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ORIGINAL RESEARCH

Genetic relatedness and host specificity of *Pseudomonas aeruginosa* isolates from cystic fibrosis and non-cystic fibrosis patients

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Background: *Pseudomonas aeruginosa* is one of the primary pathogens isolated more frequently in cystic fibrosis (CF) and it exhibits innate resistance to a wide range of antibiotics.

Purpose: We sought to determine whether the highly prevalent genotypes of *P. aeruginosa* are specifically linked to CF patients and have any related multidrug antibiotic resistance. Isolates from hospitalized non-CF patients and from environmental sources were also genotypically analyzed.

Methods: Collections of *P. aeruginosa* from lower respiratory secretions (n=45) were genotyped using pulsed-field gel electrophoresis (PFGE). Phenotypic screening for antibiotic susceptibility was performed for the common antimicrobial agents by E-test and automated Phoenix method.

Results: *P. aeruginosa* isolates from CF (n=32), hospitalized non-CF patients (n=13), and environment sources (n=5) were analyzed. The population structure of *P. aeruginosa* is highly diverse and population-specific. All PFGE results of *P. aeruginosa* isolates fall among four major clusters. Cluster 1 contained 16 *P. aeruginosa* isolates from CF patients and two from environmental sources; cluster 2 contained 11 *P. aeruginosa* isolates from CF and one each from non-CF and environmental sources; cluster 3 contained 12 *P. aeruginosa* isolates from hospitalized non-CF patients and two *P. aeruginosa* isolates from one CF patient and one environmental source; and cluster 4 consisted of three isolates from CF patients and one from the environment. The majority of multidrug-resistant *P. aeruginosa* isolates were in clusters 3 and 4. *P. aeruginosa* isolates from CF patients were resistant to ciprofloxacin (34.4%) followed by resistance to amikacin and gentamicin (each 28%), whereas the majority of isolates from non-CF patients were resistant to meropenem (69%) and were grouped in cluster 3.

Conclusion: PFGE of *P. aeruginosa* isolates from CF patients shows a high degree of similarity, suggesting specific adaptation of these clones to CF-affected lungs. The hospitalized non-CF cluster has a different clonal origin, indicating specific clustering in a specific location, suggesting hospital-acquired *P. aeruginosa* infections.

Keywords: cystic fibrosis, drug susceptibility testing, *Pseudomonas aeruginosa,* pulsed-field gel electrophoresis

Introduction

Pseudomonas aeruginosa is the main bacterial pathogen that infects the lungs of patients with cystic fibrosis (CF).[1] CF patients chronically infected with *P. aeruginosa* show a greater loss of lung function and a higher overall morbidity and mortality in comparison with patients with intermittent or no colonization.[1–4]

Initial pulmonary infection with *P. aeruginosa* in CF is thought to occur through the acquisition of unique environmental strains of *P. aeruginosa*, which persist and

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undergo phenotypic changes over time in the lungs of CF patients.[5] However, *P. aeruginosa* can spread between people with CF.[6] Some so-called transmissible or epidemic strains are associated with a worse clinical outcome and can be associated with a more rapid annual loss of lung function, worse nutritional status, and even an increased rate of death or lung transplantation.[7] An outbreak of colistin-resistant *P. aeruginosa* in a UK pediatric CF clinic was reported, describing the transmission of a genotypically indistinguishable *P. aeruginosa* strain between three patients in a nosocomial setting.[8]

Typing of strains of *P. aeruginosa* is important for specific characterization of the species, the choice of antibiotic regimen, the detection of unusual traits, and the recognition of a potential cluster of a single clone within patients.[9]

Several comprehensive molecular typing techniques for discriminating among *P. aeruginosa* strains have been developed, based either on DNA banding patterns such as restriction fragment length polymorphism and pulsed-field gel electrophoresis (PFGE), DNA sequencing (eg, multilocus sequence typing and genome sequencing), or on DNA hybridization (DNA macro- and microarrays).[10] PFGE typing is considered a common genotyping method for *P. aeruginosa* fingerprinting and is sometimes mentioned as the gold standard method for this bacterium, useful for hospital epidemiologists who need to monitor the effectiveness of infection control measures.[11–13] A recent study by Waters et al[14] demonstrated the highest level of concordance between PFGE and multilocus sequence typing results during the genotyping of a blinded sample of well-characterized *P. aeruginosa* isolates from CF patients across multiple laboratories. The aim of this study is to determine whether the highly prevalent genotypes of *P. aeruginosa* are specifically linked to CF patients and related to multidrug antibiotic resistance. Isolates from hospitalized non-CF patients and environment sources were also genotypically analyzed.

