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***Pseudomonas aeruginosa* isolates from a cohort of Mexican children with CF show adaptation to a chronic phenotype**

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Key words: Cystic fibrosis, bacterial pathogens, antimicrobial resistance, polymyxin resistance, virulence factors

Abbreviated title: *P. aeruginosa* from children with CF are chronicity-inducers

Running head title: *P. aeruginosa* in Mexican children with CF

Abstract

Background: Long-term persistence of *Pseudomonas aeruginosa* in the lung of individuals with cystic fibrosis is associated with progressive selection of diverse genotypes and phenotypes. This bacterial adaptation leads to chronic infection, and increased morbidity and mortality. The aim of this study was to establish the prevalence, clonal relatedness, antimicrobial susceptibility and virulence-associated phenotypes of *P. aeruginosa* isolates in a cohort of 50 Mexican children with CF-associated chronic lung infection.

Methods: Clonal relatedness of *P. aeruginosa* isolates was verified by pulsed-field gel electrophoresis. The antimicrobial susceptibility was determined by an automated VITEK 2 system and/or broth micro-dilution method. Biofilm formation was quantified by the crystal violet method; swarming motility was measured on soft agar, and susceptibility to normal human serum determined by reduction of CFUs.

Results: High prevalence of *P. aeruginosa* colonization among Mexican children with CF was confirmed; 20% (10/49) of clones identified showed a multidrug-resistant phenotype and 8.2% (4/49) an extensive drug resistance phenotype; 26.5% (13/49) of the isolates were resistant to colistin, 42.9% (21/49) presented a phenotype of adaptation associated with chronic infection, and 79.6% (39/49) showed increased ability to survive in normal human serum.

Conclusions: This cohort of children with cystic fibrosis reveals that colonizing *P. aeruginosa* strains predominantly display resistance to several first-line antibiotics although most isolates were susceptible to meropenem and tobramycin; 42.9% of isolates showed a phenotype consistent with adaptation to chronic lung infection.

1 **Introduction**

2 Cystic fibrosis (CF) is an autosomal recessive disease¹ characterized by multiple organ
3 dysfunction including the lung, gastrointestinal tract, liver, pancreas and male reproductive
4 organs.² Organ dysfunction is associated with mutations in the cystic fibrosis transmembrane
5 conductance regulator (*cfr*) gene³, which modulates chloride, sodium, HCO₃ and water
6 transport across epithelial membranes.⁴ In particular, CF lungs have a defective mucociliary
7 clearance that results in airway obstruction by production of thick and dehydrated
8 secretions,^{2,5} which in turn leads to chronic bacterial infections.⁶ Chronic infection and
9 persistent inflammation gradually induce structural lung damage.⁶ Early in life, the lungs of
10 affected children are initially colonized by *Haemophilus influenzae* and *Staphylococcus*
11 *aureus*⁶⁻¹⁰ and progressively acquire *P. aeruginosa*, which increases from 10-30% at ages 0-
12 5 years to 80% at ages ≥18 years.¹¹ With less frequency, the lungs of individuals with CF are
13 colonized by members of the *Burkholderia cepacia* complex, *Achromobacter xylosoxidans*,
14 *Stenotrophomonas maltophilia* and non-tuberculous mycobacteria.¹² Although the true
15 incidence of CF in Mexico is unknown, the Mexican Cystic Fibrosis Society¹³ estimates that
16 350 children with CF are born every year. Unfortunately, only 15% of them are detected
17 before the first four years of life, and the others die due to respiratory complications and
18 malnutrition. The life expectancy of children with CF in Mexico is 19 years. Until now, the
19 prevalence of bacterial pathogens associated to pediatric patients with CF was unknown. In
20 this study, we report a cohort of Mexican children with CF-associated lung disease that are
21 mainly colonized by strains of *P. aeruginosa* with phenotypes consistent with bacterial
22 adaptation to chronic infection.

Material and Methods

Sample collection and bacterial identification

This study was approved by the Institutional Review Board (IRB) of the School of Medicine at UNAM (Protocol FMED/CI/RGG/022/2016). Bacterial strains were isolated from lung infections in 50 subjects (0 to 18 years of age) that were diagnosed with CF by screening of the immunoreactive trypsinogen and by sweat chloride test and followed at the CF clinic at “Hospital Infantil de México Federico Gómez” (HIMFG), Mexico City, Mexico from August 2016 to January 2018. The parents of the children that accepted to participate in the study signed a letter of consent authorizing the collection of samples and the laboratory procedures to identify bacterial pathogens. The staff of the CF clinic collected information on each patient including name, age, sex, place of residency, pulmonary function and pancreatic function, sweat chloride test, and mutations associated to *cfr* gene status. Patient samples were collected as part of routine care based on scheduled appointments every 3-6 months (however, some patients do not always assist to their scheduled appointments at the CF clinic). Samples were handled anonymously. Sputum samples or cough swabs (children of <2 years of age) were transported to our laboratory and processed immediately. Briefly, sputum samples were dissolved with sputolysin weight/volume (1:1) (Merk-Millipore) during 30 min at 37°C. Dissolved sputum and cough swabs were subsequently inoculated onto MacConkey, salt and mannitol, chocolate, blood (DIBICO) and cetrimide (Becton Dickinson) agar media plates. Inoculated plates were incubated aerobically at 37°C for up 48 h. Chocolate and blood agar plates were also incubated under microaerophilic conditions by 24 h at 37°C. Initial bacterial identification was performed by standard microbiological

1 methods, and the species confirmed with the API20NE (bioMérieux®SA) system. All
2 samples were stored at -70°C until analysis.

