse CF model, and NHmuc and DK1, with a smaller genome than the previously described transmissible DK2 strain [47, 48], were isolated from human CF patients. Interestingly, they are all from the ST387.

The diversity of colony morphology variants observed in our case report suggest the existence of strong and multiple selective pressures. The mutations linked to the emergence of SCV that were most identified are those that induce the loss of function in repressor proteins that regulate the diguanylate cyclase (DGC) activity, occurring in genes which control intracellular levels of c-di-GMP (cyclic-di-GMP), a signaling molecule involved in mobility, biofilm formation, production of bacterial exopolysaccharides (EPS), adhesins, and virulence [16, 49].

When comparing the nucleotide sequences of the rsmA, yfiN, yfiR, fleQ, wspF, mutS, and accBC genes, associated with the SCV in *P. aeruginosa* [16, 49, 50], the same mutations were observed in isolates 21114NM, 21113MUC, 21167NM, and 21168MUC, which did not express this phenotype. When the mutS nucleotide sequence was analyzed, numerous mutations in the two SCV isolates were observed, although those were not exclusive to this phenotype. This seems to exclude a relationship of mutS mutations with the emergence of the SCV phenotype, at least in the present study.

The mutS is an important mutator target in the HPM, associated with the chronicity, increased antimicrobial resistance, multidrug resistance and to SCV phenotype emergence. Mutations detected in this gene may explain the rate of increased mutations demonstrated phenotypically by the six isolates, since genetic changes in the mutS, a central part of the mismatch repair system, are linked to favor the adaptation of this microorganism to the airways of CF patients [11, 33, 51].

Conclusion

The phenotypic characteristics of antimicrobial resistance were supported by the results of in silico genomic analyses. On the other hand, no relevant genotypic differences expected and related to small colony variant (SCV) were observed, which could be attributed to factors such as the short period between sample collections, a limited number of isolates from a single patient, or the absence of mutations in the specific genes under investigation. These findings suggest the involvement of alternative mechanisms or potentially unknown genes in the observed phenotype.

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Author Contributions All authors contributed to the study conception and design. Study conception and design were performed by EAM and RSL. Most of material preparation, data collection, phenotypic and genotypic analysis was performed by MMA and LRB. Molecular assays and analysis were performed by RMA, MMA and LRB. Material preparation, data collection and phenotypic analysis were performed by MMA. Clinical data collection and analysis were performed by MMA and MCF. The first draft of the manuscript was written by MMA, EAM and RSL and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data Availability All data generated or analyzed during this study are included in this published article.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in this study were in accordance with the ethical standards of the institutional research committee (CAAE: 79547616.1.0000.5259), and the approval was waived by the local Ethics Committee of Universiade do Estado do Rio de Janeiro, in view of the retrospective nature of the study and all the procedures were performed with samples stored in a bacteriological collection.

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