Genetic Variations in MexAB-OprM Efflux Pump Regulators and Their Association with Antibiotic Resistance and Sequence type in Clinical and Epidemiologically High-risk Clones of Pseudomonas Aeruginosa.

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Abstract

Background: *Pseudomonas aeruginosa* is a major opportunistic pathogen involved in healthcare-associated infections with high mortality rates. This bacterium exhibits elevated resistance to a wide range of antibiotics, resulting in part from the overexpression of efflux pumps, among which MexAB-OprM stands out as constitutive. Antibiotic resistance in clinical isolates is associated with mutations in the *mexR*, *nalC*, and *nalD* repressors that modulate the expression of this efflux pump. This study identifies point mutations in the *mexR, nalC,* and *nalD* genes and investigates their associations with antibiotic resistance and sequence type in clinical and epidemiologically high-risk clones of *P. aeruginosa*.

Results: A total of 91 *P. aeruginosa* strains isolated at a pediatric hospital in Mexico (2007–2015) were classified according to their resistance to antibiotics. The strains were typed by multilocus sequencing of 7 genes. The MexAB-OprM efflux pump phenotype was determined using the minimal inhibitory concentration for the reporter antibiotic carbenicillin in the presence/absence of the efflux pump inhibitor Phe-Arg-β-naphthylamine. Sequencing of the *mexR, nalC,* and *nalD* genes to identify mutations was performed. Genetic relationship among the strains was evaluated by a phylogenetic inference analysis using maximum likelihood to construct a phylogenetic network. The relationship between variables was determined by a principal component analysis. STs revealed six main complexes. Mutations in the *mexR, nalC,* and *nalD* genes revealed 27 different haplotypes. Pan-drug and extensive drug resistant profiles were associated with specific STs with haplotypes 1 (ST1725, endemic clone), 8, 12 (ST233, epidemiologically high-risk clone), and 5 [related to dead when compared to ST1725 and ST233 (RRR 23.34; p=0.009 and RRR 32.01; p=0.025)], however the resistance in these strains was not mainly attributed to the MexAB-OprM phenotype. Strains with the same haplotype and resistant profile showed different pump behavior.

Conclusions: A significant relationship between ST and resistant profiles was observed; on one hand, the *mexR-nalC-nalD* haplotypes were not related to the MexAB-OprM efflux pump phenotypic behavior. On the other hand, the relationship between *mexR-nalC-nalD* haplotypes and phylogenetically related ST, suggest mutations in these repressors are highly maintained within these STs.

Background

Pseudomonas aeruginosa, a free-living microorganism, is a major opportunistic pathogen involved in healthcare-associated infections with high mortality rates [1, 2]. Worldwide, *P. aeruginosa* is of major public health importance because it exhibits elevated resistance to nearly all types of antibiotics used for its mitigation [3, 4] and the distribution of epidemiologically high-risk clones is increasing [5].

The multi-antibiotic resistance of clinical *P. aeruginosa* strains lies from the combined effects of its intrinsic resistance, attributed to the low permeability of its external membrane and efflux pumps [6], and acquired resistance, principally through mutational changes or genetic material uptake [7]. The MexAB-OprM efflux pump is a major contributor to multi-resistance against commonly used antibiotics, and its overexpression has been observed in strains bearing mutations in its regulatory genes (*mexR, nalC*, and *nalD*) [8, 9, 10].

The MexAB-OprM efflux pump belongs to the resistance–nodulation–cell division family and is a complex that comprises 3 components: a cytoplasmic membrane transporter (MexB), a membrane fusion protein (MexA), and an external membrane pore (OprM). This system allows antimicrobials and other toxic compounds transportation from the cytoplasm to the extracellular environment [6, 11].

Constitutive expression of the MexAB-OprM efflux pump maintains basal levels of the pump and is mediated by the repressor genes *mexR, nalC*, and *nalD*. The *mexAB-oprM* operon that encodes the efflux pump is regulated directly by the transcriptional repressors MexR and NalD and indirectly by NalC, which represses ArmR protein expression (MexR anti-repressor) to de-repress efflux pump expression. Mutations in the *mexR, nalC*, and *nalD* genes (mainly *mexR* and *nalD*) can impair their function, favoring MexAB-OprM efflux pump overexpression with a consequent increase in bacterial resistance [6, 12, 13]. Such overexpression confers advantages to *P. aeruginosa* by expelling a diverse range of agents: fluoroquinolones, β-lactamics, β-lactamase inhibitors, extended spectrum cephalosporins, carbapenems, tetracycline, macrolides, chloramphenicol, novobiocin, trimethoprim, sulfonamides, dyes, detergents, fatty acids biosynthesis inhibitors, organic solvents, and homoserine lactones associated with cell–cell signaling systems and virulence determinants [14, 15, 16, 17, 18].

P. aeruginosa is known to have a non-clonal population structure with some outstanding sequence types (ST). The constant appearance of new variants has allowed for the observation that while this non-clonal structure is maintained in sensitive strains, multi-resistant strains primarily exhibit a clonal structure [5, 19]. Worldwide, ST111, ST146, ST175, ST233, and ST235 are the major epidemiologically high-risk clones associated with multidrug resistance (MDR) and extensive drug resistance (XDR); other clones, including ST357 and ST664, have also been described but with less frequency [5, 20, 21, 22].

Recent studies suggest that mutations in efflux pumps and porins are responsible for the generation of epidemiologically high-risk clones with MDR phenotypes [5, 23, 24] however, in the case of the MexAB-OprM efflux pump, only a small number of studies have investigated the relationship between mutations in the 3 repressor genes (*mexR, nalC*, and *nalD*) and the MDR phenotype [25, 26].

This study aims to investigate point mutations in the *mexR, nalC*, and *nalD* repressor genes of the MexAB-OprM efflux pump in clinical and epidemiologically high-risk clones of *P. aeruginosa* and their associations with: 1) ST (phylogeny), 2) antibiotic susceptibility (MDR, XDR, and pan drug resistance (PDR)), and 3) the survival outcome of infected patients in a level 3 health care facility in Mexico.

Results

Bacterial isolates

Of the 91 *P.aeruginosa* isolates investigated, 77 were of nosocomial origin (1H–77H), and 14 were from different environmental sources (1A–14A) (Table 1). The nosocomial isolates were principally recovered from urine (62.34%; n = 48) and blood (19.48%; n = 15). The environmental isolates were mainly obtained

from soil (42.86%; n = 6), water (35.71%; n = 5), and plants (21.43%; n = 3). The majority of strains were obtained from the following hospital wards: nephrology, 19.49% (n = 15); emergency, 15.58% (n = 12); and surgical therapy, 11.69% (n = 9). Among these patients, the mortality rate was 18.64% (11/59).

Table 1

Classification of the P. aeruginosa strains (origin, haplotype, susceptibility, MexAB-OprM phenotype, and carbapenemase product

	Haplotype		Antibiotics														Susceptibility	MexAB
ID	MexAB-OprM	ST	GEN	TOB	AK	IMI	MEM	CAZ	CPM	P/T	AZT	CIP	LEV	CB*	FOS*	CS		CB - PaβN
18H	1	1725	S	S	S	S	S	S	S	I	R	I	S	R	R	S	MDR	*2048*
54H	1	1725	R	R	R	R	R	R	R	R	R	R	R	R	R	S	XDR	*2048*
3H †	1	1725	R	R	R	R	R	R	R	I	R	R	R	R	R	R	XDR	*2048*
14H †	1	1725	R	R	I	R	R	R	R	I	R	R	R	R	R	S	XDR	*2048*
15H †	1	1725	R	R	R	R	R	R	R	I	R	R	R	R	R	S	XDR	*2048*
39H	1	1725	R	R	R	S	R	I	R	R	R	R	S	R	R	R	XDR	*2048*
55H	1	1725	R	R	R	R	R	R	R	I	R	R	R	R	R	R	XDR	*2048*
8H	1	1725	R	R	R	R	R	R	R	I	R	R	R	R	R	R	XDR	**1024**
53H	1	1725	R	R	R	R	R	R	R	R	R	R	R	R	R	S	XDR	64
38H	1	1725	R	R	R	R	R	R	R	R	R	R	R	R	R	S	XDR	**2048**
43H	1	1725	R	R	R	R	R	R	R	R	R	R	R	R	R	S	XDR	**2048**
5H	1	1725	R	R	R	R	R	R	R	I	R	R	R	R	R	R	XDR	**2048**
25H	1	1725	R	R	R	R	R	R	R	R	R	R	I	R	R	R	XDR	**2048**
6H	1	1725	R	R	R	R	R	R	R	R	R	R	R	R	R	R	PDR	*2048*
7H	1	1725	R	R	R	R	R	R	R	R	R	R	R	R	R	R	PDR	*2048*
30H	1	1725	R	R	R	R	R	R	R	R	R	R	R	R	R	R	PDR	*2048*
31H	1	1725	R	R	R	R	R	R	R	R	R	R	R	R	R	R	PDR	*2048*
37H	1	1725	R	R	R	R	R	R	R	R	R	R	R	R	R	R	PDR	*1024*
11H	1	1725	R	R	R	R	R	R	R	R	R	R	R	R	R	R	PDR	*2048*
12H	1	1725	R	R	R	R	R	R	R	R	R	R	R	R	R	R	PDR	*2048*
26H	1	1725	R	R	R	R	R	R	R	R	R	R	R	R	R	R	PDR	*2048*
36H	1	1725	R	R	R	R	R	R	R	R	R	R	R	R	R	R	PDR	*1024*
40H	1	1725	R	R	R	R	R	R	R	R	R	R	R	R	R	R	PDR	*2048*
41H	1	1725	R	R	R	R	R	R	R	R	R	R	R	R	R	R	PDR	**1024**
50H	1	1725	R	R	R	R	R	R	R	R	R	R	R	R	R	R	PDR	**2048**
33H	1	1725	R	R	R	R	R	R	R	R	R	R	R	R	R	R	PDR	**2048**
48H	1	1725	R	R	R	R	R	R	R	R	R	R	R	R	R	R	PDR	**2048**
4H	1	1725	R	R	R	R	R	R	R	R	R	R	R	R	R	R	PDR	**2048**
16H	1	1725	R	R	R	R	R	R	R	R	R	R	R	R	R	R	PDR	**2048**
42H	1	1725	R	R	R	R	R	R	R	R	R	R	R	R	R	R	PDR	**2048**
49H	1	1725	R	R	R	R	R	R	R	R	R	R	R	R	R	R	PDR	**1024**
35H †	1	1725	R	R	R	R	R	R	R	R	R	R	R	R	R	R	PDR	**2048**
51H	1	1725	R	R	R	R	R	R	R	R	R	R	R	R	R	R	PDR	**2048**

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Haplotype: MexAB-OprM was considered as the DNA sequence from the concatenation of *mexR-nalC-nalD* genes; **ST**: Sequence Type. The ST1725 (most frec (Epidemiological high risk clone) are highlighted.

