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CLINICAL MICROBIOLOGY – RESEARCH PAPER

Genetic determinants of antimicrobial resistance in polymyxin B resistant *Pseudomonas aeruginosa* isolated from airways of patients with cystic fibrosis

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Abstract

Pseudomonas aeruginosa is the main pathogen associated with pulmonary exacerbation in patients with cystic fibrosis (CF). CF is a multisystemic genetic disease caused by mutations in the cystic fibrosis transmembrane conductance regulator gene, which mainly affects pulmonary function. *P. aeruginosa* isolated from individuals with CF in Brazil is not commonly associated with multidrug resistance (MDR), especially when compared to global occurrence, where the presence of epidemic clones, capable of expressing resistance to several drugs, is often reported. Due to the recent observations of MDR isolates of *P. aeruginosa* in our centers, combined with these characteristics, whole-genome sequencing was employed for analyses related to antimicrobial resistance, plasmid identification, search for phages, and characterization of CF clones. All isolates in this study were polymyxin B resistant, exhibiting diverse mutations and reduced susceptibility to carbapenems. Alterations in *mexZ* can result in the overexpression of the MexXY efflux pump. Mutations in *oprD*, *pmrB*, *parS*, *gyrA* and *parC* may confer reduced susceptibility to antimicrobials by affecting permeability, as observed in phenotypic tests. The phage findings led to the assumption of horizontal genetic transfer, implicating dissemination between *P. aeruginosa* isolates. New sequence types were described, and none of the isolates showed an association with epidemic CF clones. Analysis of the genetic context of *P. aeruginosa* resistance to polymyxin B allowed us to understand the different mechanisms of resistance to antimicrobials, in addition to subsidizing the understanding of possible relationships with epidemic strains that circulate among individuals with CF observed in other countries.

Keywords *Pseudomonas aeruginosa* · Cystic fibrosis · Polymyxin · Antimicrobial resistance · Whole genome sequencing

Introduction

Pseudomonas aeruginosa is a ubiquitous microorganism capable of adapting to several environments. It is an opportunistic pathogen associated with airway infections, which, when well established, is practically impossible to eradicate, especially the chronicity. This morbidity can culminate in pulmonary exacerbations and irreversible lung damage in patients with cystic fibrosis (CF) [1].

CF is a multisystemic, autosomal recessive disease caused by cystic fibrosis transmembrane conductance regulator (*CFTR*) gene mutations located on the long arm of chromosome 7, involving mucus and sweat producer cells. The lungs are the most severely affected, increasing mucus viscosity and inability to clear bacteria, resulting in chronic airway inflammation. Airway damage mainly occurs due to bacterial infections caused by typical microorganisms, which can lead to reduced lung function, leading to decreased quality of life in patients with CF [2–4].

P. aeruginosa possesses a diverse pool of resistance mechanisms, such as porins, efflux pumps and enzymes, affecting its susceptibility to several antimicrobial classes, such as β-lactams, carbapenems, aminoglycosides,

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fluoroquinolones and polymyxins [5, 6]. Resistance to carbapenems, mediated by permeability alterations and carbapenemases, is a significant concern as carbapenems are considered first-line drugs in the treatment of airway infections caused by *P. aeruginosa* in CF [5, 7, 8]. Similarly, resistance to polymyxins, facilitated by alterations in two-component system (TCS) and acquisition of exogenous DNA (plasmid *mcr* gene) is also a cause for concern since polymyxins are often considered a last resort treatment for MDR *P. aeruginosa* [9–11].

One of the challenges encountered in *P. aeruginosa* infection is the existence of multidrug-resistant strains and their spread among patients with CF [12], such as the Liverpool, Manchester, Midlands, Australia, Denmark, and Canada strains [13, 14]. Phylogenetic analysis aims to identify common bacterial clones that can be used as strains with important resistance profiles against CF [3, 15].

Whole-genome sequencing (WGS) is a standard technique for investigating outbreaks and bacterial typing. In addition to the rapid identification of agents that cause infections with high accuracy, it allows the detection of genes associated with antimicrobial resistance and relevant virulence factors. Despite the high cost and requirement for specialized professionals, it has been adopted by several laboratories and hospital institutions. *In silico* analysis of bacterial data obtained from WGS guarantees speed in the characterization and management of outbreaks, personalized therapy, and facilitates approaches in the control of nosocomial infections [16].

In recent years, an increase in *P. aeruginosa* isolates obtained from individuals with CF, followed up in two centers in Rio de Janeiro, showed decreased susceptibility to carbapenems, polymyxin B, and other antimicrobials [17]. This results in difficulties in the treatment and eradication of the pulmonary environment; therefore, it is necessary to analyze the phenotypic and genotypic characteristics of these isolates. The aim of this study was to perform a genetic analysis of *P. aeruginosa* resistance to polymyxin B, which may contribute to understanding the mechanisms of antimicrobial resistance, its phylogeny, and other important genomic aspects.