4 **Pulsed-field gel electrophoresis (PFGE)**

5 Pulsed-field gel electrophoresis (PFGE) was used to genotype bacterial isolates. Bacterial
6 genomic DNA was prepared as previously described.¹⁴ For *P. aeruginosa*, the genomic DNA
7 was digested with *SpeI*, *XbaI* (Invitrogen) and *ApaI* (New England Biolabs, every, MA).
8 Digested DNAs were resolved by PFGE analysis using a Gene Path system (BioRad®). The
9 relatedness between clinical isolates was determined using the Tenover criteria¹⁵ and Dice
10 coefficient.¹⁶ Every isolate yielding a correlation >85% was deemed as member of the same
11 clone.

13 **Antimicrobial susceptibility testing**

14 *P. aeruginosa* isolates were tested for susceptibility to amikacin, aztreonam, cefazolin,
15 cefepime, gentamicin, meropenem, piperacillin/tazobactam and tobramycin using the
16 VITEK 2 system (bioMérieux®SA). The susceptibility to colistin and polymyxin B was
17 determined by broth microdilution according to Clinical Laboratory Standards Institute
18 (CLSI) guidelines 2019.¹⁷ *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922
19 were used as controls.

21 **Pigment and alginate production**

Pigment and alginate production by *P. aeruginosa* isolates were determined on cetrimide agar as described.¹⁸ Alginate production was observed on cetrimide or MacConkey agar plates.

Biofilm assays

Biofilm production was evaluated on polystyrene tubes, as previously described.¹⁴ *P. aeruginosa* PAO1, kindly donated by Dr. Maria del Rosario Morales-Espinoza from the “Facultad de Medicina, Universidad Nacional Autónoma de México”, was used as reference strain.¹⁹

Motility assays

For swarming motility, *P. aeruginosa* clinical isolates were grown in 5 ml Luria broth (LB) at 37°C, 180 RPM for 24 h. Bacterial cultures were adjusted to 1×10^7 cells/ml in fresh LB. Ten- μ l aliquots were carefully inoculated on a semi-solid LB plates containing 0.3 % of agar (Bacto), once the agar plates with bacteria was dried, the plates were incubated at 37°C during exactly 24 h. The diameter of the motility zone was measured. *Acinetobacter baumannii* ATCC 17965 and *Salmonella typhimurium* ATCC 14028 were used as non-motile and motile control strains, respectively.

Serum resistance assay

Survival of *P. aeruginosa* isolates in normal human serum (NHS) was evaluated as previously reported.¹⁴ Briefly, every *P. aeruginosa* isolate was grown in Müller Hinton (MH) broth overnight. The bacterial culture was adjusted to 1×10^7 cells/ml using 40% of normal

human serum (NHS) (Cedarlane Laboratories Limited, Ontario, Canada) or with 40% of heat-inactivated NHS (HIS). Samples were incubated during 3 h at 37°C without shaking. After incubation, the number of surviving bacteria in each sample was determined by serial dilution and plating in MH-agar and incubated at 37°C for 18 h. The survival of *P. aeruginosa* to NHS activity was calculated with the formula: % survival = (CFUs-NHS / CFUs-HIS) *100. The efficiency of NHS and HIS were assessed with *Salmonella enterica* Seroovar Typhi 9:12:Vid as a sensitive strain to NHS.

Results

Prevalence of *P. aeruginosa* in pediatric individuals with Cystic Fibrosis

We enrolled 50 subjects (0-18 years of age) attending the CF clinic; 52.0% (26/50) were males. Thirty individuals of this cohort had documented mutations within the *cfr* gene, while the other 20 did not have a sequenced *cfr*. Of sequenced genes, 26.6% (8/30) carried the most frequent homozygotic mutation: ΔF508, followed by 36.6% (11/30) carrying ΔF508/x, 10.0% (3/30) ΔF508/other mutation and 26.6% (8/30) carrying other types of mutations (Table 1). Twenty-one patients were chronically infected by *P. aeruginosa* at the time of enrollment (defined as at least three of six positive cultures over one year), 13 had a history of intermittent infection (defined as one of six positive culture), and 16 did not have a positive culture (Table 2, Supplementary Figure 1A). Two patients died during the study period. The patients with chronic infection and lower lung function were older (Table 2, Supplementary Figure 1A and 1B). Further, during the study, we detected 10/16 patients with good lung function in whom *P. aeruginosa* had not been previously identified (Table 2 and Supplementary Figure 1A and 1B).