Antibiotic categories: Aminoglycosides (GEN: gentamicin, TOB: tobramycin, AK: amikacin), Carbapenems (IMI: imipenem, MEM: meropenem), Cephalosporins cefepime), Penicillins (CB: carbenicillin), Penicillins + β-lactamase inhibitors (P/T: piperacillin-tazobactam), Monobactams (AZT: aztreonam), Fluoroquinolon levofloxacin), Phosphonic Acids (FOS: fosfomycin), Polymyxins (CS: colistin);

SusceptibilityS: sensitive, **I**: intermediate resistant, **R**: resistant; **S**: sensitive, **MDR**: multidrug resistant, **XDR**: extensively drug resistant, **PDR** (highlighted): pan

	Haplotype		Antibiotics														Susceptibility	MexAB
ID	MexAB-OprM	ST	GEN	TOB	AK	IMI	MEM	CAZ	CPM	P/T	AZT	CIP	LEV	CB*	FOS*	CS		CB - PaβN
46H	**1**	2244	**R**	**R**	S	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	XDR	1024
47H †	**1**	2245	**R**	**R**	**R**	**R**	**R**	**R**	**R**	I	**R**	**R**	**R**	**R**	**R**	**R**	XDR	**2048**
52H	**1**	2247	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	XDR	**1024**
45H	**1**	2243	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**PDR**	**512**
1H	**1**	1723	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**PDR**	**2048**
32H	**1**	1730	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**PDR**	**2048**
12A	**1**	111	S	S	S	S	I	S	S	S	S	I	S	**R**	**R**	**R**	MDR	512
56H	2	1725	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**PDR**	**512**
44H	3	2246	**R**	**R**	**R**	**R**	**R**	**R**	**R**	I	**R**	**R**	**R**	**R**	**R**	**R**	XDR	*2048*
10A	4	2566	S	S	S	S	I	S	**R**	S	S	S	S	**R**	**R**	**R**	MDR	128
10H †	**5**	1726	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	XDR	*2048*
27H †	**5**	1727	**R**	S	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	XDR	*2048*
9H	**5**	1724	**R**	**R**	**R**	**R**	**R**	**R**	**R**	I	**R**	**R**	**R**	**R**	**R**	**R**	XDR	*2048*
2H †	**5**	1724	**R**	**R**	**R**	**R**	**R**	**R**	**R**	I	**R**	**R**	**R**	**R**	**R**	S	XDR	**2048**
28H †	**5**	1728	**R**	**R**	**R**	**R**	**R**	**R**	**R**	I	**R**	**R**	**R**	**R**	**R**	S	XDR	**2048**
17H	6	1733	S	S	S	S	**R**	S	S	S	S	S	S	**R**	**R**	**R**	MDR	**2048**
11A	7	2567	S	S	S	S	S	S	S	S	I	S	S	**R**	**R**	**R**	MDR	256
68H	**8**	2710	**R**	**R**	**R**	**R**	**R**	**R**	**R**	I	**R**	**R**	**R**	**R**	**R**	**R**	XDR	*1024*
70H	**8**	2716	**R**	**R**	**R**	**R**	**R**	**R**	**R**	I	**R**	**R**	**R**	**R**	**R**	**R**	XDR	*1024*
63H	**8**	2704	**R**	**R**	**R**	**R**	**R**	**R**	**R**	I	**R**	**R**	**R**	**R**	**R**	**R**	XDR	*2048*
67H	**8**	2710	**R**	**R**	**R**	**R**	**R**	**R**	**R**	R	**R**	**R**	**R**	**R**	**R**	**R**	**PDR**	*1024*
72H	**8**	2731	**R**	**R**	**R**	**R**	**R**	**R**	**R**	R	**R**	**R**	**R**	**R**	**R**	**R**	**PDR**	*2048*
69H	9	2713	**R**	**R**	**R**	**R**	**R**	**R**	**R**	I	**R**	**R**	**R**	**R**	**R**	**R**	XDR	*1024*
73H	10	2732	S	S	S	**R**	**R**	S	I	I	**R**	**R**	**R**	**R**	**R**	**R**	MDR	512
2A	11	2249	S	S	S	S	S	S	S	S	S	S	S	**R**	**R**	**R**	MDR	128
9A	11	2565	S	S	S	S	R	S	S	S	I	**R**	I	**R**	**R**	**R**	MDR	256
71H	**12**	2559	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	**R**	**R**	**R**	**R**	**R**	XDR	1024
65H	**12**	**233**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	**R**	**R**	**R**	**R**	**R**	XDR	*2048*
75H	**12**	**233**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	I	S	**R**	**R**	**R**	**R**	**R**	XDR	*2048*
76H	**12**	**233**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	**R**	**R**	**R**	**R**	**R**	XDR	*2048*
57H	**12**	**233**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	**R**	**R**	**R**	**R**	**R**	XDR	*2048*
59H	**12**	**233**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	**R**	**R**	**R**	**R**	**R**	XDR	*2048*
77H	**12**	**233**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	**R**	**R**	**R**	**R**	**R**	XDR	*2048*

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74H	**12**	2560	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	I	**R**	**R**	**R**	**R**	**R**	XDR	*2048*
66H †	**12**	2559	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	**R**	**R**	**R**	**R**	**R**	XDR	*2048*
1A	13	2561	S	S	S	S	**R**	S	**R**	I	**R**	S	S	**R**	**R**	S	MDR	512
23H	14	1736	S	S	S	S	S	S	S	S	I	I	I	**R**	**R**	**R**	MDR	*128*
24H	14	1736	S	S	S	S	I	S	S	S	S	S	S	**R**	**R**	**R**	MDR	*128*
61H †	15	2557	S	S	S	S	S	S	I	S	S	S	S	**R**	**R**	**R**	MDR	*2048*
13A	16	2568	S	S	S	S	**R**	S	**R**	S	S	**R**	S	**R**	**R**	S	MDR	128
64H	17	2709	**R**	**R**	S	**R**	S	**R**	S	I	I	S	**R**	**R**	**R**	**R**	XDR	*1024*
60H †	18	2248	**R**	**R**	S	**R**	**R**	**R**	**R**	I	**R**	I	**R**	**R**	**R**	S	XDR	*2048*
62H	18	2558	I	S	**R**	**R**	**R**	**R**	**R**	I	**R**	I	**R**	**R**	**R**	S	XDR	*2048*
58H	19	112	**R**	**R**	**R**	I	**R**	**R**	**R**	S	S	S	S	**R**	**R**	**R**	MDR	**1024**
22H	20	1735	S	S	S	S	S	S	S	S	S	S	S	**R**	**R**	**R**	MDR	*2048*
34H †	21	561	S	S	S	S	S	S	S	S	S	S	S	**R**	**R**	**R**	MDR	128
13H †	21	1737	S	S	S	S	S	S	S	S	S	S	S	**R**	**R**	**R**	MDR	*2048*
19H	22	1731	S	S	S	S	S	S	I	S	S	S	R	**R**	**R**	S	MDR	**256**
4A	23	2563	S	S	S	**R**	**R**	S	S	S	**R**	S	S	**R**	**R**	**R**	MDR	*2048*
21H	24	1734	S	S	S	S	S	S	S	S	S	S	S	**R**	**R**	**R**	MDR	**512**
3A	25	2562	S	S	S	**R**	S	S	S	S	S	S	S	**R**	**R**	**R**	MDR	64
7A	**26**	2250	S	S	S	S	S	S	S	S	I	S	S	**R**	**R**	S	S	64
6A	**26**	540	S	S	S	S	S	S	S	S	S	S	I	**R**	**R**	S	S	128
8A	**26**	2251	S	S	S	S	I	S	S	S	S	R	S	**R**	**R**	**R**	MDR	64
5A	**26**	2564	S	S	S	S	**R**	S	S	S	S	S	S	**R**	**R**	**R**	MDR	128
29H †	**26**	1729	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	R	R	**R**	**R**	**R**	XDR	*2048*
14A	**26**	1729	S	S	**R**	S	**R**	S	**R**	I	R	R	S	**R**	**R**	**R**	XDR	*64*
20H †	27	2226	S	S	S	S	S	S	S	S	S	S	S	**R**	**R**	S	S	1024
PAO1		549	S	S	S	S	S	S	S	S	S	S	S	S	**R**	S	S	64

ID: origin (H: nosocomial strains; A: environmental strains); patient death outcome: †

Haplotype: MexAB-OprM was considered as the DNA sequence from the concatenation of *mexR-nalC-nalD* genes; **ST**: Sequence Type. The ST1725 (most frec (Epidemiological high risk clone) are highlighted.

Antibiotic categories: Aminoglycosides (GEN: gentamicin, TOB: tobramycin, AK: amikacin), Carbapenems (IMI: imipenem, MEM: meropenem), Cephalosporins cefepime), Penicillins (CB: carbenicillin), Penicillins + β-lactamase inhibitors (P/T: piperacillin-tazobactam), Monobactams (AZT: aztreonam), Fluoroquinolone levofloxacin), Phosphonic Acids (FOS: fosfomycin), Polymyxins (CS: colistin);

SusceptibilityS: sensitive, **I**: intermediate resistant, **R**: resistant; **S**: sensitive, **MDR**: multidrug resistant, **XDR**: extensively drug resistant, **PDR** (highlighted): pan

All strains evaluated in this study were taxonomically identified using the automated system MALDI-TOF (Biomerieux Marcy l'Etoile, France) and additionally were positive for phenotype tests of *P. aeruginosa*, including large, smooth colonies with flat edges and an elevated appearance; gram-negative bacillus; positive for oxidase, catalase, and β-hemolysis; pyocyanin and pyoverdine pigments produced; grape-like odor; non-fermenter of glucose or other carbohydrates such as lactose according to Kliger biochemical test; and growth in cetrimide agar at 42°C.

P. aeruginosa **nosocomial strains exhibit more multi-drug resistance than environmental strains**

The 91 strains were classified as S, R, MDR, XDR, or PDR according to their susceptibility profile (Table 1) as determined by the CLSI 2019 standard values and criteria established by Magiorakos et al., 2012 [27, 28]. Of the nosocomial strains, 49.35% (38/77) were classified as XDR; 10 strains showed intermediate

resistance to P/T, 8 strains were AZT sensitive, and 6 strains were CS sensitive. In addition, 33.77% of the nosocomial strains were classified as PDR (26/77), 15.58% as MDR (12/77), and 1.30% (1/77) as sensitive. Of the environmental strains, 78.57% were classified as MDR (11/14), 7.14% as XDR (1/14), and 14.29% as sensitive (2/14). *P. aeruginosa* nosocomial strains were highly associated with the XDR profile compared to the environmental strains and taking the MDR strains as reference (RRR = 34.82; p = 0.001). The susceptibility of the studied strains to 14 antibiotics in 9 categories is shown in Table 1.

Mexab-oprm Efflux Pump Contributes To Mic Rising Behavior

Of the 91 *P. aeruginosa* strains, 56.04% [51/91: 9 MDR, 23 XDR, and 19 PDR] demonstrated high phenotypic activity of the MexAB-OprM efflux pump. This activity was observed as a 4-fold decrease in MIC value for the reporter antibiotic carbenicillin (CB) in the presence of PaβN inhibitor (CB + PaβN) compared with the MIC value in the absence of the inhibitor (CB- PaβN) (Table 1). However, the MexAB-OprM efflux pump was considered as the most likely cause of the elevation of the MIC (+); if the MIC value for CB -PaβN was at least 2 log2 dilutions higher than in the wild type strain (PAO1: MIC 64 µg/ml), and the MIC for CB + PaβN was lower than the measured in the wild type strain or ± 1 dilution; 52.9% of the strains [27/51: (4 MDR, 9 XDR, and 14 PDR)] showed this phenotype. If the MIC values for CB + PaβN remained elevated compared with the wild type strain, the MexAB-OprM efflux pump was contributing in the elevation of the MIC (*); 47% of the strains [24/51: (5 MDR, 14 XDR, and 5 PDR)] showed this phenotype. Finally, if there was a difference of 1 dilution between CB -PaβN and CB + PaβN or no difference, the MexAB-OprM efflux pump was not the cause of the elevation of the MIC (-); 43.96% of the strains [40/91: (3 S, 14 MDR, 16 XDR,7 PDR)] showed this phenotype.

Considering the phenotypic positive-strains (+ and *) (Table 1): a significant difference was observed between nosocomial (64.93%, 50/77) and environmental strains (7%, 1/14) ($p < 0.0001$), nosocomial strains were associated with the MexAB-OprM contribution (*) when compared to the environmental strains and taking the negative MexAB-OprM efflux pump strains as reference (RRR = 11.07; p = 0.025); and between the strains classified as PDR (73%; 19/26), XDR (58.97%; 23/39), MDR (39.1%; 9/23) and S (0%; 0/3) ($p = 0.016$), where positive MexAB-OprM efflux pump strains were mainly associated with PDR compared to negative MexAB-OprM efflux pump strains and taking the MDR strains as reference (RRR = 7; p = 0.008) (Table 1).