Table 1 Distribution of patients, isolates and colonial morphology of *Pseudomonas aeruginosa* strains from cystic fibrosis patients

Patient	Number of isolates	Isolate number	Isolation date
1	1	9876^{NM}	01/27/2010
2	4	10,705^{NM}	08/16/2010
		17,138^{M}	11/12/2013
		17,749^{NM}	03/31/2014
		17,801^{NM}	04/07/2014
3	3	11,227^{NM}	12/03/2010
		14,297^{NM}	02/23/2012
		17,973^{M}	05/21/2014
4	1	14,339^{NM}	03/01/2012
5	1	17,828^{NM}	04/24/2014

M: mucoid; NM: non-mucoid

Materials and methods

Clinical specimens

Isolation and identification

We selected ten *P. aeruginosa* isolates from microbiological cultures of the respiratory secretions of five patients with CF (1 to 4 isolates per individual), obtained between 2010 and 2014 (Table 1), and stored in the bacteriological collection at the State University of Rio de Janeiro. Isolates from archived strains were used and no patient data were consulted for this study, therefore, informed consent was not obtained. 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 Universidade do Estado do Rio de Janeiro.

Biochemistry test evaluations of the oxidative metabolism of glucose (non-fermentative), oxidase production (positive), arginine decarboxylation (positive), and physiological tests, such as motility and growth at 42ºC were conducted as previously described [18]. The sample inclusion criteria was a non-susceptibility profile to polymyxin B using the supplemental colistin agar test [19].

Antimicrobial susceptibility test (AST)

Disk-diffusion test (DDT) was performed for the following antimicrobials: piperacillin-tazobactam (PPT) (100/10 µg), ceftazidime (CAZ) (30 µg), cefepime (FEP) (30 µg), meropenem (MER) (10 µg), imipenem (IMP) (10 µg), doripenem (DOR) (10 µg), aztreonam (ATM) (30 µg), ciprofloxacin (CIP) (5 µg), amikacin (AMI) (30 µg) and tobramycin (TOB) (10 µg) (Becton, Dickinson and Company, BD, Sparks, NV, USA) as described in Clinical and Laboratory Standards Institute (CLSI) [19]. The *P. aeruginosa* ATCC 27,853 strain was used as a quality control.

Minimum inhibitory concentration

Minimum inhibitory concentration **(**MIC) was determined by broth microdilution technique [20] only to polymyxin B (10.000 µg/mL, Sigma, St. Louis, USA) using the quality control strain *P. aeruginosa* ATCC 27,853, and the breakpoints of ≤ 2 µg/mL as intermediate and ≥ 4 µg/mL as resistant [19].

Bacterial resistance to multiple antimicrobial agents

P. aeruginosa isolates were classified as multidrug-resistant (MDR) when they were resistant to ≥ 1 agent in ≥ 3 antimicrobial categories tested; and as extensively drug-resistant (XDR) when they were resistant to ≥ 1 agent in all but ≤ 2 categories tested as it was described by Magiorakos and collaborators [21].

Genomic analysis and bioinformatic tools (*in silico* analysis)

Bacterial DNA were extracted and purified from recent bacterial cultures using a QIAmp DNA Mini Kit (QIAGEN, Hilden, Germany). DNA was quantified by the Quanti-Fluor® (Promega, Madison, Wisconsin, USA) system, genomic libraries were created using Nextera XT DNA Library Preparation (Illumina Inc, California, USA) kit, and then sequenced on the Illumina MiSeq System (Illumina Inc, California, USA).

Reads quality control were carried out in FastQC v.0.11.9 (usergalaxy.org.au) program and then assembled in contigs using Unicycler v.0.4.8 [22] in Bacterial and Viral Bioinformatics Resource Center (BV-BRC) (bv-brc.org) platform. Annotations were performed using Rapid Annotation Subsystem Technology (rast.nmpdr.org/rast.cgi) and BV-BRC (bv-brc.org). The Basic Local Alignment Search Tool (blast.ncbi.nlm.nih.gov/Blast.cgi) database was used to ensure the integrity of the sequences using BLASTn and BLASTp tools. Extrinsic resistance genes were identified using the Center for Genomic Epidemiology tool, ResFinder (cge.food. dtu. dk/services/Resfinder/), and intrinsic resistance genes were identified using the BV-BRC databases. The sequences were aligned using the *P. aeruginosa* PAO1 reference strain in Bioedit (bioedit.software.informer.com/7.2/). Plasmids were assembled and annotated using Galaxy Australia (usergalaxy.org.au) tools, plasmidSPAdes v.3.9.0 [23] and Bakta v.1.5.0 [24]. The online tool PHAge Search Tool Enhanced Release was used for phages [25]. Molecular typing was performed using the Public Database for Molecular Typing and Microbial Genome Diversity (pubmlst. org/), and the ST relation of the samples was compared with epidemic CF clones using the Grapetree tool [26].