Materials and methods

Patients and sampling

Sputum samples or deep-pharyngeal swabs were prospectively collected and cultured over a period of 6 months from CF patients attending the CF clinic or hospitalized at the Hamad Medical Corporation, Doha, Qatar. The diagnosis of CF was based on one or more clinical features consistent with CF, positive family history of CF in siblings and close relatives, elevated sweat chloride (>60 mmol/L) on two separate occasions, in addition to the presence of two disease-causing mutations in the *CFTR* gene. Patients with *P. aeruginosa* isolates cultured from sputum or deep-pharyngeal swab samples were enrolled in the study (18 CF patients with chronic *P. aeruginosa* and 3 CF patients with intermittent *P. aeruginosa* infections). Chronic *P. aeruginosa* infection was defined as persistent presence of *P. aeruginosa* for at least 6 consecutive months.[15] *P. aeruginosa* isolates from the lower respiratory secretions of hospitalized non-CF patients during the same period and *P. aeruginosa* isolates from environmental samples obtained from a water basin were also included in the study.

Sputum samples or deep-pharyngeal swabs from patients not producing sputum were inoculated onto MacConkey agar, Columbia blood agar, and Columbia chocolate agar (Mast Diagnostics Ltd, Bootle, UK). Blood and chocolate agar media were incubated in a 5% CO_2 atmosphere. After 24 hours of incubation at 37°C, lactose-negative and oxidase-positive colonies were selected from among the different colony morphology types – mucoid, nonmucoid, and characteristic-pigment-producing colonies – subcultured on 5% Columbia sheep blood agar, and identified up to the species level using the Phoenix 100 (Becton, Dickinson and Co, Franklin Lakes, NJ, USA). Mucoid colonies were further confirmed by growth on nutrient agar, including a control reference *P. aeruginosa* strain at 42°C. Phenotypically different isolates were preserved in cryotubes at −70°C until analysis. This study was reviewed and approved by the local ethics committee at Hamad Medical Corporation (number 13103/13).

PFGE analysis

PFGE was performed according to a previously described protocol.[16] PulseNet standard *Salmonella enterica* serovar Braenderup H9812 strain was used for running the gels in accordance with the PulseNet protocol.[17] Two *S. enterica* serovar Braenderup H9812 precast plugs were digested with 50 IU of XbaI restriction enzyme (isolated from *Xanthomonas badrii*) alongside *Pseudomonas* plugs and loaded on the gel in the first and sixth lanes in order to normalize for analysis using BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium).

The electrophoresis was performed with the Bio-Rad CHEF-DR III system (Bio-Rad Laboratories Inc, Hercules, CA, USA). Electrophoresis run conditions were designed for a run time of 19 hours with 14°C cooling. The CHEF-DR III apparatus was then programmed to initial switch time 2.2 seconds, final switch time 54.2 seconds, gradient 6 V, and

an angle of 120°. All other parameters remained identical with those of the standard procedure.

Following electrophoresis, the gels were stained for 45 minutes in 600 mL of sterile distilled water containing 50 µL of ethidium bromide (10 mg/mL) and destained in three washes of 30 minutes each in 600 mL of distilled water. The gels were analyzed using a gel documentation system (Bio-Rad) and saved as TIFF (tagged-image file format) files for further analysis. The same TIFF files were later imported to the BioNumerics software version 6.0 (Applied Maths). All the software settings were used as defaults, and the gel was analyzed to generate a dendrogram describing the relationship among the pulsotypes.

Drug susceptibility testing

Drug susceptibility testing and interpretation were performed according to Clinical and Laboratory Standards Institute guidelines using the E-test and the automated Phoenix method for nine antimicrobial agents, including amikacin, gentamicin, ceftazidime, cefepime, ciprofloxacin, imipenem, meropenem, piperacillin/tazobactam, and colistin. *P. aeruginosa* ATCC 27853 was used as a control.