Carbapenemases are not the main contributor of resistance in P. aeruginosa strains

Carbapenemase expression was evaluated in 74 strains confirmed to be meropenem and/or imipenem resistant. The commercial kit β CARBA Test showed invalid results for 15 strains (Table 1), which produced an orange coloration instead of red (positive result) or yellow (negative result). For the remaining 59 strains, carbapenemase typing (serine carbapenemase or metallo-β-lactamase) was conducted (Table 1) and showed that 8 strains were positive for serine carbapenemases and 11 strains were positive for metallo-β-lactamases.

Multilocus sequence typing verifies the diversity of most P. aeruginosa strains, and reveals the emergence of outstanding Sequence types.

The ST for 58 *P. aeruginosa* strains were acquired from the MLST data base entry http://pubmlst.org/paeruginosa/ (Supplementary Table 1), being the endemic clone ST1725 the most frequent and persistent for over 7 years in the hospital. The remaining 33 strains isolated during 2013–2015 were analyzed here, revealing 3 new alleles: allele 233 for *aroE*, 147 for *guaA*, and 157 for *mutL;* in addition to 23 new ST that were integrated into the worldwide *P. aeruginosa* MLST database (Supplementary Table 1). During the same period, 6 ST233 strains and one ST111 strain (both reported worldwide as epidemiologically high-risk clones) were isolated, with ST111 identified in the environment. Of the 48 ST studied, the environmental strains showed the greatest diversity, with a different ST in each strain (Table 1). Hospital strains had the most STs (n = 35). The nucleotide and gene diversity was greatest among environmental strains (Pi, 0.0074; Hd, 0.0071), with the greatest diversity observed in the *aroE* and *trpE* genes (Pi, 0.010; Hd, 0.007). Of the 94 mutations identified, 81 were in hospital strains, with the greatest number seen in the *aroE* (n = 18) and trpE (n = 19) genes. Relevant genetic data, including the number of haplotypes, nucleotide diversity, gene diversity, and substitutions identified by MLST genotyping are summarized in Supplementary Table 2.

Mutations in the regulatory mexR, nalC, and nalD genes show nalC gene as the most diverse

It was observed a total of 62 mutations (49 synonymous; 13 non-synonymous) in the *mexR, nalC*, and *nalD* genes (Table 2). The *mexR* gene had 13 synonymous substitutions in 74.7% of the strains (68/91) and 3 non-synonymous substitutions in 71.42% (65/91) of the strains, with the V126E amino acid variation being the most frequent (69.23%; 63/91). The 268C→T nonsense mutation, the only mutation encoding a stop codon (Q90*), was observed in 2 strains. The *nalC* gene had 19 synonymous substitutions in 76.92% (70/91) of the strains and 9 non-synonymous substitutions in 98.90% (90/91) of the strains, with G71E being the most frequent 96.70%; (88/91). Additionally, one strain had a 12-bp deletion from position 105 to 116. Finally, the *nalD* gene had 17 synonymous substitutions in 67.03% of the strains (61/91) and 1 non-synonymous (A46T) in 6.59% of the strains (6/91). The *nalC* gene had the highest number of substitutions.

Table 2
Genetic variations identified in the *mexR, nalC* and *nalD* repressor genes in *Pseudomonas aeruginosa* strains.

Repressor gene	Genetic variation	H (n = 77)	A (n = 14)	Total (n = 91)	Nucleotide Variations	Amino acid Variation
mexR	No mutation	16	5	21	-	-
	Synonymous mutation (n = 13)	59	9	68	15G→C, 18T→C, 33C→T, 60G→A, 96A→G, 168C→T, 201G→A, 264C→T, 327G→A, 378G→T, 384G→A, 411G→A	**V5V, N6N, P11P**, V20V, T22T, **R32R**, D56D, **L67L**, **S88S, E109E**, V126V, **Q128Q**, Q137Q
	Nonsynonymous mutation (n = 3)	57	8	65	170T→C, 268C→T, 377T→A	L57P, Q90*, **V126E**
	Amino acid substitution	55	8	63	170T→C, 377T→A	L57P, **V126E**
	Amino acid deletion	0	0	0	-	-
	Amino acid insertion	0	0	0	-	-
	Frameshift	0	0	0		
	Stop	2	0	2	268C→T	Q90*
nalC	No mutation	0	1	1	-	-
	Synonymous mutation (n = 19)	61	9	70	12T→G, 15T→C, 69T→C, 123A→T, 129G→A, 147G→A, 177G→A, 186C→T, 258G→A, 294T→C, 354C→T, 358C→A, 369G→A, 411T→C, 435C→A, 441C→T, 444T→C, 447T→C, 558G→A	A4A, S5S, A23A, I41I, R43R, G49G, E59E, F62F, **T86T**, F98F, S118S, R120R, A123A, Y137Y, A145A, **V147V**, A148A, **P149P**, A186A
	Nonsynonymous mutation (n = 9)	77	13	90	△105–116, 130G→A, 212G→A, 237T→A, 434C→T, 457G→C, 459G→T, 556G→A, 625A→C	(T35△, T36△, L37△, D38△, M39△), A44T, **G71E**, D79E, A145V, E153Q, E153D, A186T, **S209R**
	Amino acid substitution	77	13	90	130G→A, 212G→A, 237T→A, 434C→T, 457G→C, 459G→T, 556G→A, 625A→C	A44T, **G71E**, D79E, **A145V**, E153Q, E153D, A186T, **S209R**
	Amino acid deletion	1	0	1	△105–116	T35△, T36△, L37△, D38△, M39△
	Amino acid insertion	0	0	0	-	-
	Frameshift	0	0	0	-	-
	Stop	0	0	0	-	-
nalD	No mutation	18	6	24	-	-
	Synonymous mutation (n = 17)	53	8	61	78G→A, 120C→T, 135C→A, 153C→T, 165C→T, 169C→T, 231C→A, 276C→T, 295T→C, 297G→A, 303G→T, 333C→T, 450T→C, 477G→A, 504G→A, 540C→T, 555T→C	**K26K**, A40A, G45G, F51F, A55A, L57L, S77S, C92C, L99L, **L99L**, T101T, I111I, R150R, P159P, A168A, D180D, **D185D**
	Nonsynonymous mutation (n = 1)	6	0	6	136G→A	A46T
	Amino acid substitution	6	0	6	136G→A	A46T
	Amino acid deletion	0	0	0	-	-
	Amino acid insertion	0	0	0	-	-

H: nosocomial strains, A: environmental strains. Nucleotide variation: the number indicates the nucleotide position in the gene where the change occurs, the first letter indicates reference strain (*P. aeruginosa* PAO1) nucleotide and the second letter indicates the nucleotide that substitutes the original. The symbol △nt means nucleotide deletion. Amino acid variation: A: alanine, C: cysteine, D: aspartic acid, E: glutamic acid, F: phenylalanine, G: glycine, H: histidine, I: isoleucine, K: lysine, L: leucine, M: methionine, N: asparagine, P: proline, Q: glutamine, R: Arginine, S: serine, T: threonine, V: valine, W: tryptophan, Y: tyrosine. The * means stop codon. First letter indicates reference strain (*P. aeruginosa* PAO1) amino acid, the number indicates the amino acid position where the change occurs and the second letter indicates the amino acid that substitutes the original amino acid. In bold are mutations previously reported by Quale et al., 2006 and Suresh et al., 2018 [13, 47].

Repressor gene	Genetic variation	H (n = 77)	A (n = 14)	Total (n = 91)	Nucleotide Variations	Amino acid Variation
	Frameshift	0	0	0	-	-
	Stop	0	0	0	-	-

H: nosocomial strains, A: environmental strains. Nucleotide variation: the number indicates the nucleotide position in the gene where the change occurs, the first letter indicates reference strain (*P. aeruginosa* PAO1) nucleotide and the second letter indicates the nucleotide that substitutes the original. The symbol △nt means nucleotide deletion. Amino acid variation: A: alanine, C: cysteine, D: aspartic acid, E: glutamic acid, F: phenylalanine, G: glycine, H: histidine, I: isoleucine, K: lysine, L: leucine, M: methionine, N: asparagine, P: proline, Q: glutamine, R: Arginine, S: serine, T: threonine, V: valine, W: tryptophan, Y: tyrosine. The * means stop codon. First letter indicates reference strain (*P. aeruginosa* PAO1) amino acid, the number indicates the amino acid position where the change occurs and the second letter indicates the amino acid that substitutes the original amino acid. In bold are mutations previously reported by Quale et al., 2006 and Suresh et al., 2018 [13, 47].

Table 2

Haplotype was defined as the DNA sequence of the concatenated *mexR-nalC-nalD* genes. A total of 27 different haplotypes were identified in the 91 strains, including 26 haplotypes with substitutions and one haplotype with a 12-bp deletion (Table 3). The hospital strains showed the largest number of haplotypes (n = 19) (Table 1), while the environmental strains had the greatest diversity in nucleotides (Pi) and genes (Hd) (Pi, 0.00922; Hd, 0.879), with the greatest diversity observed for the *nalC* gene (Pi, 0.01184; Hd, 0.771) (Table 3). We observed a total of 62 mutations, of which 61 were in hospital strains and most were located in the *nalC* gene (n = 28) (Table 2). Data including the number of haplotypes, nucleotide diversity, gene diversity, and nucleotide substitutions in the *mexR, nalC, and nalD* repressor genes are summarized in Table 3.

Table 3
Genetic variations (haplotypes) identified in the *mexR, nalC* and *nalD* repressor genes in *P. aeruginosa* strains.

Haplotype	*mexR*	*nalC*	*nalD*	Total
1	S88S, E109E, Q128Q, Q137Q	A4A, S5S, A23A, I41I, R43R, G49G, E59E, S118S, Y137Y, A145A, A148A, P149P	L57L, L99L	18
	V126E (1)	**G71E, S209R (1)**	**(1)**	**3**
2	S88S, E109E, Q128Q, Q137Q	A4A, S5S, A23A, I41I, R43R, G49G, E59E, S118S, Y137Y, A145A, A148A, P149P	L57L, L99L	18
	V126E	**(T35△, T36△, L37△, D38△, M39△), G71E, S209R (2)**	**(1)**	**4**
	(1)			
3	S88S, E109E, Q128Q, Q137Q	A4A, S5S, A23A, I41I, R43R, G49G, E59E, S118S, A123A, Y137Y, A145A, A148A, P149P	K26K, L99L, R150R, P159P	21
	V126E (1)	**G71E, D79E, S209R (3)**	**(2)**	**4**
4	V20V, E109E, Q128Q, Q137Q	A4A, S5S, A23A, I41I, R43R, G49G, E59E, S118S, A123A, Y137Y, A145A, A148A, P149P	G45G	18
	V126E (2)	**G71E, S209R (4)**	**(3)**	**3**
5	V20V, E109E, Q128Q, Q137Q	A4A, S5S, A23A, I41I, G49G, E59E, S118S, R120R, A123A, Y137Y, A145A, A148A, P149P	C92C, L99L, I111I, D180D	21
	V126E (2)	**G71E, E153Q, S209R (5)**	**(4)**	**4**
6	V5V, N6N, P11P, T22T, R32R, S88S, E109E Q128Q, Q137Q	A4A, S5S, A23A, I41I, G49G, E59E, S118S, Y137Y, A145A, A148A, P149P	L57L, C92C, L99L, D180D	24
	V126E (3)	**G71E, A145V, S209R (6)**	**(5)**	**4**
7	V5V, N6N, P11P, R32R, D56D, S88S, E109E, Q128Q, Q137Q			9
	V126E (4)	**G71E, S209R (7)**	**(6)**	**3**
8	V5V, N6N, P11P, R32R, S88S, E109E, Q128Q, Q137Q	A23A		9
	V126E (5)	**G71E, S209R (8)**	**A46T (7)**	**3**
9	V5V, N6N, P11P, R32R, S88S, E109E, Q128Q, Q137Q	A23A		9
	V126E (5)	**G71E (9)**	**A46T (7)**	**3**
10	S88S, E109E, Q128Q, Q137Q			4
	L57P, V126E (6)	**G71E, S209R (7)**	**(6)**	**4**
11				0
	(7)	**G71E, S209R (7)**	**(6)**	**2**
12				0
	(7)	**G71E, E153D, A186T (10)**	**(6)**	**3**
13				0
	(7)	**(11)**	**(6)**	**0**
14				0
	(7)	**A44T (12)**	**(6)**	**1**
15	V126V, Q137Q			2
	(8)	**G71E (13)**	**(6)**	**1**
16	L67L			1
	(9)	**G71E, S209R (7)**	**(6)**	**2**