The WGS Shotgun projects have been deposited at the National Center for Biotechnology Information (NCBI) under the following accession numbers: JAPTHH000000000 (9876, BioProject PRJNA890687), JAOYMC000000000 (10,705, BioProject PRJNA890688), JAOYMD000000000 (11,227, BioProject PRJNA890692), JAOYME000000000 (14,297, BioProject PRJNA890694), JAOYMF000000000 (14,339, BioProject PRJNA890695), JAOYMG000000000 (17,138, BioProject PRJNA890696), JAOYMG000000000 (17,749, BioProject PRJNA890697), JAOYMI000000000 (17,801, BioProject PRJNA890698), JAOYMJ000000000 (17,828, BioProject PRJNA890700) and JAOYMK000000000 (17,973, BioProject PRJNA890701).

Table 2 Antimicrobial susceptibility in *Pseudomonas aeruginosa* isolates

Isolate	Patient	Antimicrobial resistance profile	Polymyxin B (μg/mL)
9876^{MDR}	1	MER-IMP-CIP	4
10,705	2	IMP	4
$11,227^{MDR}$	3	MER-IMP-TOB-AMI	4
$14,297^{XDR}$	3	PPT-CAZ-FEP-MER-IMP-ATM-CIP-AMI	4
$14,339^{MDR}$	4	IMP-TOB-AMI	8
$17,138^{XDR}$	2	PPT-CAZ-FEP-MER-IMP-DOR-TOB-ATM-CIP-AMI	4
$17,749^{XDR}$	2	PPT-CAZ-FEP-MER-IMP-DOR-TOB-ATM-CIP-AMI	4
$17,801^{XDR}$	2	PPT-FEP-MER-IMP-DOR-TOB-ATM-CIP-AMI	4
$17,828^{MDR}$	5	PPT-FEP-MER-DOR	4
$17,973^{MDR}$	3	PPT-CAZ-FEP-MER-IMP-DOR-ATM	4

Table 2: PPT: piperacillin-tazobactam; ATM: aztreonam; CAZ: ceftazidime; FEP: cefepime; MER: meropenem; IMP: imipenem; DOR: doripenem; AMI: amikacin; CIP: ciprofloxacin; TOB: tobramycin; MDR: multidrug-resistant; XDR: extensively drug-resistant.

Results

The 10 *P. aeruginosa* isolates were resistant to polymyxin, as noticed by screening tests, with MIC ranging from 4 to 8 μg/mL, highlighting that almost all had an MIC of 4 μg/mL. All isolates were tested against 10 different antibiotics using DDT. Full or intermediate resistance was observed for all antimicrobials evaluated, with the highest marker resistance observed for carbapenems (IMP and MER) and the lowest for CAZ. Five isolates were categorized as MDR, and four as XDR (Table 2).

After annotation of the genomes, the contigs per isolate ranged from to 45–120 with an average genome size of 6.434.232 kpb (6.250.921–6.738.939 kpb) and 72–58 RNA genes (Table 3).

According to molecular typing, the isolates were classified as ST252 (isolate 9876), ST865 (10,705), ST871 (14,297), ST2211 (17,138 and 17,749) and ST1560 (17,801). The three new STs were identified as ST4051 (11,227), ST4052 (14,339), and ST4053 (17,828 and 17,973) (Table 4). Using a phylogenetic tree, the following CF-transmissible strains of *P. aeruginosa* were compared with clinical isolates: Liverpool, Manchester, Midlands, Praire, Australian 1, 2, and 3, Denmark, Clone C, and Dutch strains. Through

Table 3 Genomic composition data of *Pseudomonas aeruginosa* isolates

Isolates										
	9876	10,705	11,227	14,297	14,339	17,138	17,749	17,801	17,828	17,973
Access number	NA890687	NA890688	NA890692	NA890694	NA890695	NA890696	NA890697	NA890698	NA890700	NA890701
Genome size (bp)	6,303kpb	6,339kpb	6,476kpb	6,421kpb	6,250kpb	6,447kpb	6,439kpb	6,738kpb	6,476kpb	6,448kpb
GC content (%)	66.46	66.46	66.29	66.41	66.57	66.52	66.52	66.13	66.34	66.35
Contigs number	77	65	72	75	45	72	88	120	70	81
Reads	990,698	1,058,748	1,201,396	1,464,916	1,178,615	1,288,790	1,075,183	815,261	1,011,630	1,278,221
CDS	5,970	5,997	6,145	6,110	5,944	6,142	6,143	6,566	6,150	6,138
RNAs	61	62	63	72	64	62	58	62	64	62

Table 3: GC: guanine and cytosine; bp: base pairs; CDS: coding sequences; kpb: kilobase pairs.