We analyzed 106 sputum samples collected during the medical assessment (50 were obtained at time of patient enrollment, 33 from a second appointment, 19 from a third and 3 from a fourth appointment, respectively). We identified the presence of one bacterial pathogen in 50.9% (54/106) samples, two bacterial pathogens in 29.2% (31/106), three in 6.6% (7/106) and four in 1.9% (2/106). No pathogenic bacteria were isolated in 11.3% (12/106) samples. We identified 160 bacterial species; 47.5% were *P. aeruginosa*, 31.9% *Staphylococcus aureus*, 9.4% *Escherichia coli*, 1.9% coagulase-negative Staphylococci, 1.9% group A *Streptococcus*, 1.3% group B *Streptococcus*, 1.3% *Serratia marcescens*, 1.3% *Klebsiella pneumoniae*, 1.3% *Klebsiella oxytoca*, 1.3% *Enterobacter cloacae*, 0.6% group C *Streptococcus* and 0.6% *Burkholderia cepacia* (Figure 1A and Table 2). *P. aeruginosa* was the most frequently isolated pathogen from samples of individuals ranging from 0-18 years of age, and *Staphylococcus aureus* was the second most frequent pathogen in individuals ranging from 6-12 years of age (Figure 1A and 1B).

Genotyping of *P. aeruginosa* isolates

we analyzed 76 *P. aeruginosa* isolates by PFGE to investigate the genetic relatedness of isolates and determine a possible clonal dissemination within individuals attending the HIMFG (Figure 2). Forty-nine unrelated clones were identified, indicating unique colonization events, while one of the isolates was non-PFGE typeable. Of the related clones present in different individuals, *Psa01* was isolated from individual CF002 and CF003, *Psa02* from CF003 and CF014, and *Psa17* from CF013 and CF028. Twelve subjects (24%) were colonized by different clones of *P. aeruginosa*. For example, individual CF002 was colonized by *Psa01* and *Psa18*, CF003 by *Psa01*, *Psa02* and *Psa40*, whereas individual

CF008 was colonized by strains *Psa05* and *Psa34*. The rest of combinations are presented in Figure 2.

Antibiotic susceptibility to *P. aeruginosa* isolates

One representative member of each *P. aeruginosa* PFGE clone was assessed for the antimicrobial susceptibility. We did not consider in this analysis the intrinsic resistance exhibited by *P. aeruginosa*.¹⁷ Twenty % (10/49) of these clones presented a multidrug-resistance (MDR) phenotype (non-susceptible to $\geq$ one agent in $\geq$ three antimicrobial categories)²⁰ whereas 8.1% (4/49) showed an extensively drug resistance (XDR) phenotype (non-susceptible to $\geq$ one agent in all but $\geq$ two antimicrobial categories)²⁰ (Table 3 and Supplementary Figure 1). We also found that 26.5% (13/49) of strains were resistant to the last resort antibiotic colistin and 10.2% (5/49) to polymyxin B.

Adaptation to a chronic infection phenotype by clones of *P. aeruginosa*

During the course of lung infection in people with CF, *P. aeruginosa* undergoes adaptive changes including reduced flagella and pili expression, reduced production and release of some proteases and pyoverdine, and downregulation of the type 3 secretion system.²¹ Other changes involve overexpression of alginate, increased biofilm formation, and increased antibiotic resistance.²¹ These changes are characteristic of *P. aeruginosa* strains establishing chronic infections that persist until the end of the patient's life.²² In Table 3, we show that 42.9% (21/49) of representative members of *P. aeruginosa* clones were high biofilm producers, 48.9% (24/49) of them produced moderated biofilm and 8.2% (4/49) were weak biofilm producers. The quantification of bacterial motility shows that only 10.2% (5/49) were non-motile, 81.6% (40/49) had reduced motility and only 8.2% (4/49) were motile. 34.7%

(17/49) of the isolates produced alginate, 69.4% (34/49) produced pyoverdine, 16.3% (8/49) pyocyanin and 8.2% (4/49) pyomelanin (Table 3). Particularly, 21/49 clones; *Psa03*, *Psa05*, *Psa06*, *Psa10*, *Psa12*, *Psa13*, *Psa17*, *Psa18*, *Psa26*, *Psa28*, *Psa30*, *Psa31*, *Psa33*, *Psa34*, *Psa35*, *Psa37*, *Psa40*, *Psa42*, *Psa44*, *Psa47* and *Psa49* have at least three phenotypes associated with chronic lung infection (biofilm production, decreased motility, alginate production and MDR/XDR resistance). *PsaNT* was not included in the analysis. Clones *Psa02*, *Psa04*, *Psa07*, *Psa09*, *Psa11*, *Psa15*, *Psa16*, *Psa20*, *Psa21*, *Psa22*, *Psa23*, *Psa24*, *Psa25*, *Psa27*, *Psa29*, *Psa32*, *Psa36*, *Psa38*, *Psa41*, *Psa43*, *Psa45* and *Psa48* showed two phenotypes associated with chronic lung infection. Clone *Psa04* was isolated three times from the same patient, *Psa06* four times, *Psa09* two times, *Psa14* two times, *Psa17* three times in the same patient and two times in an unrelated patient, *Psa23* two times, *Psa24* two times, *Psa25* three times, *Psa31* two times, and *PsaNT* two times, suggesting these strains persisted in the lung environment allowing them to persist over the time (Figure 2). Nevertheless, clone *Psa01* was isolated three times (two times in CF002 and one in CF003) and *Psa14* (two times in CF048) that did not show a characteristic phenotype of adaptation. The remaining clones isolated showed one phenotype associated with persistence in the lung environment. In summary, 42.9% (21/49) of these clones mentioned above showed a phenotype characteristic of bacteria establishing chronic infections.