First letter indicates reference strain (*P. aeruginosa* PAO1) amino acid. The number indicates the amino acid position where the change occurs and the second letter indicates the amino acid that substitutes the original amino acid. The symbol △ means nucleotide deletion. The * means stop codon. Amino acid variation: A: alanine, C: cysteine, D: aspartic acid, E: glutamic acid, F: phenylalanine, G: glycine, H: histidine, I: isoleucine, K: lysine, L: leucine, M: methionine, N: asparagine, P: proline, Q: glutamine, R: Arginine, S: serine, T: threonine, V: valine, W: tryptophan, Y: tyrosine. In bold are non-synonymous mutations. Pi: nucleotide diversity, Eta: theta (per site), Hd: gene diversity, S: nucleotide substitutions. In haplotype 13 (no mutations identified), the *mexR, nalC* and *nalD* repressor genes of the MexAB-OprM efflux pump sequences were identical to those of *P. aeruginosa* PAO1. The number of haplotypes (specific mutation or combined mutations) by gene is also described; in parenthesis, the number of haplotype.

Haplotype	*mexR*	*nalC*	*nalD*	Total
17	L67L		A40A	1
	(9)	**G71E, S209R (7)**	**(8)**	**2**
18				0
	Q90* (10)	**G71E, S209R (7)**	**(6)**	**3**
19	D56D	T86T, V147V, P149P		4
	(11)	**G71E (14)**	**(6)**	**1**
20		T86T, V147V, P149P	F51F, L57L, L99L	6
	(7)	**G71E, S209R (15)**	**(9)**	**2**
21		A23A		1
	(7)	**G71E, S209R (8)**	**(6)**	**2**
22	D56D	A23A	D185D	3
	(11)	**G71E, S209R (8)**	**(10)**	**2**
23		A23A		1
	(7)	**G71E (9)**	**(6)**	**1**
24		A4A, S5S	A168A	2
	(7)	**G71E, S209R (16)**	**(11)**	**2**
25		A4A, S5S, A23A, I41I, R43R, G49G, E59E, S118S, A123A, Y137Y, A145A, A148A, P149P	R150R, P159P, D185D	16
	(7)	**G71E, S209R (4)**	**(12)**	**2**
26	V20V, E109E, Q128Q, Q137Q	A4A, S5S, A23A, I41I, G49G, E59E, F62F, F98F, S118S, Y137Y, A145A, A148A, P149P, A186A	K26K, A55A, T101T, R150R, P159P	23
	V126E (2)	**G71E, A145V, S209R (17)**	**(13)**	**4**
27		A4A, S5S, A23A, I41I, G49G, E59E, S118S, R120R, A123A, Y137Y, A145A, A148A, P149P	S77S, L99L	15
	(7)	**G71E, E153Q, S209R (5)**	**(14)**	**3**
Number of haplotypes	11	17	14	**27**
Pi	0.00745	0.01184	0.00376	0.00769
Eta	0.00709	0.00906	0.00585	0.00735
Hd	0.718	0.771	0.723	0.781
S	16	28	18	62

First letter indicates reference strain (*P. aeruginosa* PAO1) amino acid. The number indicates the amino acid position where the change occurs and the second letter indicates the amino acid that substitutes the original amino acid. The symbol △ means nucleotide deletion. The * means stop codon. Amino acid variation: A: alanine, C: cysteine, D: aspartic acid, E: glutamic acid, F: phenylalanine, G: glycine, H: histidine, I: isoleucine, K: lysine, L: leucine, M: methionine, N: asparagine, P: proline, Q: glutamine, R: Arginine, S: serine, T: threonine, V: valine, W: tryptophan, Y: tyrosine. In bold are non-synonymous mutations. Pi: nucleotide diversity, Eta: theta (per site), Hd: gene diversity, S: nucleotide substitutions. In haplotype 13 (no mutations identified), the *mexR, nalC* and *nalD* repressor genes of the MexAB-OprM efflux pump sequences were identical to those of *P. aeruginosa* PAO1. The number of haplotypes (specific mutation or combined mutations) by gene is also described; in parenthesis, the number of haplotype.

Table 3.

Phylogenetic Analysis arranges P. aeruginosa strains into genetic complexes that share the same characteristics

The phylogenetic network based on the MLST genotyping (ST) of the *P. aeruginosa* strains is shown in Figs. 1 and 2. Although both figures refer to the same phylogenetic network, different information is highlighted. The phylogenetic relationships, evolutionary history, clonal complexes identified in the 48 STs and the 27 *mexR-nalC-nalD* haplotypes are shown in Fig. 1. In this network, the data indicated 6 important clonal complexes (CC); in each complex, all STs have a close phylogenetic relationship as follows:

1) Complex I: Includes strains ST1725 as the most prevalent (n = 34), followed by ST1723, ST1730, ST2243, ST2244, ST2245 and ST2247 (n = 1 strain each), all of nosocomial origin. If considering a profile match at n-3 loci (n = number of loci in the scheme, MLST = 7), ST2248 clone can be included in this complex. The ST1725 was identified by the BURST analysis as the potential Ancestral Type (AT) of this clonal complex. Globally reported data in the PubMLST *P.*

aeruginosa database describe this complex as part of the CC309. Haplotype 1 was present in all the STs that make up this complex (39/41 strains), except for a ST1725 strain that presented haplotype 2 (the same mutations in the repressors as haplotype 1 plus a 12-bp deletion), and the ST2248 that presented haplotype 18. Haplotype 1 was highly associated with Complex I compared to other haplotypes and taking the singletons STs as reference (RRR = 409.53; p = 0.000).

2) Complex II: Includes ST1724, ST1726, ST1728 and ST1727, all of nosocomial origin with the ST1724 identified as the AT. This complex forms part of the globally CC235. All STs that make up this complex presented haplotype 5. This haplotype is possibly associated with fatal patient outcomes. Complex II was associated with death when compared to the singletons STs (RRR = 40.01; p = 0.006), to complex I (RRR = 23.34; p = 0.009), and to complex 4 (RRR = 32.01; p = 0.025).

3) Complex III: Includes ST1729, ST540, ST2250, and ST2251, all of environmental origin except for one ST1729 strain. If considering a profile match at n-3 loci, ST2564 clone can be included in this complex. No AT was identified within these STs, however they are part of the global CC253. All STs that make up this complex presented haplotype 26.

4) Complex IV: Includes ST2559, ST2560, and ST233 (n = 6), all of nosocomial origin. ST233 (an epidemiologically high-risk clone) is considered the AT of this complex, conforming the CC233 worldwide. All STs that make up this complex presented haplotype 12.

5) Complex V. Includes ST1737 and ST561, both of nosocomial origin and being part of the CC245 worldwide. Both STs that make up this complex presented haplotype 21 and are possibly associated with fatal patient outcomes.

6) Complex VI: Includes ST2710, ST2704, ST2713, and ST2731. If considering a profile match at n-3 loci, ST2716 clone can be included in this complex. All of these STs were of nosocomial origin without AT neither global CC identified. All STs that make up this complex presented haplotype 8, except for a ST2313 strain that presented haplotype 9, being the only difference between these haplotypes a mutation in the *nalC* gene S209R.

The rest of the STs are considered singletons since more than 3 locus variants are noticed between them and other STs. The remaining 19 *mexR-nalC-nalD* haplotypes were identified in the STs considered singletons.

Close phylogenetic relationship between CCI and CCII is evident, while the CCIV is the most distant complex. However, all CCs appear to be related somehow to the CCI.

The second phylogenetic network is focus in the relationship between the 27 identified *mexR-nalC-nalD* haplotypes, the 48 STs, and the susceptibility profiles of the *P. aeruginosa* strains (Fig. 2):

1) Complex I: All of the STs (haplotypes 1, 2 and 18) in this complex showed MDR (4.76% MDR, 38.10% XDR and 57.14% PDR); this complex was mainly associated with XDR compared to the singletons STs and taking the MDR strains as reference (RRR = 96.04; p = 0.000). In 51.22% of the strains in this complex [21/41: (14 PDR and 7 XDR)] the MexAB-OprM efflux pump was the most likely cause of the elevation of the MIC (RRR = 5.73; p = 0.011, if compared to the singletons STs and taking the negative MexAB-OprM efflux pump strains as reference); (RRR = 5.45; p = 0.008 associated to Haplotype 1 compared to other haplotypes and taking the negative MexAB-OprM efflux pump strains as reference), and in 21.95% of the strains [9/41: (1 MDR, 3 XDR, 5 PDR)] the pump was contributing in the elevation of the MIC, highlighting the ST1725 when compared to the negative MexAB-OprM efflux pump strains and taking the environmental STs as reference (RRR = 6.50; p = 0.036). Serine carbapenemases were identified in 3 strains (ST1725 and ST2244) and two strains produced metallo-β-lactamase (ST2248).

2) Complex II: All STs grouped in this complex were haplotype 5 and XDR. In 2 strains (2/5), efflux pump activity was the most likely cause of the elevation of the MIC, and in 3 strains (3/5) efflux pump was contributing in the elevation of the MIC.

3) Complex III: This complex shows variations in antimicrobial susceptibility (S, MDR, and XDR). The only strain of nosocomial origin in this group, ST1729, is XDR and associated with a fatal outcome in 2009. All of the strains were haplotype 26. In 2 strains (2/6), efflux pump activity was contributing in the elevation of the MIC.

4) Complex IV: All STs (haplotype 12) that make up this complex were XDR and produced metallo-β-lactamase. In 3 strains (3/9), the activity of the pump was contributing to the elevation of the MIC.

5) Complex V: Both STs (haplotype 21) that make up this complex presented MDR. The MexAB-OprM efflux pump was not the cause of the elevation of the MIC

6) Complex VI: All STs grouped in this complex showed MDR (66.66% XDR and 33.33% PDR). Only in one strain, the MexAB-OprM efflux pump was contributing to resistance; in the remaining strains of this complex, the pump was not the cause of the elevation of the MIC. All haplotype 8 strains produced serine carbapenemases (ST2710, ST2731 and ST2716) and the haplotype 9 strain (ST2713) produced metallo-β-lactamase.

Most of the STs (different haplotypes) considered singletons were MDR and only one strain (ST2568) produced serine carbapenemases.

Furthermore, the genetic relationship between different STs and the *mexR-nalC-nalD* haplotypes were corroborated with the phylogenetic neighbor-net network (Fig. 3), in which the 6 important clonal complexes previously described were observed highly conserved. The phylogenetic network showed a close relationship between CCI, CC2 and CC3, and a distant relationship between these clonal complexes and CCIV. However, close relationship between CCIV and

CCVI was only evident in this network. The presence of rectangular boxes in the network represents the high probability of extensive homologous recombination, which was corroborated with the PHI test that revealed statistically significant recombination events ($p < 0.05$).