Table 4 Sequence type identification and allele profile of *Pseudomonas aeruginosa* isolates from this study and from cystic fibrosis epidemic clones

Identification	ST	acsA	aroE	guaA	mutL	nuoD	ppsA	trpE
9876	252	6	28	4	3	3	4	7
10,705	865	15	5	83	11	4	4	7
11,227	4051*	16	3	20	71	4	7	1
14,297	871	16	3	1	5	1	55	61
14,339	4052*	125	5	5	11	4	15	19
17,138	2211	87	34	114	37	86	100	170
17,749	2211	87	34	114	37	86	100	170
17,801	1560	9	8	5	67	95	20	9
17,828	4053*	142	152	65	165	16	16	198
17,973	4053*	142	152	65	165	16	16	198
LES	146	6	5	11	3	4	23	1
MES	217	28	5	11	18	4	13	3
Midlands-1	148	17	5	1	3	13	6	7
PES	192	1	5	7	5	4	4	2
AES-01	649	11	84	11	3	4	4	7
AES-02	775	28	5	11	5	4	4	7
AES-03	242	28	5	5	11	3	15	44
DK-1	387	28	5	11	11	4	12	3
DK-2	386	17	5	11	18	4	10	3
Clone C	17	11	5	1	7	9	4	7
DES	406	40	5	11	3	4	13	7

Table 4: LES: Liverpool epidemic strain; MES: Manchester epidemic strain; PES: Praire epidemic strain; AES: Australian epidemic strain; DK: Denmark strain; DES: Dutch epidemic strain; *: New STs described.

phylogenetic analysis based on the distances calculated from the known STs and the strains included in this study, it was not possible to establish phylogenetic correlation between the described isolates and the typical transmissible strains observed around the world (Fig. 1).

The genes associated with intrinsic antimicrobial resistance were also investigated. Isolates 17,138 and 17,749, both ST2211 strains, exhibited the highest number of mutations (substitutions, "s") in the efflux pump regulators *mexR* (5 s), *nalC* (17 s), *nalD* (4 s), *nfxB* (26 s), *mexS* (6 s), and *mexZ* (18 s). The remaining isolates showed variations of 1–3 s for the same genes. In isolate 14,339, a premature stop codon (sc) was detected in the *mexZ* amino acid chain at position 134 (Supplementary Material 1).

Each isolate exhibited substitutions in at least one of the studied porin genes, with isolate 11,227 displaying the highest number of alterations in *oprD*. When compared with other porin genes, *oprD* showed the greatest number of modifications (deletions "Δ", insertions "ins" and substitutions) among the isolates, being the only gene among porins that showed deletions (Table 5). In *oprD*, isolates 9876, 10,705, 11,227 and 14,339 presented a frameshift deletion in codons 372, 373 and 383; 17,138, 17,749 and 17,801, exhibited a premature stop codon in the codon 277. Additionally, 9876, 10,705, 11,227, and 14,339 exhibited a