***P. aeruginosa* survives in normal human serum**

Bacteremia by *P. aeruginosa* has been demonstrated in individuals with CF.²³ To determine the ability of clones of *P. aeruginosa* isolated from our pediatric cohort to survive the bactericidal activity of normal human serum (NHS), we performed a conventional assay to determine their susceptibility to NHS.²⁴ Our results demonstrated that 28.6% (14/49) of the

clones were highly resistant to the NHS ($\geq 60.0\%$ of survival), 22.4% (11/49) showed moderated resistance ($\geq 20.0\%$ but $< 60\%$ of survival), 24.4% (12/40) showed low resistance to NHS activity ($< 20.0\%$ but $\geq 1.0\%$ of survival) and 24.4% (12/29) were highly susceptible to HNS activity ($< 1.0\%$ of survival) (Table 3 and Supplementary Figure 2).

Discussion

Prior to this study, the incidence of bacterial pathogens associated with respiratory complications in Mexican children with CF was unknown. In this study, we found that *P. aeruginosa* was the most prevalent pathogen associated with the lung micro-environment of individuals with CF. The *P. aeruginosa* adaptation to this adverse lung environment is key for bacterial persistence.²⁵ It is believed that the continuous and frequent rate of mutation leads to the adaptation of *P. aeruginosa* to the CF lung environment and results in phenotypes characteristic of chronic infection, they exhibit reduced bacterial motility, increased biofilms formation, and increased antimicrobial resistance.^{21,26} Consistent with this notion, 32% of *P. aeruginosa* clones in our study had reduced motility ($< 50\%$ of PAO1 motility), 6% were non-motile, 44% were higher biofilm producers (more than PAO1) and 34% produced alginate. However, 12.2% (6/28) of isolated clones persisted in the lung without a clear-cut chronic phenotype, suggesting that other mechanism of adaptation could be involved. The possibility also exists that these children are directly exposed in their households to *P. aeruginosa* strains and given their impaired lung activity, they become colonized. Unfortunately, two individuals (CF008 and CF014) died during this study; CF008 was infected by *Psa05* and *Psa34*, isolates that showing a classical adaptation phenotype to chronic infection (e.g., MDR, higher biofilm production, reduced motility, alginate producer but susceptible to

normal human serum), whereas CF014 was infected by *Psa02* and *Psa09*, clones with moderated adaptation phenotype (e.g., MDR, biofilm producer, non-motile, alginate producer and moderated resistance to normal human serum). These two clones were not isolated from any other patient.

Our study underscores the importance of maintaining epidemiological surveillance of the bacterial colonization in CF patients in order to avoid or limit the possible spreading of *P. aeruginosa* among these patients in the hospital environment.²⁷⁻²⁹ We found that shared clones of *P. aeruginosa* were not associated with geographic distribution of the subjects; therefore, a common source of contamination is unlikely. During clinical assessment, each CF subject is seen, one at a time, in separate examining rooms with exhaustive cleaning between visits. However, we suspect that the spreading of clones between the subjects enrolled could have occurred in common areas of the hospital in which they could gather. Bloodstream infections caused by *P. aeruginosa* may be an important risk factor associated to mortality in CF patients.^{30,31} Our results show that fourteen clones are highly resistant to anti-bacterial activity of normal human serum while nine strains presented moderate resistance. However, no correlation with lung function or antimicrobial resistance was detected. Overall, the identification of clones of *P. aeruginosa* that present phenotypes associated with chronic lung infections of pediatric people with CF help us understand pathogenic mechanisms associated with the establishment of chronic infection.

Conclusions

This is the first study reporting the bacterial colonization of Mexican children with CF and most surprisingly that 42.9% of the patients were colonized with a *P. aeruginosa* phenotype associated with chronic infection and an ability to survive the bactericidal effect of normal

human serum. Rapid bacterial detection in the respiratory secretions of newly diagnosed patients with CF is of paramount importance for the early treatment to prevent the irreversible pulmonary damage. Our study underscore the importance of prospectively characterizing the associated respiratory bacterial pathogens beyond a simple identification of genus and species. As we have clearly shown, while *P. aeruginosa* is a common colonizer, the simple phenotype does not predict its relationship with chronic lung infection. We believe that there is a need for the development of simpler diagnostic test to identify those phenotypes associated with chronic lung infections.

Figure legends

Figure 1. Frequency of bacterial pathogens isolated from sputum samples of pediatric patients with CF. The samples were analyzed for frequency of bacterial pathogens isolated (A) and by frequency of bacterial pathogens identified over a range of ages from 1-18 years of age (B).