Principal component analysis reveals correlation between ST and mexR-nalC-nalD haplotype

PCA analysis of variables associated with each strain showed that the variability of component 1 is 47.81% and that of component 2 is 18.34% (Fig. 4); combined, these 2 components have a variability of 66.15%. PCA also revealed a strong relationship between resistance and ST (Fig. 4). ST233 strains and 41.18% of strains with other STs were XDR; of the ST1725 strains, 61.76% were PDR and 35.29% were XDR ($p < 0.0001$); ST1725 was the main ST associated with XDR when compared to the environmental STs and taking the MDR strains as reference (RRR = 65.98; p = 0.001); and with PDR when compared to the hospital STs and taking the MDR strains as reference (RRR = 46.19; p = 0.001).

In addition, the MexAB-OprM haplotype (*mexR-nalC-nalD*) correlated with the ST ($p < 0.0001$) and resistance ($p < 0.0001$); ST1725 was highly associated with Haplotype 1 compared to other environmental STs and taking other haplotypes as reference (RRR = 264.02; p = 0.000). Haplotype 1 was highly associated with PDR (RRR = 218.51; p = 0.000) and XDR (RRR = 28.50; p = 0.000) compared to other haplotypes and taking the MDR strains as reference.

PCA showed a strong relationship between the strain isolation site, isolation date, and hospital ward. However, these variables were inversely proportional to ST and haplotype. On the other hand the ST and haplotype variables showed closeness and the same direction. The outcome variable (fatal outcome) was inversely proportional to all analyzed variables, although an association was observed between haplotype 5 strains (*mexR-nalC-nalD*) (Fig. 4, green dots), haplotype 1 strains (blue dots), and fatal outcomes. Fatal outcomes were observed in 12.5% of haplotype 1 strains, 80% of haplotype 5 strains, 11.11% of haplotype 12 strains, and 16.67% of haplotype 26 strains ($p = 0.051$) (Fig. 4; Fig. 1, see haplotype colors). However, it should note that patients' underlying conditions were not considered in this study.

Statistical analysis of the relationship between the mutations in the *mexR-nalC-nalD* genes, the sequence type, the resistance, the MexAB-OprM phenotype, and patient death outcome gave the following results:

- **Sequence type (ST)**: It was observed that phylogenetically related sequence types presented equal or similar *mexR-nalC-nalD* haplotypes ($p < 0.05$) (Fig. 1, see color by haplotype and phylogenetic relationships between STs; Table 3, see similar *mexR-nalC-nalD* haplotypes).
- **Resistance**: It was observed that 27 mutations were associated with the resistance profiles (XDR and PDR) of the *P. aeruginosa* strains, including 6 in the *mexR* gene (V20V, **S88S, E109E, Q128Q**, Q137Q and **V126E)**, 18 in the *nalC* gene (A4A, S5S, A23A, I41I, R43R, G49G, E59E, S118S, R120R, Y137Y, A145A, A148A, **P149P**, **G71E**, E153Q, E153D, A186T, and **S209R)** and 3 in the *nalD* gene (**K26K**, L57L, and L99L**)** ($p < 0.05$). Specific mutations were identified in the strains: in all the sensitive (*nalD* gene: S77S and **L99L**), MDR (*mexR* gene: D56D) and XDR (*nalC* gene: E153D and A186) (Table 3). No PDR-associated mutations were detected at 100%.
- **MexAB-OprM phenotype**: It was observed that 19 mutations were associated with the MexAB-OprM phenotype, including 4 in the *mexR* gene (**E109E**, **Q128Q**, Q137Q, and **V126E)**, 11 in the *nalC* gene (A4A, S5S, I41I, R43R, G49G, E59E, S118S, Y137Y, A145A, A148A, and **P149P)**, and 4 in the *nalD* gene (L57L, C92C, L99L, and D180D) ($p < 0.05$) (Table 3).
- **Patient death outcomes**: It was observed that 7 mutations were associated with patient death outcomes (all identified in haplotype 5 strains), including one in the *mexR* gene (V20V**)** 3 in the *nalC* gene (R120R, A123A, and E153Q), and 3 in the *nalD* gene (C92C, I111I, and D180D**)** ($p < 0.05$) (Table 3). However, it should note that patients' underlying conditions were not considered in this study.

Discussion

This study identifies point mutations in the regulatory *mexR, nalC, and nalD* genes and their associations with antibiotic resistance and sequence type in clinical and epidemiologically high-risk clones of *P. aeruginosa*. STs revealed six complexes. Mutations in the *mexR, nalC*, and *nalD* genes revealed 27 different haplotypes. Pan-drug and extensive drug resistant profiles were associated with specific STs with haplotypes 1 (ST1725, endemic clone), 8, 12 (ST233, epidemiologically high-risk clone), and 5 (related to dead), however the resistance in these strains was not mainly attributed to the MexAB-OprM phenotype. Strains with the same haplotype and resistant profile showed different efflux pump behavior. The results suggest a significant relationship between *mexR-nalC-nalD* haplotypes and phylogenetically related ST; and a significant relationship between ST and high-drug resistance in *P. aeruginosa* strains. However, no statistically significant relationship between *mexR-nalC-nalD* haplotypes and positive MexAB-OprM phenotypes (to which the resistance was completely attributed +) was revealed.

In our previous study, we identified and characterized *P. aeruginosa* strains with MDR, XDR, and PDR susceptibility profiles [19]. Of these strains, the ST1725 endemic clone stands out for its frequency and persistence, with characteristics that strongly suggest high adaptation to the nosocomial environment and potential for epidemiological risk. This study also identified the ST233 clone, reported in other parts of the world as an epidemiologically high-risk clone [29, 30] and reported in a study in Mexico as resistant to colistin and sensitive to aztreonam. However, because aztreonam is not commercially available in Mexico, the ST233 clone presents a risk to patients in this country.

P. aeruginosa has a great capacity to resist adverse environments, as evidenced by its nosocomial survival. Here we observed a high fraction of PDR (33.7%) and XDR (78%) in strains isolated from a hospital setting, while those from environmental settings were only abundant in MDR (78%) (Table 1). In our work and worldwide, the worrisome observation has been made that colistin resistant strains are present in both nosocomial and other environments. Colistin is the

last therapeutic option for MDR and XDR isolates [31, 32]. Dößelmann et al. (2017) warn about the rapid acquisition of colistin resistance by 2 *P. aeruginosa* clinical strains after they observed a 10-fold increase in resistance after 10 days of exposure to this antibiotic and 100-fold increase after 20 days [32].

The presence of antibiotics or heavy metals in the environment induces MDR and even XDR environmental strains. The finding of resistant bacteria in the environment is attributed to the discharge of antibiotics into waste waters [33] and in industrial waste and the misuse of antibiotics as a preventive measure in livestock and fish farms [4]. These factors may explain our observed high resistance to numerous antibiotics in environmental and clinical strains, which may interact in nature.

The resistance of the 91 *P. aeruginosa* strains to 14 different antibiotics [28] observed in this study (Table 1) resembles the results obtained in other studies. For example, Suresh et al. (2018) identified resistance to 16 antibiotics in 27.6% (8/29) of clinical isolates from Kerala (India) [13], and a Brazilian study in 2017 reports that 71.4% (25/35) of the investigated strains exhibited MDR [34].

We identified a total of 48 different ST of *P. aeruginosa* strains of nosocomial and environmental origin (Table 1, Supplementary Table 1). One other ST was reported in Mexico (ST3351); this strain is described in the Institute of Biomedical Sciences in the *P. aeruginosa* MLST world data base (http://pubmlst.org/paeruginosa/) (Supplementary Table 1). Without further data from Mexico, we are not able to compare the frequencies or emergent cases involving these ST with those observed in other healthcare facilities or geographical zones. As expected, all the environmental strains had a different ST, representing great diversity and indicating a non-clonal population structure. In contrast, the nosocomial strains had a non-clonal population structure; however, the emergence of highly successful epidemic clones was evident [35] (ST1725 and ST233), as previously described by Aguilar-Rodea et al., 2017 [19].

Among the nosocomial strains, ST1725 stands out for its high frequency (n = 34 strains) and multidrug resistance (PDR, 21 strains; XDR, 12 strains; MDR, 1 strain). This endemic clone prevailed for over 7 years (2007–2013) in the Hospital Infantil de México Federico Gomez (Table 1; Fig. 1, Complex I). However, this strain has not been reported in other parts of the world or in Mexico. Similarly, we identified 6 *P. aeruginosa* nosocomial strains as ST233, all of which were XDR (five AZT sensitive and one with intermediate resistance to P/T) (Table 1; Fig. 1, Complex 4). Although ST233 has been previously reported worldwide as an epidemiologically high-risk clone [29, 36, 37, 38], there are no records of colistin resistant strains or even XDR or PDR strains. The AZT susceptibility of our analyzed strains may result from the lack of commercial use of this antibiotic in Mexico due to restrictions. In the United States (Northeast, Ohio), where AZT use is free of such restrictions, AZT-resistant ST233 strains have been reported [30].

We also identified ST111 in a MDR environmental strain collected from a water source (CS, FOS resistant, and intermediate resistance to MEM) (Table 1; Fig. 1). ST111 is considered an epidemiologically high-risk clone and was recently reported in 4 patients with cystic fibrosis [39]. This clone also was identified in Croatia and France, with MDR association [40, 41]. Occasionally, *P. aeruginosa* strains migrate from the environment and cause animal or human infections [24, 42]. For this reason, this type of ST is considered highly dangerous and should be kept under surveillance.

MDR *P. aeruginosa* strains likely result from several factors. The involvement of overexpression of efflux pumps has gained recognition, particularly the MexAB-OprM pump for its constitutive expression and attribution of resistance to most antibiotics [14, 15, 16, 17, 18].

Efflux pumps are part of the intrinsic protective mechanism used by bacteria to avoid stress and therefore work in response to natural signals and against antibiotics. Resistance–nodulation–cell division pumps in *P. aeruginosa* have overlapping but non-identical substrates. While MexB transports (among other antibiotics) a broad spectrum of β-lactams, MexD has a narrower spectrum, excluding some of the MexB substrates such as carbenicillin. MexY has the narrowest spectrum, further excluding other antibiotics [43]. Studies suggest that MexB accepts negatively-charged substrates that other pumps do not recognize, as in the case of carbenicillin, which has a high negative charge (-2) compared to other β-lactams [43]. The MIC of a specific substrate in the presence of the efflux pump inhibitor PaβN is one of the most widely used assessments for evaluating efflux pumps. PaβN both inhibits efflux pumps and permeabilizes the outer membrane of gram-negative bacteria in a dose-dependent manner, such that low doses inhibit efflux pumps and higher doses destabilize membranes [43, 44].

Our results corroborate the relationship between the MDR of a given *P. aeruginosa* strain and its MexAB-OprM efflux pump activity, with 66.23% of nosocomial strains demonstrating activity, principally in PDR strains (73%). Efflux pump activity was also higher in strains ST1725 (76.5%) and ST233 (50%), an epidemiologically high-risk clone, compared with other STs ($p < 0.0001$) (Table 1). Similar results were reported by Arabestani et al., (2015), who observed a significant positive correlation between pump activity and antimicrobial resistance [45]. Ozer et al., (2012) reported a consistent positive correlation between MexAB-OprM expression and multidrug resistance in 50 *P. aeruginosa* clinical strains [46]. However, in our study, of the *P. aeruginosa* strains that showed MexAB-OprM activity (phenotypic positive-strains), only in 52.9% the MexAB-OprM efflux pump was the most likely cause of resistance (+), in the remaining 47% of the strains the MexAB-OprM efflux pump was contributing to the resistance (*), which demonstrated that although the MexAB-OprM pump shows positive activity and contributes to resistance, this is not the only mechanism of resistance in these strains [13]. For example, in the remaining phenotypic negative-strains (n = 40) (3 S, 14 MDR, 16 XDR and 7 PDR), where the MexAB-OprM efflux pump was unlikely to be the cause of the resistance (Table 1); the high-drug resistance can be attributed in part to the production of carbapenemases (1 MDR, 3 XDR, and 2 PDR: serine carbapenemases, 8 XDR: metallo-.β-lactamases).