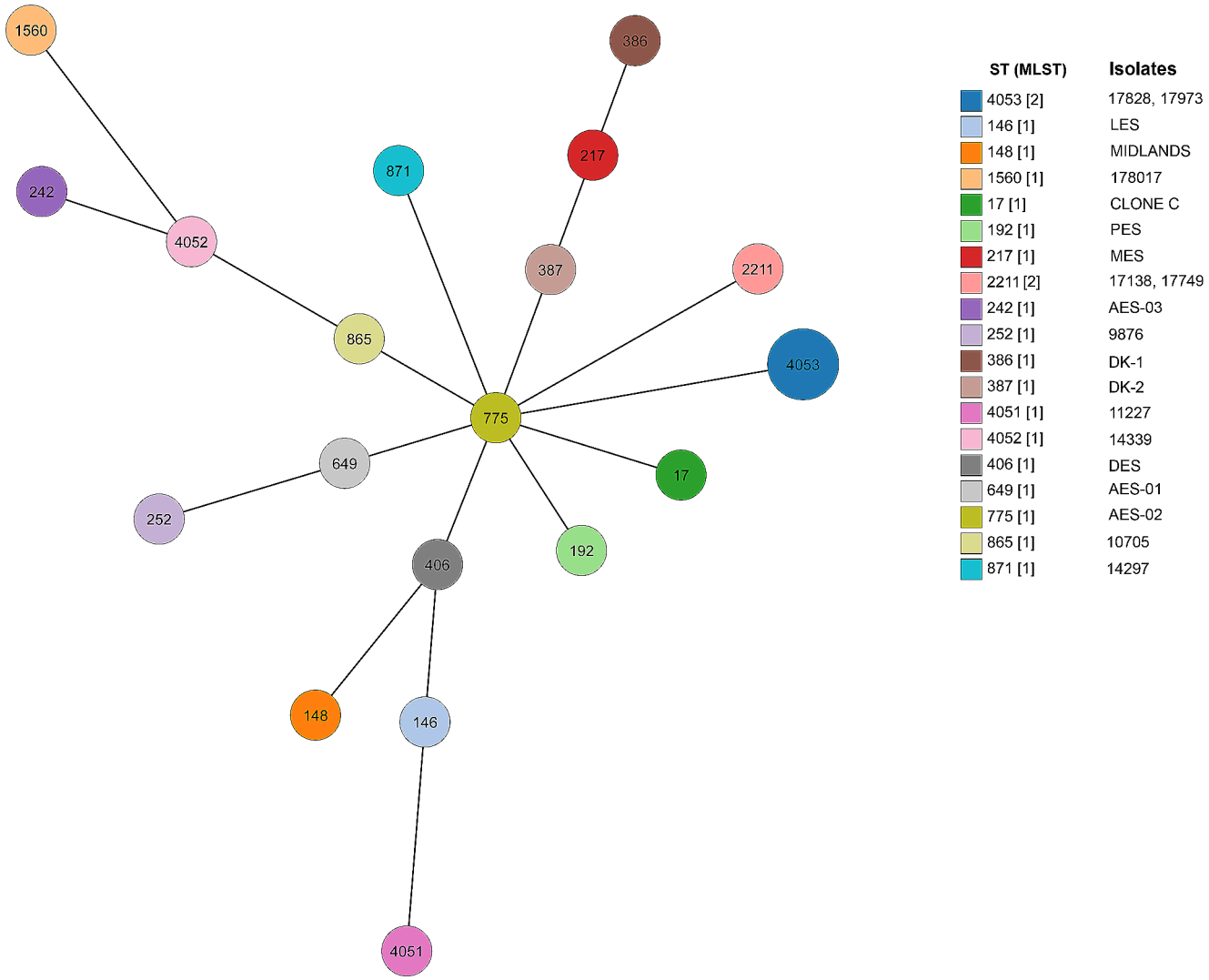

Fig. 1 – Spanning tree of *Pseudomonas aeruginosa* isolates from this study and from cystic fibrosis epidemic clones. (Figure 1: Spanning tree of *Pseudomonas aeruginosa* isolates comparing isolates in this study and cystic fibrosis epidemic clones; Figure was generated in Public Database for Molecular Typing and Microbial Genome Diversity (pubmlst. org/) platform with Grapetree [26] tool in 2023; ST: sequence type; MLST: Multi-locus Sequence Typing; LES: Liverpool epidemic strain; MES: Manchester epidemic strain; PES: Praire epidemic strain; AES: Australian epidemic strain; DK: Denmark strain; DES: Dutch epidemic strain)

sequence of mutations ranging from codon 375 to 382 (Supplementary Material 1).

Regarding TCS genes, in connection with their capacity to induce polymyxin resistance through genetic mutations, isolates 17,138 and 17,749 displayed the highest number of substitutions among all isolates. This emphasizes the involvement of *pmrB* (28 s and 1sc), *parS* (32 s), and *cprS* (25 s) genes. Furthermore, both isolates exhibited identical alterations and were the only isolates that manifested mutations in all TCS genes. Mutations in *colR* were unique to these two isolates (3 s). The other isolates displayed a variable range of alterations, ranging from one to five substitutions (Table 5).

Similar to that observed for TSC, four genes (*gyrA*, *gyrB*, *parC*, and *parE*) related to the DNA gyrase and Topoisomerase IV subunits showed the same mutations in 17,138 and 1749 isolates (Table 5).

Among the extrinsic resistance genes, oxacillinases *OXA-4* (17,828 and 17,973), *OXA-50* (14,339), *OXA-395* (14,297, 17,801, 17,828 and 17,973), and *OXA-486* (9876, 10,705, 11,227 and 14,297) were found (Table 6).

The *aph(3')-llb* and *aadA24* genes, related to aminoglycoside resistance, were exclusively detected in isolates 17,828 and 17,973, both ST4053. *crpP*, which is associated with CIP resistance, was detected in isolates 17,138, 17,749, and 17,801. The *sul1* gene, associated with sulfamethoxazole resistance, was only detected in isolate 17,801. *catB7*

Table 5 Alterations in the genes of interest in *Pseudomonas aeruginosa* isolates