Figure 2. Dendrogram generated from the analysis of 76 *P. aeruginosa* clinical isolates from pediatric patients with CF. Isolates were grouped in 49 different clones. One PFGE profile by clone was used to construct the dendrogram. NT, non-typeable. Frequency of isolates by clone is indicated (# isolates). The patient column indicates the patient in which a clone is isolated. Patients colonized by more than one clone are underlined. The number in parenthesis indicates the frequency of clone identification. *Clones identified in different patients. Dashed line indicates the 70% similarity level used in the cluster designation. Clustering groups are indicated.

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Conflict of interest statement

The authors have no conflict of interest.

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24 **Figure legends**

Figure 1. Frequency of bacterial pathogens isolated from sputum samples of pediatric patients with CF. The samples were analyzed for frequency of bacterial pathogens isolated (A) and by frequency of bacterial pathogens identified over a range of ages from 1-18 years of age (B).

Figure 2. Dendrogram generated from the analysis of 76 *P. aeruginosa* clinical isolates from pediatric patients with CF. Isolates were grouped in 49 different clones. One PFGE profile by clone was used to construct the dendrogram. NT, non-typeable. Frequency of isolates by clone is indicated (# isolates). The patient column indicates the patient in which a clone is isolated. Patients colonized by more than one clone are underlined. The number in parenthesis indicates the frequency of clone identification. *Clones identified in different patients. Dashed line indicates the 70% similarity level used in the cluster designation. Clustering groups are indicated.

Figure 1. Frequency of bacterial pathogens isolated from sputum samples of pediatric patients with CF.

Figure 2. Dendrogram generated from the analysis of 76 *P. aeruginosa* clinical isolates from pediatric patients with CF.

Table1. Demographic and clinical characteristics of enrolled patients.

Patient	Sex	State	Mutations (<i>cftr</i> gene)	Inhaled antibiotics	Sweat chloride (mEq/L)	Pancreatic insufficiency	Meconium ileus	Type 2 Diabetes	Malnutrition	Biomass	Smoke	Complete scheduled vaccine
CF001	F	CDMX	ΔF508/X	-	82.0						Y	Y
CF002	F	CDMX	ΔF508 / X	TOB	113.0	Y	Y	Y				Y
CF003	F	MOR	- / -	-	108.0	Y			Y			Y
CF004	M	OAX	ΔI507 / 165-G>A	TOB	93.0	Y						Y
CF005	F	CDMX	ΔF508 / S945L	-	69.0							
CF006	M	DGO	W1089X / E588X	CXM	88.0	Y			Y			Y
CF007	M	EMEX	- / -	-	85.0	Y			Y			Y
CF008†	F	EMEX	ΔF508/ΔF508	TOB	81.0	Y			Y		Y	
CF009	M	EMEX	ΔF508/ΔF508	TOB	84.0	Y			Y		Y	Y
CF010	F	CDMX	ΔF508 / A455E	AZM	81.0	Y			Y		Y	Y
CF011	M	GTO	ΔF508/ΔF508	-	83.0	Y			Y	Y		Y
CF012	M	PUE	ΔF508/ΔF508	AMK	103.0	Y			Y	Y		Y
CF013	M	CDMX	ΔF508 / X	AMX	104.0	Y				Y		
CF014†	F	CDMX	ΔF508 / X	TOB	106.0	Y			Y			Y
CF015	F	HGO	G542 / X	TOB	93.0	Y						Y
CF016	M	GTO	ΔI507 / X	TOB	95.0	Y						Y
CF017	F	EMEX	ΔF508/ΔF508	-	96.0	Y						Y
CF018	F	MOR	- / -	TOB	89.0	Y						Y
CF019	M	CDMX	ΔF508 / X	TOB	96.0	Y	Y		Y			Y
CF020	M	CDMX	- / -	TOB	98.0	Y			Y			Y
CF021	F	CDMX	ΔF508 / ΔF508	TOB	90.0	Y						Y
CF022	M	CDMX	- / -	TOB	91.0	Y			Y			Y
CF023	F	PUE	- / -	TOB	85.0	Y			Y	Y	Y	Y
CF024	M	CDMX	1154insAT (1288insTA) / 3232_3231delTG	AMK/AZM	101.0	Y			Y			Y
CF025	M	CDMX	ΔF508 / ΔF508	TOB	96.0	Y			Y		Y	Y
CF026	F	EMEX	- / -	TOB	92.0	Y			Y		Y	Y
CF027	F	EMEX	- / -	TOB	84.0	Y			Y			Y
CF028	F	EMEX	- / -	-	88.0	Y			Y			Y
CF029	M	PUE	- / -	-	83.0	Y				Y		Y
CF030	F	CDMX	ΔF508/ W1089X	TOB	93.0	Y			Y			Y
CF031	F	GTO	ΔF508 / X	TOB	105.0	Y						Y
CF032	F	EMEX	ΔF508 / X	-	82.0	Y						Y
CF033	M	QRO	ΔF508 / X	-	80.0	Y			Y	Y		Y
CF034	M	GRO	ΔF508 / X	TOB	92.0	Y				Y	Y	Y
CF035	M	EMEX	ΔF508 / ΔF508	AMK/AZM	107.0	Y						Y
CF036	M	EMEX	ΔF508 / X	-	97.0	Y	Y					Y
CF037	M	GTO	G542 / X	TOB	97.0	Y			Y		Y	
CF038	F	GTO	- / -	-	88.0	Y						Y
CF039	M	EMEX	- / -	-	83.0	Y	Y		Y		Y	Y
CF040	F	EMEX	- / -	-	86.0	Y						Y
CF041	F	MICH	G542X / X	TOB	96.0	Y	Y					Y
CF042	F	EMEX	- / -	-	100.0	Y			Y			Y
CF043	M	EMEX	- / -	-	99.0	Y						Y
CF044	F	EMEX	ΔF508/X	-	95.0	Y					Y	Y
CF045	F	EMEX	- / -	-	92.0	Y					Y	Y
CF046	M	SIN	N1303K / X	TOB	110.0	Y			Y			Y
CF047	F	PUE	- / -	TOB	65.0	Y				Y	Y	Y
CF048	F	CAMP	- / -	-	102.0	Y				Y		Y
CF049	M	MICH	- / -	TOB	106.0	Y			Y	Y		
CF050	M	CDMX	- / -	-	96.0	Y						Y