While recent studies have identified point mutations in the *mexR*, *nalC*, and *nalD* repressor genes [13, 25], few have investigated the relationship between these mutations and the occurrence of multidrug resistance and ST. Here, we observed 27 different haplotypes in the 3 repressor genes. Regarding the *mexR* gene 3 non-synonymous substitutions were identified in 71.42% of the strains, with the V126E amino acid variation being the most frequent, agreeing with previous studies [25]. The 268C→T nonsense mutation, the only mutation encoding a stop codon (Q90*), was observed in 2 strains (haplotype 18), however, until now we do not know its effect on the final structure of the protein. The majority of the non-synonymous substitutions were found in the *nalC* gene, as seen in 98.90% of the strains (90/91). The most frequent mutation was G71E (96.70%; 88/91) as previously described by Horna et al (2018) [25]. In addition, a 12-bp

deletion was identified in one strain (Tables 2 and 3). Another study also identifies the *nalC* gene as the main site of point mutations target, reporting relevant point mutations (non-synonymous substitutions) in 87% of nosocomial isolates (n = 77/90) as well as some deletions [25]. Most of the substitutions reported in that study differ from those we observed (Table 3).

It has been reported that genetic variations as stop codons, frameshifts or deletions lead to loss of functionality of the repressor genes and may contribute to the MexAB-OprM over expression [47]; the deletion identified in the *nalC* gene, was possibly the cause of the MexAB-OprM positive-phenotype that was associated to a PDR phenotype in the strain (haplotype 2), however, in the case of the stop codon identified in the *mexR* gene, no activity was detected from the MexAB-OprM efflux pump that contributes to the XDR shown by these strains, which is possibly partly attributed to the production of metallo-.β-lactamases.

The contribution of the MexAB-OprM efflux pump to the resistance of *P. aeruginosa* strains was evident, as 17 out of 19 statistically significant mutations associated with MexAB-OprM efflux pump activity also correlated with resistance of the studied strains ($p < 0.05$). Some authors suggest that mutations in the *mexR, nalC*, and *nalD* genes can impair their function, favoring MexAB-OprM efflux pump overexpression with a consequent increase in bacterial resistance [6, 12, 13], however, in our study, same haplotypes (mutations) lead to different MexAB-OprM efflux pump phenotypic behavior and resistance.

The haplotype networks highlight the important relationship between ST and *mexR-nalC-nalD* haplotype in *P. aeruginosa* strains. In the first haplotype network (Fig. 1) it was observed 6 important clonal complexes made up of phylogenetically related ST and its relationship with the pump haplotype, and in the second network (Fig. 2) the relationship between ST/haplotype and resistant profiles in the *P. aeruginosa* strains is evident. These relationships are further supported by principal components analysis (Fig. 4), which shows a clear correlation between ST and resistance and between ST and *mexR-nalC-nalD* haplotype, but an inversely proportional relationship to the outcome variable (patient death) with all variables analyzed.

Haplotype 1 (*mexR-nalC-nalD*) was identified with high frequency (n = 40) (Fig. 1). All ST1725 strains presented this haplotype except one isolate classified as haplotype 2 because of a deletion (△105–116). ST111, an epidemiologically high-risk clone also presented this haplotype (although it appears to be phylogenetically distant from CC1); both haplotypes (1 and 2) were associated with XDR and PDR (Fig. 2). Haplotype 1 was also identified in other strains of different ST (ST1723, ST1730, ST2243, ST2245, ST2247, and ST2244) (Fig. 1, CC1), suggesting that these substitutions are specific to phylogenetically related ST. Likewise, the neighbor-net graph based on the MLST genotyping of the *P. aeruginosa* strains showed a strong relationship between the ST mentioned above, which suggests that these ST are highly maintained (Fig. 3). However, high-drug resistance shown by haplotype 1 strains was not significantly related to the MexAB-OprM activity, since only in 47.5% of the strains the resistance was completely attributed to this efflux pump, in 22.5% the efflux pump contribute to resistance, and in 27.5% of the strains other resistance mechanisms are suggested. Association between haplotypes and high-drug resistance may be due to potential relationship between the ST and *mexR-nalC-nalD* haplotypes, as resistance is hightly related to specific STs.

Haplotype 5 (*mexR-nalC-nalD*) was identified in phylogenetically related STs, which were highly associated with XDR (Fig. 2). The high-drug resistance exhibited by these strains could be in part attributed to the MexAB-OprM efflux pump activity (Table 1), however no significant relationship between resistance due to the pump was observed, since different behavior of the pump was noticed within haplotype 5 strains. Finally, the relationship between this haplotype and fatal patient outcomes was remarkable.

Haplotypes 8 and 12 (*mexR-nalC-nalD*) correlate closely with XDR and PDR profiles in the analyzed strains (Fig. 2), with few strains exhibiting contribution of the MexAB-OprM efflux pump to resistance (20% and 33.33%, respectively) (Table 1), suggesting the relationship between haplotypes and resistance is mainly due to the potential association between haplotypes and phylogenetically related STs, and the latter with resistance (Fig. 2). In these cases, resistance could be explained by the presence of carbapenemases, other pumps, among other mechanisms, as explained by Correa et al (2015) [21]. Interestingly, the carbapenemase types differ between strains of haplotype 8 (serine carbapenemases) and those of haplotype 12 (metallo β-lactamases) [5].

In addition, the 6 clonal complexes previously identified in the phylogenetic networks stand out as groups of highly maintained STs in the neighbor-net graph (Fig. 3): CCI with haplotype 1 strains (mainly ST1725) associated with XDR and PDR. CCII with haplotype 5 strains (ST1724, ST1726, ST1727, and ST1728) were associated with XDR and fatal outcomes (Fig. 2, CC II). CCIII with haplotype 26 strains (ST540, ST2250, ST2251, and ST2564) were identified in environmental sites and in a nosocomial strain associated with a fatal outcome (Fig. 2, CC III) suggesting that nosocomial strains might originate from environmental strains, and once in hospitals, can persist for extended periods due to their highly adaptive features and that haplotypes are highly maintained within phylogenetically related STs no matter what environment they came from. CCIV with haplotype 12 strains, characterized by non-synonymous substitutions in the *nalC* repressor gene (Table 3), correlates closely with the XDR phenotype and ST233 (epidemiologically high-risk clone), ST2559, and ST2560 (Fig. 1, CCIV). CCV with haplotype 21 strains (ST1737 and ST561) both MDR. CCVI with haplotype 8 strains (ST2704, ST2710, ST2716, and ST2731) strongly associated with XDR and PDR in nosocomial strains (CCVI, Fig. 1).

According to the neighbor-net graph, CCII (Haplotype 5) and CCIII (Haplotype 26) appear to be closely associated with the CC1 (Haplotype 1), and distant from CCIV (Haplotype 12) and CCVI (Haplotype 8) (related to production of carbapenemases) which are closely related, indicating that despite the highly recombinant nature of *P. aeruginosa*, some substitutions are highly maintained among the strains (CCs) (Fig. 3, Table 3). This close relationship between specific ST that differs little in sequence (CCs) is troubling, as it raises the possibility of the eventual selection of an efficient epidemiologically high-risk clone with high dissemination capacity.

Conclusions

In this study, a significant relationship between ST and resistant profiles was observed. The *mexR-nalC-nalD* haplotypes were not related to the MexAB-OprM efflux pump phenotypic behavior, however activity of this pump was most evident in XDR and PDR strains. In addition, there was a significant relationship between *mexR-nalC-nalD* haplotypes and phylogenetically related ST, suggesting mutations in these repressors are highly maintained within these STs.

The haplotypes 1, 5, 8, and 12 stand out for their frequency, strong relationship with resistance and their association with outstanding STs, and should be under supervision, specially haplotype 5 which was closely related to the death outcome of the patients, however it should be noted that patients' underlying conditions were not considered in this study.

The MexAB-OprM efflux pump is one of the most important mechanisms, to whom *P. aeruginosa* resistance is attributed; however not in all strains analyzed in this study, the resistance is entirely caused by this pump, in many cases the MexAB-OprM pump contributes to resistance, but other resistance mechanisms must be taken into consideration in the *P. aeruginosa* strains high-drug resistance.

Methods

This study aims to identify point mutations in the regulatory *mexR, nalC,* and *nalD* genes of the MexAB-OprM efflux pump and their associations with antibiotic resistance and sequence type in clinical and epidemiologically high-risk clones of *P. aeruginosa*.

Previously, 58 MDR, XDR, and PDR strains of *P. aeruginosa* were collected from patients at the Hospital Infantil de México Federico Gomez, a level 3 health care institute. Responsible for a high mortality rate in patients during 2007–2013, these strains were biochemically and molecularly characterized by Aguilar-Rodea and colleagues (2017) [19]. These 58 previously analyzed strains plus 33 clinical and environmental isolated strains from 2014 to 2015 were analyzed in this study.

In total, 91 *P. aeruginosa* strains were analyzed: 77 strains of nosocomial origin, isolated at the Central Laboratory of the Hospital Infantil de México Federico Gomez during 2007-2015, and 14 environmental strains isolated from soil, water, and plants by the Aerobiology Laboratory at the Centro de Ciencias de la Atmósfera, UNAM during 2014. Strains were taxonomically identified using the automated system MALDI-TOF (Biomerieux Marcy l'Etoile, France). All *P. aeruginosa* strains were cultured on blood agar plates at 37ºC for 24h.

The following assessments of *P. aeruginosa* phenotypes were conducted: observation of macroscopic and microscopic morphology, oxidase and catalase tests, hemolysis test in blood agar, pigment production in Mueller Hinton agar (pyocyanin and pyoverdine), odor, Kliger biochemical test, and growth in cetrimide agar at 42ºC [48].

Susceptibility profiles

The susceptibility profiles of 58 *P. aeruginosa* strains were reported by Aguilar-Rodea *et al*., 2017 [19]. For the remaining 33strains, the susceptibility profiles were determined according to the minimal inhibitory concentration (MIC) for 9 different antibiotic categories [28] using the agar dilution method described by the Clinical and laboratory Standards Institute (CLSI) (2019) [27]. The classes and types of antibiotics tested are as follows: 1) Aminoglycosides: gentamicin (GEN), tobramycin (TOB), and amikacin (AK); 2) Carbapenems: imipenem (IMI), meropenem, (MEM); 3) Cephalosporins: ceftazidime (CAZ), cefepime (CPM); 4) Fluoroquinolones: ciprofloxacin (CIP), levofloxacin (LEV); 5) Penicillins: carbenicillin (CB); 6) Penicillin + β-lactamase inhibitors: piperacillin/tazobactam (P/T); 7) Monobactams: aztreonam (AZT); 8) Phosphonic acids: fosfomycin (FOS); and 9) Polymyxins: colistin (CS). All the antibiotics used were pure salts (Sigma-Aldrich, St. Louis, M.O). The reference strains used for validation of the technique included *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 (American Type Culture Collection, Manassas, VA, USA). The MIC interpretative criteria (µg/mL) for *P. aeruginosa* strains are as follows: GEN: S ≤4, I 8, R ≥16; TOB: S ≤4, I 8, R ≥16; AK: S ≤16, I 32, R ≥64; IMI: S ≤2, I 4, R ≥8; MEM: S ≤2, I 4, R ≥8; CAZ: S ≤8, I 16, R ≥32; CPM: S ≤8, I 16, R ≥32; CIP: S ≤0.5, I 1, R ≥2; LEV: S ≤1, I 2, R ≥4; CB: No values reported; P/T: S ≤16/4, I 32/4-64/4, R ≥128/4; AZT: S ≤8, I 16, R ≥32; FOS: No values reported; CS: S ≤2, R ≥4.