	Isolates									
	Gene	9876	10,705	11,227	14,297	14,339	17,138	17,749	17,801	17,828	17,973
Efflux pump	*mexR*	–	–	1*s*	–	–	5*s*	5*s*	1*s*	1*s*	1*s*
	nalC	1*s*	–	2*s*	1*s*	2*s*	17*s*	17*s*	2*s*	3*s*	3*s*
	nalD	–	–	–	–	–	4*s*	4*s*	–	–	–
	nfxB	–	–	–	–	1*s*	26*s*	26*s*	–	2*s*	2*s*
	mexS	1*s*	1*s*	1*s*	1*s*	1*s*	6*s*	6*s*	1*s*	1*s*	1*s*
	mexZ	–	–	–	–	1*sc*	18*s*	18*s*	2*s*	2*s*	2*s*
Porins	*oprD*	21*s*, 3Δ	21*s*, 3Δ	26*s*, 3Δ	9*s*	21*s*, 3Δ	11*s*, 1*sc*	11*s*, 1*sc*	4*s*, 1*sc*	7*s*	7*s*
	opdP	16*s*	16*s*	16*s*	16*s*	16*s*	–	–	16*s*	16*s*	16*s*
	opdH	–	–	1*s*	–	1*s*	16*s*	16*s*	–	–	–
	opdD	6*s*	6*s*	4*s*	5*s*	5*s*	**X**	**X**	2*s*	6*s*	6*s*
	oprE	–	–	–	–	–	9*s*	9*s*	5*s*, 3*ins*, 1*sc*	17*s*, 4*ins*	17*s*, 4*ins*
	oprF	–	–	1*s*	–	–	–	–	–	–	–
	oprH	–	–	–	–	–	4*s*	4*s*	–	–	–
Two component system	*phoP*	–	–	–	–	–	4*s*	4*s*	–	–	–
	phoQ	–	–	–	–	–	14*s*	14*s*	–	1*s*	1*s*
	pmrA	1*s*	1*s*	1*s*	1*s*	1*s*	4*s*	4*s*	–	1*s*	1*s*
	pmrB	1*s*	1*s*	5*s*	1*s*	1*s*	28*s*, 1*sc*	28*s*, 1*sc*	5*s*	3*s*	3*s*
	parR	–	–	2*s*	–	1*s*	13*s*	13*s*	2*s*	2*s*	2*s*
	parS	1*s*	1*s*	1*s*	1*s*	1*s*	32*s*	32*s*	1*s*	1*s*	1*s*
	colR	–	–	–	–	–	3*s*	3*s*	–	–	–
	colS	–	–	–	–	–	8*s*	8*s*	–	1*s*	1*s*
	cprR	–	–	–	–	–	4*s*	4*s*	–	–	–
	cprS	–	–	5*s*	–	1*s*	25*s*	25*s*	–	5*s*	5*s*
DNA gyrase subunits	*gyrA*	2Δ	–	–	–	–	6*s*, 2Δ	6*s*, 2Δ	1*s*	2Δ	2Δ
	gyrB	–	–	–	–	–	7*s*	7*s*	–	–	–
Topoisome-rase IV subunits	*parC*	1*s*	1*s*	–	1*s*	1*s*	7*s*	7*s*	1*s*	–	–
	parE	–	–	–	–	1*s*	3*s*	3*s*	1*s*	1*s*	1*s*

Table 5: *s*: substitution; *ins*: insertion; Δ: deletion; *sc*: stop codon; X: gene absent in annotation; –: no mutation found.

Table 6 Analysis of extrinsic resistance genes found in the investigated samples of *Pseudomonas aeruginosa* using Resfinder

	Genes	9876	10,705	11,227	14,297	14,339	17,138	17,749	17,801	17,828	17,973
β-lactamases	*OXA-4*	A	A	A	A	A	A	A	A	P	P
	OXA-50	A	A	A	A	P	A	A	A	A	A
	OXA-395	A	A	A	P	A	A	A	P	P	P
	OXA-486	P	P	P	P	A	A	A	A	A	A
Aminoglycosides	*aph(3')-llb*	A	A	A	A	A	A	A	A	P	P
	aadA24	A	A	A	A	A	A	A	A	P	P
Ciprofloxacin	*crpP*	A	A	A	A	A	P	P	P	A	A
Sulfonamides	*sul1*	A	A	A	A	A	A	A	P	A	A
Chloramphenicol	*catB7*	P	P	P	P	P	A	A	P	P	P
Fosfomycin	*fosA*	P	P	P	P	P	A	A	P	P	P

Table 6: P: present; A: absent.

and *fosA*, related to chloramphenicol and fosfomycin resistance respectively, were present in all isolates, except for 17,138 and 17,749 (ST2211). Notably, isolates 17,828 and 17,973 (ST4053) had the highest number of extrinsic resistance genes (Table 6).

Broad variations were observed in the size and presence of genes in the annotated plasmids. The smallest plasmid (15,805 bp) was found in isolate 9876, whereas the largest (414,162 bp) was found in isolate 17801. According to the Bakta database, each isolate carried one plasmid. Resistance genes were identified in the plasmid of isolates 17138 (*crpP*), 17801 (*OXA-395*), 17828, and 17973 ($OXA\text{-}1_{like}$ and *aaC(6')lb*).

Intact phages found were Pseudo_YMC11/02/R656 (9876 and 10,705 isolates), Pseudo_H66 (11,227 isolate), Pseudo_JD024 (14,297 isolate), Escher_vB_EcoM_ECO1230_10 (17,749 isolate), Pseudo_Dobby (17,828 and 17,973 isolates), Pseudo_ F10 (17,828 and 17,973 isolates).

Discussion

Pseudomonas aeruginosa is one of the main pathogens associated with CF, severely affecting the lungs and contributing to the worsening of disease prognosis through inflammation and airway damage. It is difficult to eradicate due to its ability to withstand antimicrobial agents and establish chronicity [27]. Several mechanisms of antimicrobial resistance, biofilm formation, and persistence of multidrug-tolerant cells are strongly associated with chronic infections [8, 28].

In the present study, *P. aeruginosa* polymyxin-resistant isolates demonstrated reduced susceptibility to at least one carbapenem, a concern in patients with CF, owing to the use of these first-line drugs in the treatment of chronically infected patients. This makes it even more difficult to achieve a positive therapy response and eradicate pulmonary infection [29].