CF, Cystic fibrosis; M, male; F, female; Mexico City (CDMX), Morelos (MOR), Oaxaca (OAX), Durango (DGO), Mexico State (EMEX), Guanajuato (GTO), Puebla (PUE), Hidalgo (HGO), Querétaro (QRO), Guerrero (GRO), Michoacán (MICH), Sinaloa (SIN), Campeche (CAM); Δ, deletion; ins, insertion; del, deletion; F, phenylalanine; x, wild gene; -, non-determined; TOB, Tobramycin; CXM, Cefuroxime; AMK, Amikacin; AZM, Azithromycin; †, patient deceased.

Table2. Bacterial pathogens isolated from 106 respiratory samples of pediatric patients with CF.

Patient	Psa ^a	FEV1		Pathogens isolated			
		%	(y/m)	1 st	2 nd	3 rd	4 th
CF001	III	78.3	(10/0)	<i>Sau</i>	<u><i>Psa28</i></u> , <i>Sau</i>	<i>Sau</i> , <i>Stp-A</i>	
CF002	II	79.0	(10/0)	<i>Psa01</i> *	<u><i>Psa18</i></u>	---	
CF003	III	34.1	(12/0)	<i>Psa02</i> ^ψ	<i>Psa01</i> , <i>Sau</i>	<u><i>Psa40</i></u> , <i>Sau</i>	
CF004	II	102.0	(13/0)	---	<u><i>Psa03</i></u> , <i>Sau</i>		
CF005	I	106.7	(11/0)	---			
CF006	II	97.0	(10/0)	<i>Sau</i>	<i>Sau</i>	<i>Psa43</i> ^ψ , <i>Sau</i> , <i>Stp-A</i>	
CF007	III	40.7	(17/0)	<i>Psa04</i> ^ψ , <i>Sau</i>	<i>Psa04</i> ^ψ *, CoNS		
CF008 †	III	28.1	(10/0)	<u><i>Psa05</i></u>	<u><i>Psa34</i></u> , <i>Sau</i>		
CF009	III	68.1	(9/0)	<i>Psa06</i> ^ψ	<i>Psa06</i> ^ψ , <i>Psa22</i> ^ψ , <i>Sau</i>	<i>Psa06</i> ^ψ	<i>Psa06</i> ^ψ
CF010	II	76.8	(18/0)	<i>Sau</i>	<i>Sau</i>	<i>Psa41</i> *, <i>Sau</i>	
CF011	I	121.0	(8/0)	<i>Psa07</i> , <i>Ecoli</i>	<i>Psa07</i> , <i>Sau</i>	<i>Psa07</i> , <i>Sau</i> , <i>E.coli</i>	
CF012	III	59.9	(17/0)	<i>Psa08</i> , <i>Smarc</i>	<i>Psa24</i> ^ψ	<i>Psa24</i> ^ψ *, <i>Sau</i>	
CF013	I	117.0	(8/0)	---	<i>Sau</i>	<u><i>Psa17</i></u> *	<i>Sau</i>
CF014 †	III	33.3	(9/6)	<i>Psa09</i> ^ψ , <i>Sau</i>	<i>Psa02</i> ^ψ , <i>Sau</i> , <i>Stp-C</i>	<i>Psa09</i> ^ψ , <i>Sau</i>	
CF015	II	84.5	(17/0)	---	<u><i>Psa31</i></u> *	<i>Stp-A</i>	
CF016	I	101.0	(6/0)	<i>Smarc</i> , <i>Koxy</i> , <i>Ecoli</i> , <i>Sau</i>	<i>Psa29</i> , <i>Sau</i> , <i>Ecoli</i>	<u><i>Psa47</i></u> , <i>Sau</i> , <i>Ecoli</i>	