The strains were classified as follows: sensitive (S), resistant (R), multidrug resistant (MDR), extensively drug resistant (XDR), and pan drug resistant (PDR), according to the CLSI (2019) breakpoints and the criteria described by Magiorakos *et al.,* 2012 [27, 28]. The following definitions were used to categorize *P. aeruginosa* strains as MDR, XDR, or PDR: MDR, non-susceptible to at least one agent in 3 or more antimicrobial categories; XDR, non-susceptible to at least one agent in all but 2 or fewer antimicrobial categories; PDR, non-susceptible to all agents in all antimicrobial categories.

MexAB-OprM efflux pump phenotype determination

Phenotypic activity of the MexAB-OprM efflux pump was assessed in the 91 *P. aeruginosa* strains and confirmed as a 4-fold decrease in MIC value for carbenicillin (CB) in the presence of Phe-Arg-β-naphthylamine (PaβN) inhibitor relative to that in the absence of the inhibitor. For this technique, the MexB-specific substrate CB was used as the reporter antibiotic (specific MexB substrate) [43, 44]. The MIC was determined for each strain in the absence/presence of the efflux inhibitor PaβN (50 mg/L). At this concentration, the inhibitor completely restored the susceptibility of the control strain to the reporter antibiotic and did not inhibit bacterial growth [44]. PaβN had no significant effect on carbenicillin (MexB substrate). *P. aeruginosa* ATCC 27853 (American Type Culture Collection, Manassas, VA) was used to confirm theresults. The MexAB-OprM efflux pump was considered as the most likely cause of the elevation of the MIC (+) if the MIC value for **CB -PaβN** was at least 2 $\log_2$ dilutions higher than in the wild type strain (PAO1: MIC 64 µg/ml), and the MIC for **CB +PaβN** was lower than the measured in the wild type strain (PAO1: MIC 64 µg/ml) or + 1 dilution; if the MIC values for **CB +PaβN** remained elevated compared with the wild type strain, the MexAB-OprM efflux pump was contributing in the elevation of the MIC (*); and if there was a difference of 1 dilution between **CB -PaβN** and **CB +PaβN** or no difference, the MexAB-OprM efflux pump was not the cause of the elevation of the MIC (-).

Identification of carbapenemase-producing *P. aeruginosa*

Carbapenemase-producing *P. aeruginosa* were screened using the phenotypic technique βCARBA Test (BIO-RAD, France). Rapid detection of carbapenemase-producing strains is a qualitative colorimetric test used to detect strains with decreased susceptibility to carbapenems due to carbapenemase production. This assessment is based on the color change of a pH indicator following hydrolysis of the β-lactam ring in carbapenem. For this test, all carbapenem-resistant isolates were incubated at 37ºC for 24 h on Mueller–Hinton agar plates. Isolated colonies were recovered on a calibrated loop (10 µL), resuspended in a bacterial protein extraction reagent, and further incubated for 30 min at 37ºC. Red color indicates a positive test, while no color change indicates negative. *Escherichia coli* ATCC 35218 was used as negative control, and *Klebsiella pneumoniae* NCTC 13438 (KPC-3 carbapenemase) served as the positive control [49].

In carbapenem-positive strains, serine carbapenemase and metallo-β-lactamase were evaluated using the modified carbapenem inactivation method (mCIM) and the EDTA-modified carbapenem inactivation method (eCIM) as described by the CLSI, 2019 [27].

For the mCIM test, a 10-µL loop of *P. aeruginosa* from an overnight blood agar plate was resuspended in 2 mL of Mueller Hinton broth, and a 10-µg meropenem disk was added following incubation for 4 h at 37ºC. A suspension of *E. coli* ATCC 25922 (0.5 McFarland, meropenem susceptible MIC ≤ 2 µg/mL) then was inoculated onto a Mueller–Hinton plate, and the previously-treated meropenem disk was added. For the eCIM test, 2 mL Mueller–Hinton broth with *P. aeruginosa* was also prepared, and a 10-µg meropenem disk with 20 µL of 0.5 M EDTA was added and incubated for 4 h at 37ºC. Both meropenem disks were placed on one Mueller–Hinton plate inoculated with the meropenem susceptible reference strain and incubated again for 24 h at 37ºC. The inhibition zones were measured and interpreted using the following guidelines established by CLSI, 2019 [27]. mCIM: carbapenemase^{+}, meropenem hydrolyzes and does not inhibit the growth of *E. coli* ATCC 25922; carbapenemase^{-}, meropenem inhibits the growth of E. coli ATCC 25922; eCIM: metallo-β-lactamase^{+}, carbapenemase activity is inhibited by EDTA, meropenem is not hydrolyzed, resulting in inhibition of *E. coli* ATCC25922 growth; serine carbapenemase^{+}, carbapenemase activity is not affected by EDTA, and *E. coli* ATCC 25922 growth is not inhibited.

Bacterial DNA isolation and quantification

p.aeruginosa was cultured in Mueller Hinton broth at 37ºC for 18–20h. Chromosomal DNA was isolated using the Wizard Genomic DNA purification kit (Promega, USA), following the manufacturer´s protocol. DNA quality, integrity, and concentration were confirmed by agarose (1%) gel electrophoresis. DNA concentration and purity were evaluated using an EPOCH spectrophotometer (Biotek, Vermont, USA). High quality DNA was stored at 4ºC until used.

Genotyping via Multilocus Sequence Typing (MLST)

The ST of 58 of the *P. aeruginosa* strains was previously determined [19]; for the remaining 33 strains, the same genotyping procedure via MLST was performed. Nested PCR for the metabolic genes *acsA, aroE, guaA, mutL, nuoD*, *ppsA,* and *trpE* was carried out using the primers described by Curran *et al.,* 2004 [50]. Sequencing of the PCR products was performed in both senses. The obtained sequences were edited and aligned as previously described. The ST of each strain was obtained by BLAST analysis (nucleotide) of each gene compared with the *P. aeruginosa* MLST database [51], http://pubmlst.org/paeruginosa/. The new STs were deposited in the *P. aeruginosa* MLST data base. Variability parameters were determined as previously described.

Identification of mutations in the MexAB-OprM efflux pump repressor genes *mexR, nalC,* and *nalD*

The *mexR*, *nalC*, and *nalD* repressor genes were amplified by PCR in a Thermo Hybaid Thermal cycler (PCR Express, California). The following primers were designed using the Primer3 program [52], http://bioinfo.ut.ee/primer3-0.4.0/. *mexR* (597 bp): F´5´-CAGTAAGCGGATACCTGAAAC-3´, R´5´-GGTTGATAAGGTCAACTAAAATAAGC-3´; *nalC* (998 bp): F´5´-GAAACGCTCTCAGCAAACC-3´, R´5´-CACCGAGATCCACCTCAC-3´; and *nalD* (1,035 bp): F´5´-GCATTAGACAAAGGTGGTGTCG-3´, R´5´-GGCAATACCATGCAAGTTTTCAA-3´. All genes were amplified under the following conditions: 96ºC initial denaturation for 1 min, followed by 30 cycles: 96ºC denaturation 1 min, alignment (*mexR*: 57 °C, *nalC*: 57.7 °C and *nalD*: 57 °C) for 1 min, 72ºC extension 1 min and a 72ºC final extension for 5 min. PCR products were purified using the ExoSap IT enzyme (Affymetrix, Cleveland OH, USA) according to the manufacturer's instructions. PCR products were preserved at −20ºC.

PCR products were sequenced in both senses using the primers for each gene described above using a Genetic Analyzer 310 sequencer (Applied Biosystems, Foster City, California, USA). Sequence analysis was performed using ClustalW ver. 2.0 [53], http://www.clustal.org/clustal2/, Seaview ver. 4 [54], http://pbil.univ-lyon1.fr/software/seaview.html and FinchTV ver. 1.4.0 [55], http://www.softpedia.com/get/Science-CAD/FinchTV.shtml. *P. aeruginosa* PAO1 was used as reference. Nucleotide sequences were translated into amino acid sequences to determine the variability parameters (ratio of non-synonymous to synonymous substitutions, sites of mutational changes, and polymorphisms) using DnaSP ver 5.10.01 [56], http://www.softpedia.com/get/Science-CAD/DnaSP.shtml.

Phylogenetic Analysis

The phylogenetic relationship and evolutionary history of the 91 nosocomial and environmental *P. aeruginosa* strains were evaluated by the construction of a phylogenetic network using maximum likelihood. A minimum-spanning tree was built from the MLST (ST) sequences using the GrapeTree (visualization of genomic relationships) [57] and PhyloViz Online (visualization and phylogenetic inference) [58], https://online.phyloviz.net/index softwares. In addition,

groups of related STs (clonal complexes) were identified using the BURST analysis. A group of related STs was defined as a profile match at n-2 loci to any other member of the group (n= number of loci in the scheme, MLST=7); default settings were used to achieve the most stringent definition. The GrapeTree, PhyloViz Online and BURST analysis softwares are available at the *Pseudomonas aeruginosa* PubMLST database [51], http://pubmlst.org/paeruginosa/.

Furthermore, to determine the evolutionary relationships and events of recombination between the STs a phylogenetic network was built from the MLST (ST) of the *P. aeruginosa* strains using the neighbor-net algorithm (distance-based-method) implemented in SplitsTree ver.4.0. [59]. The robustness of the network was calculated with a bootstrap test after 1000 pseudo replicates and the inference of recombination events during the generation of allelic variation was estimated with the pairwise homoplasy index test (PHI).

Genetic Diversity

For each of the MLST and MexAB-OprM efflux pump repressor genes, the number and frequency of haplotypes was determined, as well as the estimated nucleotide diversity, including the nucleotide diversity per site (Pi) and expected variation per site assuming a neutral evolution (Eta). The number of substitutions (S) for each gene is reported as well. All data were obtained using DnaSP ver 5.10.01 [56]. The DnaSP program allows the analysis of DNA polymorphisms using data from several loci by estimating several measures of DNA sequence variation within and between populations.

Statistical Analysis

The nature of the data determined the type of statistical analysis used. Qualitative variables defined subgroups of the total cohort; therefore, associations between variables required the construction of contingency tables to identify association patterns from the counts within these values. Statistical significance was considered as p <0.05 as determined by Fisher´s exact test using STATA/MP 14.1 [60]. To investigate the effects of explanatory variables on a binary response variable we used logistic regression models, while for a categorical dependent variable with outcomes that have no natural ordering, multinomial logit models were used. All procedures were done with the STATA/MP 14.1 program [60].

For principal component analysis (PCA), a graphic was constructed using RStudio software [61], http://www.rstudio.com/ to evaluate the relationships between variables (outcome, resistance, ST, MexAB-OprM haplotype, site, year, and ward). Through: (1) determining the covariance matrix of the normalized data, (2) finding the characteristic root and the characteristic vector, (3) determining the contribution rate of the variance of the principal components, (4) removing the main components, and (5) obtaining the principal component value and the integral score.

Nucleotide sequence accession numbers

The nucleotide sequences obtained in this study were deposited in the GenBank database under the following accession numbers: mexR gene sequences, MT188163–MT188173; nalC gene sequences, MT188174–MT188190; nalD gene sequences, MT188191–MT188204.

Accession numbers for the *Pseudomonas aeruginosa* isolates used in this work are available at the public database for molecular typing: PubMLST.org. See supplementary material, Table 1.

List Of Abbreviations

ST	Sequence Type
RRR	Relative Risk Ratio
MDR	Multidrug resistance
XDR	Extensive drug resistance
PDR	Pan drug resistance
S	Sensitive
I	Intermediate resistant
R	Resistant
CLSI	Clinical and Laboratory Standards Institute
H	Nosocomial strains
A	Environmental strains
GEN	Gentamicin
TOB	Tobramycin
AK	Amikacin
IMI	Imipenem
MEM	Meropenem
CAZ	Ceftazidime
CPM	Cefepime
CIP	Ciprofloxacin
LEV	Levofloxacin
CB	Carbenicillin
P/T	Piperacillin/Tazobactam
AZT	Aztreonam
FOS	Fosfomycin
CS	Colistin
MIC	Minimal Inhibitory Concentration
PaβN	Phe-Arg-β-naphthylamine
mCIM	Modified Carbapenem Inactivation Method
eCIM	EDTA-Modified Carbapenem Inactivation Method
MLST	Multilocus Sequence Typing
Pi	Nucleotide Diversity
Eta	Theta (per site)
S	Nucleotide Substitutions
Hd	Gene Diversity

A	Alanine
C	Cysteine
D	Aspartic acid
E	Glutamic acid
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
Y	Tyrosine
CC	Clonal Complex
AT	Ancestral Type
PHI	Pairwise Homoplasy Index
PCA	Principal Component Analysis

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication.