Studies have highlighted the emergence of carbapenem-resistant *P. aeruginosa* in CF in several regions of the world [29–32]. However, in Brazil, carbapenem-resistant *P. aeruginosa* reports on CF remain scarce [17, 33, 34].

One treatment option for carbapenem-resistant *P. aeruginosa* in CF is the use of polymyxins; however, monotherapy may lead to resistance [35, 36]. Variations in polymyxin susceptibility among patients with CF have also been observed in Europe. A study conducted in Italy reported an increase in polymyxin susceptibility, from 93 to 98% [37]. However, an investigation in Denmark from 2008 to 2016 identified an increase in polymyxin resistance from 7 to 13% among individuals with CF colonized with *P. aeruginosa* [38]. In Brazil, data on *P. aeruginosa* polymyxin resistance in individuals with CF are limited. In a recent study of 179 isolates from individuals with CF, 7.2% exhibited polymyxin resistance [17].

The detection of *P. aeruginosa* polymyxin resistance in individuals with CF is uncommon; however, resistance to polymyxins has been increasingly reported in non-CF cases [39–42]. This could be related to mutations in the TCS genes or the presence of plasmid genes that alter the polarity of lipid A, such as *mcr* [43–46]. To date, *P. aeruginosa* polymyxin resistance harboring *mcr* has not yet been described in individuals with CF, including those in our study.

The emergence of MDR *P. aeruginosa* isolates from patients with CF has been described for more than two decades [47–50]. Nevertheless, cases of MDR isolates from CF centers in Brazil have been reported over the last decade [17, 33, 34, 51].

In the present study, most isolates were classified as either MDR or XDR strains. This result was expected because of the study's inclusion criteria, which required the isolates to exhibit resistance to polymyxin B. Based on the criteria established by Magiorakos and collaborators [21], isolate 10,705 was not classified as MDR or XDR despite exhibiting resistance to IMP and polymyxin B. This still draws our attention as isolate 10,705 exhibited mutations in *mexS*, *oprD*, *opdP*, *opdD*, *pmrA*, *pmrB*, *parS* and *parC* genes. All these genes, except for *parC*, can mediate resistance to carbapenems or polymyxins through mechanisms such as efflux pumps (overexpression of MexEF), porins (OprD, OpdP and OpdD) and lipid A modification (PmrAB and ParRS). In the case of *parC*, which can confer resistance to fluoroquinolones through change in the target site [52], our study only tested ciprofloxacin. This may present a methodology gap, as we cannot conclusively determine the isolate's resistance to other fluoroquinolones, which could classify this isolate as MDR. Nevertheless, numerous definitions outlining the phenomenon of MDR in Gram-negative organisms are prevalent worldwide. These definitions exhibit variations contingent on their intended applications and originating country or institution. The choice of specific definitions of MDR should facilitate uniformity in epidemiological surveillance practices [53–55].

Through molecular typing, the isolates were categorized into eight ST, among which three new STs were identified. Notably, among the 10 isolates analyzed, 17,138 and 17,749, both recovered from the same individual with a one-year interval between isolations, shared the same ST (2211), and exhibited resistance to all tested antimicrobials. This is an important issue because multidrug resistance is often induced by the exposure of individuals with CF to multiple antimicrobials, thereby reducing therapeutic options and life expectancy [56].

Using a comparative analysis to assess the relationship between these STs and ST related to CF-transmissible strains, no similarity was observed, similar to previous studies conducted by our group [34, 57]. The existence of these transmissible strains has initiated debates concerning infection control protocols and the management of patients with CF. Continuous surveillance is imperative due to their considerable potential for global dissemination and their ability to harbor multiple resistance and virulence genes.

Genes associated with intrinsic and extrinsic antimicrobial resistance were investigated by WGS. Notably, isolates 17,138 and 17,749, which were observed in the same patient, exhibited the highest number of mutations in the same gene (Supplementary Material 1). The mutations observed in these isolates, such as the efflux pump regulators MexAB (*mexR*, *nalC* and *nalD*), MexCD (*nfxB*), MexEF (*mexS*), porins (*opdP*, *opdD*, *opdH*, and *oprE*), TCS (*phoP*, *phoQ*, *pmrA*, *parR*, *cprR*, *cprS*, *colR*, and *colS*), and the enzymatic DNA gyrase and Topoisomerase IV subunits (*gyrB* and *parE*), did not correlate with previously described mutations in MDR strains.

The *oprH* porin gene, involved in polymyxin resistance [8], was searched in all isolates in this study. The gene sequences were aligned with the reference strain *P. aeruginosa* PAO1, revealing that, with exception of isolates 17,138 and 17,749, all isolates exhibited 100% similarity with the *oprH* from *P. aeruginosa* PAO1. Blast comparison showed that *oprH* from isolates 17,138 and 17,749, shared 96.52% similarity with the *oprH* from *P. aeruginosa* PAO1, indicating the presence of the same protein function, with only allele variations.

mexZ, a MexXY efflux pump regulator, displayed a premature stop codon at codon 134 in isolate 14,339. Although alterations at this position have not been published, loss of protein function and impairment of repressor function have been reported when mutations occur at other positions in the amino acid chain [58, 59]. This may be related to the overexpression of the efflux pump, a determinant of resistance to aminoglycosides in the CF *P. aeruginosa* [60], similar to the resistance profile observed in AST.