CF017	III	75.7	(13/0)	<u><i>Psa10</i></u>			
CF018	II	101.9	(17/5)	<i>Bcepacia</i>			
CF019	II	ND	(2/3)	<i>Sau</i> , <i>Ecoli</i> *	<u><i>Psa23</i></u> *, <i>Sau</i>	<i>Sau</i> , <i>Ecoli</i> *	<i>Psa36</i> ^ψ , <i>Sau</i>
CF020	I	ND	(0/11)	<i>Psa11</i> ^ψ	<i>Ecoli</i>		
CF021	II	94.2	(12/0)	---	---		
CF022	III	76.2	(9/0)	<i>Sau</i>	<i>Sau</i>	<i>Sau</i>	
CF023	III	71.0	(10/0)	<u><i>Psa12</i></u>	<u><i>Psa37</i></u>	<u><i>Psa42</i></u>	
CF024	III	40.7	(18/0)	<u><i>Psa13</i></u>	<u><i>Psa35</i></u>		
CF025	I	ND	(0/9)	<i>Psa15</i> ^ψ , <i>Kpn</i>	<i>Sau</i>		
CF026	II	80.7	(6/0)	<i>Ecoli</i>			
CF027	III	72.1	(18/0)	<i>Psa16</i> ^ψ			
CF028	III	62.1	(8/0)	<u><i>Psa17</i></u> *	<i>Sau</i> , <u><i>Psa17</i></u>	<i>Sau</i>	
CF029	III	76.9	(13/0)	<i>Psa19</i> , <i>Sau</i>	<i>Sau</i>		
CF030	II	ND	(2/6)	<u><i>PsaNT</i></u> *	<i>Sau</i>	<i>Sau</i>	
CF031	III	67.6	(11/0)	<i>Psa20</i> ^ψ			
CF032	II	121.1	(11/0)	<i>Sau</i>			
CF033	III	43.0	(12/0)	<i>Spt-B</i>			
CF034	III	103.0	(6/6)	<i>Psa21</i> ^ψ *, <i>Sau</i>	<i>Psa21</i> ^ψ , <i>Sau</i>		
CF035	III	18.1	(13/0)	<i>Psa25</i> ^ψ *	<i>Psa25</i> ^ψ		
CF036	I	116.2	(5/5)	<u><i>Psa26</i></u> , <i>Sau</i> , <i>Stp-B</i>			
CF037	I	ND	(1/0)	<i>Psa27</i> ^ψ			
CF038	III	56.3	(21/0)	<u><i>Psa30</i></u>			
CF039	I	ND	(0/4)	<i>Ecoli</i>	---		
CF040	I	103.7	(7/0)	---			
CF041	I	ND	(2/9)	---	<i>Sau</i>	<i>Psa39</i> , <i>Sau</i>	
CF042	II	92.8	(5/6)	<i>Psa32</i> ^ψ , <i>Sau</i>			
CF043	I	ND	(2/4)	<i>Koxy</i> , CoNS	<i>Ecoli</i> , <i>Ecloac</i>	---	
CF044	I	ND	(0/3)	<i>Ecoli</i>			
CF045	II	99.3	(7/0)	<u><i>Psa33</i></u>			
CF046	III	96.8	(5/6)	<i>Psa38</i> ^ψ	<u><i>Psa49</i></u>		
CF047	I	89.7	(6/5)	CoNS			
CF048	III	68.2	(17/0)	<i>Psa14</i> *, <i>Sau</i>	<u><i>Psa44</i></u> , <i>Sau</i>		
CF049	I	ND	(1/1)	<i>Psa45</i>	<i>Psa48</i> ^ψ , <i>Sau</i>		
CF050	I	ND	(0/8)	<i>Psa46</i> , <i>Ecoli</i> , <i>Ecloac</i> , <i>Kpn</i>			