Not applicable

Availability of data and materials

The nucleotide sequences obtained in this study were deposited in the GenBank database under the following accession numbers: mexR gene sequences, MT188163–MT188173; nalC gene sequences, MT188174–MT188190; nalD gene sequences, MT188191–MT188204.

Accession numbers for the *Pseudomonas aeruginosa* isolates used in this work are available at the public database for molecular typing: PubMLST.org. See supplementary material, Table 1.

Competing interests

The authors declared that this research was conducted in the absence of any personal, professional, commercial, or financial relationships that could potentially be construed as a conflict of interests.

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Authors' contributions

PAR and NVG conceived and planned the study. PAR, BR and MCCC performed experiments and generated the database. PAR, NVG, GZ, AR, and RC analyzed and interpreted data. GR, AG, IPO, HOC and IR reviewed and revised the manuscript. The manuscript was prepared by PAR, RC, JCVG and NVG. All authors revised and agreed on the final version of the manuscript.

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Figures

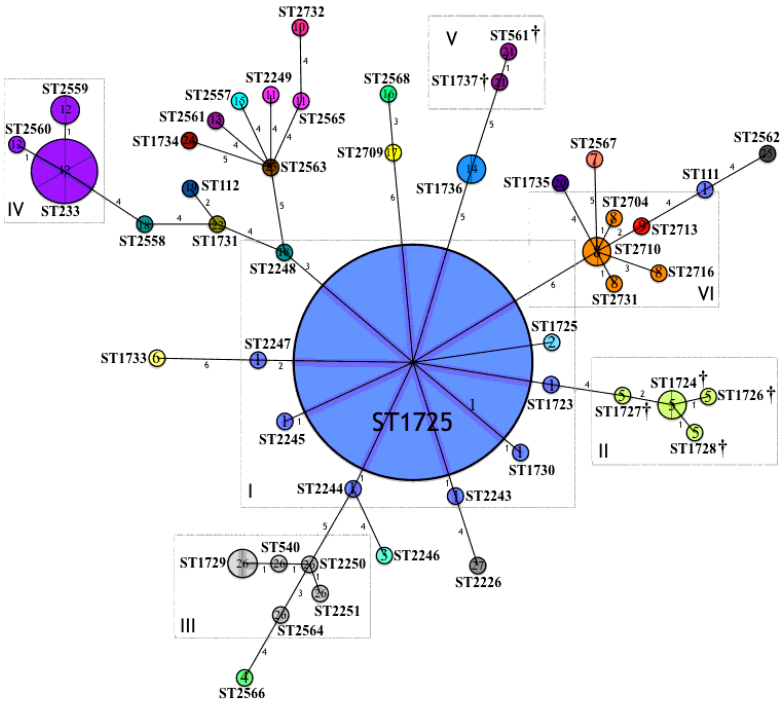

Figure 1

Phylogenetic network based on the MLST genotyping of the P. aeruginosa strains (ST/haplotypes). Phylogenetic relationships, evolutionary history, clonal complexes and relationship between mexR-nalC-nalD haplotypes and STs are shown. P. aeruginosa isolates (n= 91; 48 STs; 27 mexR-nalC-nalD haplotypes). Circles represent sequence types (STs); Circumference of the circle is based on ST frequency; Two or more strains with the same ST are depicted as fractions in each circle [in addition ST1725 (n= 34 strains)]; Lines connect locus variants; Numbers indicate the number of locus variants among the connected STs. Clonal complexes (CC) formed are highlighted in rectangles and described as (I, II, III, IV, V and, VI).STs not grouped into a CC are considered singletons (>3 locus variants with other STs). Number inside the circles (1-27) corresponds to mexR-nalC-nalD haplotype and these are differently colored. †: Fatal patient outcome.

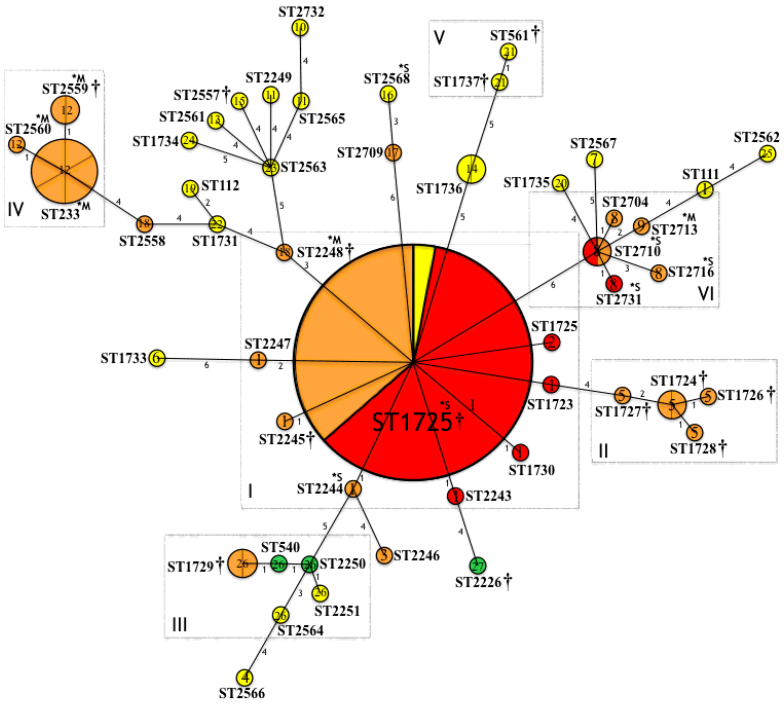

Figure 2

Phylogenetic network based on the MLST genotyping of the P. aeruginosa strains (ST/haplotypes/resistance). Relationship between mexR-nalC-nalD haplotypes, STs and susceptibility profiles are shown. P. aeruginosa isolates (n= 91; 48 STs; 27 mexR-nalC-nalD haplotypes). Circles represent sequence types (STs); Circumference of the circle is based on ST frequency; Two or more strains with the same ST are depicted as fractions in each circle (in addition ST1725 (n= 34 strains)); Lines connect locus variants; Numbers indicate the number of locus variants among the connected STs. Clonal complexes (CC) formed are highlighted in rectangles and described as (I, II, III, IV, V and, VI). STs not grouped into a CC are considered singletons (>3 locus variants with other STs). Number inside circles (1-27) corresponds to mexR-nalC-nalD haplotype and these are differently colored. †: Fatal patient outcome. Susceptibility profiles: S (green), sensitive; MDR (yellow), multidrug resistant; XDR (orange), extensively drug resistant; PDR (red), pan drug resistant. *S: Serine carbapenemase; M*: Metallo-b-lactamase.

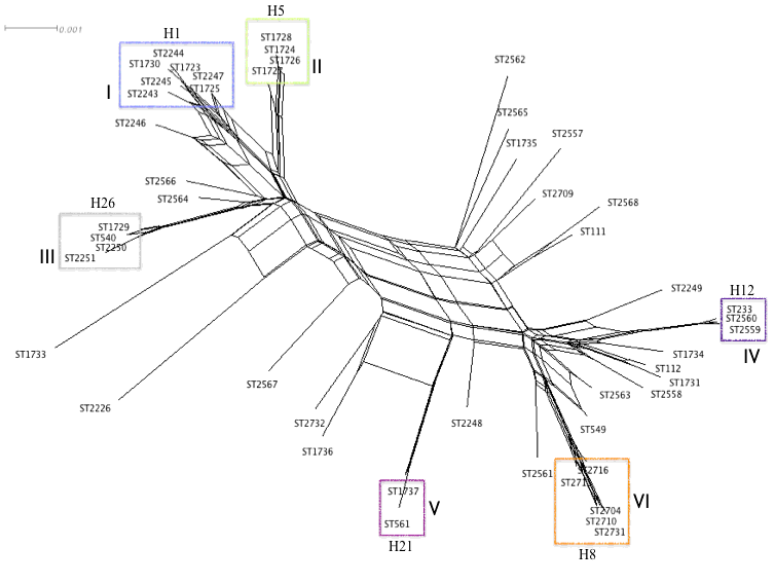

Figure 3

Neighbor-net graph based on the MLST genotyping of the P. aeruginosa strains. P. aeruginosa isolates (48 STs). Rectangular boxes represent the high probability of extensive homologous recombination. The PHI test detected statistically significant evidence of recombination (p = 0.0). Clonal complexes (CC) formed are highlighted in rectangles and described as (I, II, III, IV, V, and VI). The mexR-nalC-nalD haplotype of each CC is shown.

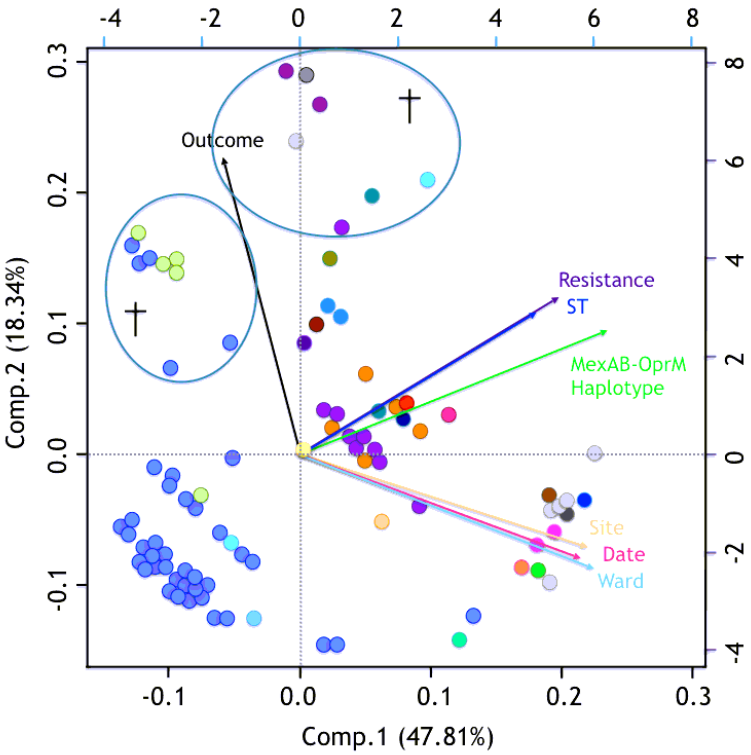

Figure 4

Principal component analysis of the 91 P. aeruginosa strains. P. aeruginosa isolates (n=91). Each strain is represented by a colored dot according to mexR-nalC-nalD haplotype (Figure 1). †: Fatal patient outcome. Variables included: Outcome (black vector): living, died; Resistance (purple vector): sensitive (S), multi-drug resistant (MDR), extensively drug resistant (XDR), and pan drug resistant (PDR); ST (blue vector) (n = 48); MexAB-OprM haplotype (mexR-nalC-nalD) (green vector) (n = 27); Isolation site (yellow vector): hospital, environmental; Date (pink vector): 2007–2013; Hospital ward (aqua vector): Stx, surgical therapy; E, emergency room; N, neurology; PICU, pediatric intensive care unit; P, pediatrics; S, surgery; Neph, nephrology; O, oncology; It, internal therapy; C, cardiology; NICU, neonatal intensive care unit; Ne, necropsy; IMed, internal medicine.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable2.docx
- SupplementaryTable1..docx