Results

This study included 21 CF patients – 14 males and 7 females – from 15 families, with a median age of 15 years (range: 4–36 years). Thirteen CF families with *CFTR* I1234V mutation, belonging to a single large Arab kindred tribe, and constituted of two families with three CF siblings and two families with two CF siblings were enrolled. The remaining families contributed one sibling each. Three closely related families were cousins to each other. Two CF patients had mutations other than *CFTR* I1234V. Furthermore, 13 CF patients provided one respiratory sample, six CF patients gave two samples each, one CF patient had three samples, and one CF patient provided four samples in different periods of time. Six CF patients were hospitalized for acute chest exacerbation during the study period. Hospitalized non-CF patients included two patients on mechanical ventilation support, one with tracheostomy and the other with chest infections.

All the *P. aeruginosa* strains were isolated from 45 specimens (41 sputum samples and four deep-pharyngeal swabs) and five environmental sources over a 6-month period of the study. *P. aeruginosa* isolates from CF (n=32), hospitalized non-CF patients (n=13), and environment sources (n=5) were analyzed. All *P. aeruginosa* isolates were successfully typed by PFGE, and the clusters of isolates obtained thereby are illustrated in the resulting dendogram (Figure 1). The population structure of *P. aeruginosa* is highly diverse and population-specific. PFGE analysis showed that the levels of genetic variation within *P. aeruginosa* were different among different clusters, with genetic relatedness within the same cluster. All PFGE results of *P. aeruginosa* isolates fall among four major clusters. Cluster 1 contained 16 *P. aeruginosa* isolates from CF patients and two from environmental sources; cluster 2 contained 11 *P. aeruginosa* isolates from CF and one each from non-CF and environmental sources; cluster 3 contained 12 *P. aeruginosa* isolates from hospitalized non-CF patients and two *P. aeruginosa* isolates from one CF patient and one environmental source; and cluster 4 consisted of three isolates from CF patients and one from the environment (Table 1). *P. aeruginosa* isolates from one CF family with three siblings were displayed in cluster 1, two CF families with siblings and closely related patients had *P. aeruginosa* isolates displayed within PFGE cluster 2, and one CF family with two siblings having two respiratory samples had *P. aeruginosa* isolates displayed within PFGE clusters 2 and 4. The PFGE analysis of one CF patient with four respiratory samples showed genetic variation of *P. aeruginosa*, which was distributed in PFGE clusters 1 and 2 during different periods. One CF patient hospitalized with acute chest exacerbation, in whom both mucoid and nonmucoid *P. aeruginosa* isolates were evaluated, showed highly close genetic relatedness to hospitalized non-CF *P. aeruginosa* isolates in cluster 3.

P. aeruginosas isolates from CF patients showed high susceptibility to antipseudomonal agents and relatively low rates of antimicrobial resistance (Table 2). *P. aeruginosa* strains in CF patients were resistant to ciprofloxacin (34.4%), followed by resistance to amikacin and gentamicin (each 28%), whereas the majority of isolates from non-CF patients were resistant to meropenem (69%), followed by resistance to ciprofloxacin (30.7%), amikacin, and gentamicin (each 23%) and were grouped in cluster 3 (Table 1). None of the CF isolates were resistant to meropenem. The majority of multidrug-resistant *P. aeruginosa* (MRPA) samples were in clusters 3 and 4. Two CF siblings with advanced lung disease had the same *P. aeruginosa* multidrug resistance to fluoroquinolones, amikacin, gentamicin, and cefepime and were within the same cluster 4, indicating high *P. aeruginosa* genetic relatedness. Another CF patient with advanced lung disease had similar multidrug resistance to antipseudomonal agents in his four sputum samples over the 6-month period, with the samples clustered in two different clusters, ie, clusters 1 and 2, upon PFGE analysis for *P. aeruginosa*.