CF, Cystic Fibrosis; *Sau*, *Staphylococcus aureus*; *Psa*, *Pseudomonas aeruginosa*; *Stp-A*, *Streptococcus* group A; *Stp-B*, *Streptococcus* group B; *Stp-C*, *Streptococcus* group C; CoNS, Coagulase Negative *Staphylococcus*; *Ecoli*, *Escherichia coli*; *Smarc*, *Serratia marcescens*; *Koxy*, *Klebsiella oxytoca*; *Bcepacia*, *Burkholderia cepacia*; *Kpn*, *Klebsiella pneumoniae*; *Ecloac*, *Enterobacter cloacae*; NT, non-typeable; ---, None bacterial pathogen isolated, *Two different colonies that belongs to the same clone; †, patient deceased; I, non-colonized; II, Intermittent colonization; III, Chronical colonization. FEV1, Forced Expiratory Volume.; (y/m), age (year/month). ^ψtwo phenotypes associated to chronical infection; underlined, three or four phenotypes associated with chronic infections. Bold, patients colonized with *P. aeruginosa* with phenotype of chronic infections.

Table 3. Phenotype of *P. aeruginosa* associated with chronic infections.

Clone	Antimicrobial drug resistance	Biofilm (Index)	Swarming (Index)	Alginate (production)	Pigment (production)	Serum (% Resistance)
PAO1		1.00	1.00		PV	
<i>Psa01</i>		0.67	1.14		PV	26.1
<i>Psa02</i> ^ψ		0.76	1.46	Y	PV	7.1
<u><i>Psa03</i></u>	MDR	1.61	0.59		PV	81.3
<u><i>Psa04</i></u> ^ψ		1.23	0.94	Y	PV	50.0
<u><i>Psa05</i></u>		1.53	0.50	Y	PV	0.0
<i>Psa06</i> ^ψ		0.88	0.73	Y	PM	0.2
<i>Psa07</i>		1.35	0.88		PV	1.0
<i>Psa08</i>		1.00	1.81		PV	0.1
<i>Psa09</i> ^ψ		0.63	0.00		PV	0.2
<u><i>Psa10</i></u>		0.59	0.00	Y	PV	0.5
<i>Psa11</i> ^ψ		1.29	0.44		PM	0.4
<u><i>Psa12</i></u>	XDR	2.36	0.27		PM	4.7
<u><i>Psa13</i></u>	MDR	0.72	0.56			1.1
<i>Psa14</i>		1.00	0.89		PC	70.3
<i>Psa15</i> ^ψ		2.27	0.06		PC	0.5
<i>Psa16</i> ^ψ		1.07	0.21		PC	0.8
<u><i>Psa17</i></u>	MDR	0.88	0.60		PV	12.7
<u><i>Psa18</i></u>	MDR	0.93	0.22		F	1.4
<i>Psa19</i>		0.77	1.64		PV	32.1
<i>Psa20</i> ^ψ		0.55	0.17		PV	5.0
<i>Psa21</i> ^ψ	XDR	0.30	0.74			85.3
<i>Psa22</i> ^ψ		0.70	0.70		PV	0.3
<u><i>Psa23</i></u>		0.72	0.70	Y	PV	69.0
<i>Psa24</i> ^ψ		0.31	0.16		PV	3.8
<i>Psa25</i> ^ψ		0.84	0.11			84.1
<u><i>Psa26</i></u>	MDR	0.41	0.30	Y	PV	81.5
<i>Psa27</i> ^ψ		0.78	0.41		PC	0.1
<u><i>Psa28</i></u>	XDR	0.95	0.21		PM	20.9
<i>Psa29</i>		0.58	1.53		PV	88.5
<u><i>Psa30</i></u>	MDR	1.06	0.30	Y	PC	0.0
<u><i>Psa31</i></u>		1.05	0.24	Y	PV	2.3
<i>Psa32</i> ^ψ		0.64	0.64		PV	60.4
<u><i>Psa33</i></u>	MDR	1.34	0.32		PV	0.1
<u><i>Psa34</i></u>		1.63	0.33	Y	PV	1.4
<u><i>Psa35</i></u>	MDR	1.73	0.54		PV	69.9
<i>Psa36</i> ^ψ		0.28	0.59	Y	PV	74.9
<u><i>Psa37</i></u>	MDR	1.94	0.00		PV	73.1
<i>Psa38</i> ^ψ		0.87	0.53		PV	79.8
<i>Psa39</i>		1.30	2.26		PV	52.0
<u><i>Psa40</i></u>		1.34	0.62	Y	PC	34.4
<i>Psa41</i>		1.05	0.77		PV	21.9
<u><i>Psa42</i></u>	XDR	1.07	0.53		PV	38.9
<i>Psa43</i> ^ψ		0.90	0.61		PC	29.6
<u><i>Psa44</i></u>	MDR	0.77	0.95	Y	PC	60.5
<i>Psa45</i>		3.28	0.81		PV	37.8
<i>Psa46</i>		0.69	1.19			59.9
<u><i>Psa47</i></u>		0.46	0.70	Y	PV	17.8
<i>Psa48</i> ^ψ		0.95	1.67	Y	PV	58.0
<u><i>Psa49</i></u>		1.12	0.52	Y	PV	66.3
<u><i>PsaNT</i></u>		1.13	0.70	Y	PV	36.1

MDR, multidrug resistance [19], XDR, extensively-drug resistance [19]; Y, Yes; PV, pyoverdine; PC, pyocyanin; PM, pyomelanin; Bold numbers indicates a higher biofilm production or decreased motility. ^ψtwo phenotypes associated to chronic infections; underlined, three or four phenotypes associated to chronic infections.

Supplementary Figure 1. Association of *P. aeruginosa* colonization with age and lung function of children with CF.

Patients enrolled in this study were classified, on the basis of having previous isolation of *P. aeruginosa*, as non-colonized (I), intermittent colonization (II) and chronic colonization (III). A) Patient age in relation to *P. aeruginosa* colonization. B) Lung function (FEV1, %) in relation to *P. aeruginosa* colonization. White symbols indicate clones of *P. aeruginosa* without phenotype of chronic colonization and red symbols indicate clones of *P. aeruginosa* with phenotype of chronic colonization.

Supplementary Figure 2. Antimicrobial susceptibility data of *P. aeruginosa* clones.

One representative isolate of each clone was assessed for the MIC determination. Broth microdilution was used for Polymyxin B and Colistin. White squares indicate susceptibility, green squares indicate intermediate susceptibility and red squares show resistance to the antibiotic assessed. The antibiotic family is indicated.

Supplementary Figure 3. Resistance to normal human serum by *P. aeruginosa* clones.

One representative isolate from each clone was assessed for resistance to the bactericidal activity of normal human serum (NHS). A) Determination of the resistance to NHS by each clone of *P. aeruginosa*, B) Heat map of susceptibility to NHS.