Analyses of *oprD*, related to carbapenem resistance in *P. aeruginosa*, showed frameshift deletions and premature stop codons, previously reported as modifications promoting resistance to these antimicrobials and porin suppressors [61, 62].

In previous studies, defective or absent *oprD* was reported in non-CF *P. aeruginosa*, and some of these alterations were similar to those observed in the present study [63, 64]. Isolates 9876, 10,705, 11,227, and 14,339 showed a sequence of mutations (372 M-382Y) with alternating deletions and substitutions, and some similar previously described cases demonstrated that changes in these amino acids were associated with increased susceptibility to MER [62, 65]. Notably, this was true for two isolates (10,705 and 14,339) in this study.

Substitutions in *pmrB* (15 V-I, 68G-S, and 343T-A), observed in isolates 11,227, 17,138, 17,749, and 17,801, were thought to be associated with colistin non-susceptibility in non-CF *P. aeruginosa*, as these are common mutations described solely in non-susceptible isolates [66]. Additionally, a substitution in *parS* (398 H-R), detected in all isolates in this study, has been linked to colistin resistance, as it occurs under external stress conditions, thereby affecting colistin resistance [67]. Both genes are known to be involved in lipid A polarity modifications which can cause polymyxin resistance.

Substitutions in *gyrA* (83T-I) and *parC* (87 S-L) were identified in isolate 17,801, resistant to CIP, already well established as causing high-level resistance to fluoroquinolones in non-CF *P. aeruginosa* [52, 68, 69].

crpP, which encodes extrinsic resistance to CIP, was identified in three phenotypically resistant isolates (17,138, 17,749, and 17,801), which are not always correlated [70]. Corroborating the findings of the authors, two isolates were resistant to CIP, but did not have *crpP*, which can be explained by another mechanism of resistance, such as the overexpression of efflux pumps [71].

A correlation between the results of phenotypic resistance obtained in the AST and the presence of *OXA-4*, *OXA-50*, *OXA-486*, and *crpP* was observed. However, for *OXA-395*, *aph(3')-llb*, *aadA24* it was not possible to establish the same association, possibly due to not being expressed, expressed at a very low level, or another mechanism [72]. Sulfamethoxazole, chloramphenicol, and fosfomycin were not tested in this study; therefore, the relationship between *sul1*, *catB7*, and *fosA* cannot be reported.

In Brazil, the presence of carbapenemases in *P. aeruginosa* strains isolated from patients with CF is rare. This was highlighted in a report of a single isolate carrying the bla_{SPM-1} gene [33]. Furthermore, the presence of bla_{KPC} has been documented in *Klebsiella pneumoniae* and *Enterobacter cloacae* among individuals with CF under surveillance in facilities within the same city. This observation has raised concerns regarding the potential transmission of these genes, including those within *P. aeruginosa* [73, 74].

In the plasmid analysis, the presence of *mcr* was not observed in any of the isolates, which may suggest a different origin of resistance to polymyxin B. Nonetheless, the *crpP*, *OXA-395*, and *aaC(6')lb* plasmid genes were identified in different isolates of *P. aeruginosa* polymyxin resistance in CF; to our knowledge, this study is the first time this has been described in Brazil.

Most isolates showed at least one intact phage. Notably, YMC11/02/R656 was present in two isolates (9876 and 10,705) from individuals with CF treated at the same follow-up center. The same phage was described in a study by our group with *Achromobacter ruhlandii* in a different CF center [75]. This is concerning since lung infections via CF are polymicrobial, and the potential of *P. aeruginosa* to acquire transmissible genetic information allows the dissemination of resistance factors, including those from different bacterial species [76, 77].

Conclusion

Among the isolates, a high resistance rate was observed, with many multi-resistance profiles. WGS is fundamental for the identification of mutations related to permeability and alterations in the outer membrane and target site, which could influence the resistance of the isolates. Additionally, it allows the localization of plasmid resistance genes and bacteriophages, which may spread and perform horizontal gene transfer between other bacteria in the same center.

Furthermore, molecular typing described new STs but did not correlate with previously described epidemic clones.

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Author contributions All author contributed to the study conception and design. In terms of the manuscript composition and information acquisition, FAS played a primary role as the main contributor. Bioinformatics assistance and writing were provided by MMA, while HSR facilitated the search process and comprehension of bioinformatics tools. Corrective measures, content structuring, and information consolidation were carried out with the contributions of EAM and RSL. The first draft of the manuscript was written by FAS, MMA, EAM and RSL, and all authors commented on previous versions of the manuscript. All authors read and approved the final version of the manuscript.

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Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential 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 Universidade 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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