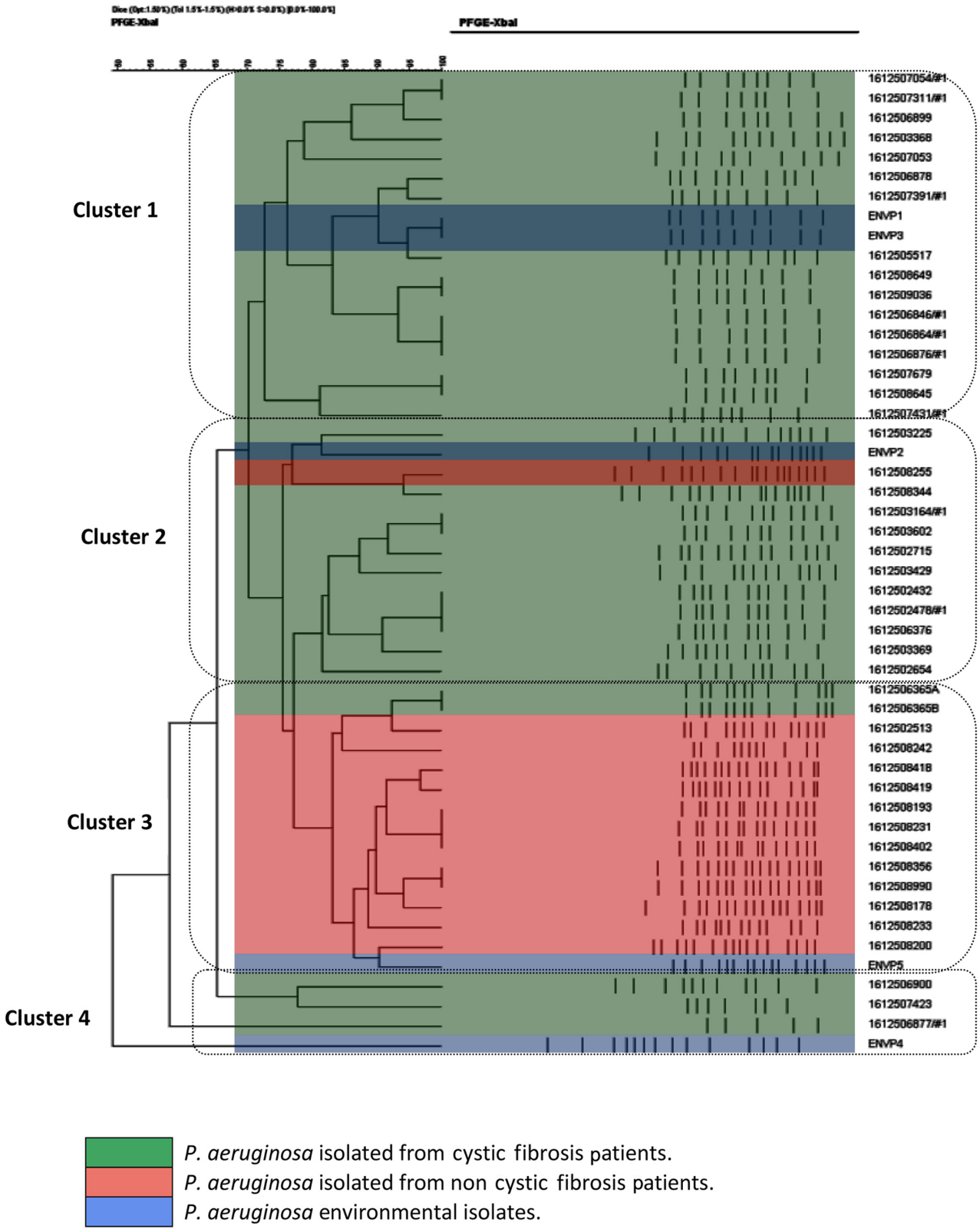

Figure 1 Dendrogram based on percentage similarities among *P. aeruginosa* isolates obtained using PFGE fingerprinting.
Abbreviations: *P. aeruginosa*, *Pseudomonas aeruginosa*; PFGE, pulsed-field gel electrophoresis; XbaI, restriction enzyme isolated from *Xanthomonas badrii*.

Table 1 Number of *P. aeruginosa* isolates and their respective clusters on PFGE typing

Patient number	Source of *P. aeruginosa*	Number of *P. aeruginosa* isolates per patient	Cluster 1: CF (n=16) and environment (n=2)	Cluster 2: CF (n=11), non-CF (n=1), and environment (n=1)	Cluster 3: CF (n=2), non-CF (n=12), and environment (n=1)	Cluster 4: CF (n=3) and environment (n=1)
1	CF	4	2	2		
2	CF	1	1			
3	CF	1	1			
4	CF	1	1			
5	CF	2	1	1		
6	CF	2	1	1		
7	CF	2	2			
8	CF	2	2			
9	CF	1	1			
10	CF	1	1			
11	CF	1	1			
12	CF	1	1			
13	CF	1	1			
14	CF	1		1		
15	CF	1		1		
16	CF	3		1	2	
17	CF	1		1		
18	CF	1		1		
19	CF	2		1		1
20	CF	2		1		1
21	CF	1				1
22	Non-CF	1		1		
23	Non-CF	1			1	
24	Non-CF	1			1	
25	Non-CF	1			1	
26	Non-CF	1			1	
27	Non-CF	1			1	
28	Non-CF	1			1	
29	Non-CF	1			1	
30	Non-CF	1			1	
31	Non-CF	1			1	
32	Non-CF	1			1	
33	Non-CF	1			1	
34	Non-CF	1			1	
Environmental isolate number						
1	Environment	1	1			
2	Environment	1	1			
3	Environment	1		1		
4	Environment	1			1	
5	Environment	1				1

Abbreviations: *P. aeruginosa*, *Pseudomonas aeruginosa*; PFGE, pulsed-field gel electrophoresis; CF, cystic fibrosis.

Discussion

P. aeruginosa is ubiquitous in the environment, the principal respiratory pathogen in CF patients, and continues to be the principal cause of morbidity and mortality among CF patients. *P. aeruginosa* colonization and infection may either be community acquired or health care associated. We identified a considerable genetic heterogeneity of *P. aeruginosa* in our patient population, and this study demonstrates a unique cohort of CF and hospitalized non-CF patients from a single center, by exploring the genetic relatedness of *P. aeruginosa* isolates from respiratory secretion using PFGE, which is capable of discriminating between genotypes of different *P. aeruginosa* isolates.

Interestingly, *P. aeruginosa* isolates from siblings of CF families were displayed within one PFGE cluster, suggesting that cross-transmission of *P. aeruginosa* among CF patients occurs more frequently when contact is more intense, especially among siblings. The ability of *P. aeruginosa* to be transmitted

Table 2 Number of *P. aeruginosa* isolates resistant to common antimicrobial agents in vitro and their respective clusters after PFGE typing

Antibiotics	Distribution of resistant *P. aeruginosa* – PFGE clusters*			
	Cluster 1: CF resistance (n=6/16)	Cluster 2: CF resistance (n=5/11)	Cluster 3: CF resistance (n=2/2) and non-CF resistance (n=8/12)	Cluster 4: CF resistance (n=3/3)
Ciprofloxacin	3	4	6	2
Amikacin	2	4	4	2
Gentamicin	2	4	4	2
Cefepime	1	1	2	1
Ceftazidime	0	0	2	1
Meropenem/imipenem	0	0	8 (non-CF)	0
Piperacillin/tazobactam	0	0	1	0
Colistin	1	0	0	0

Note: *Some isolates resistant to ≥2 antibiotics.
Abbreviations: *P. aeruginosa*, *Pseudomonas aeruginosa*; PFGE, pulsed-field gel electrophoresis; CF, cystic fibrosis.

from person to person with CF has been recognized as early as the 1980s.[18–26] By employing amplified fragment-length polymorphism molecular fingerprinting, a previous study has shown similar distribution of *P. aeruginosa* isolates among siblings and closely related families, indicating person-to-person transmission of *P. aeruginosa* within the same family or infection of *P. aeruginosa* possibly acquired from common environmental exposure.[26] The PFGE of *P. aeruginosa* isolates was largely in agreement with the diversity found by amplified fragment-length polymorphism fingerprinting in several previous studies that showed that siblings with CF frequently carry identical *P. aeruginosa* isolates.[18,27–29]

One hospitalized CF patient with advanced lung disease had high similarity of genetic relatedness of both mucoid and nonmucoid *P. aeruginosa* isolates to the samples from hospitalized non-CF patients in cluster 3 indicating specific clustering in the specific location and suggesting hospital-acquired *P. aeruginosa* infection.

There were variations of the different clusters of *P. aeruginosa* among CF patients having more than one sample in different periods of time. These findings were similar to previously published studies demonstrating that the predominant clone of *P. aeruginosa* would vary over time, with one clone predominating in one period and another clone predominating in another period. This event suggests that *P. aeruginosa* infection of the CF patient's lung is a very dynamic process, as proposed by Renders et al.[30] *P. aeruginosas* isolates from CF patients showed high susceptibility to antipseudomonal agents and relatively low rates of antimicrobial resistance. Fluoroquinolones are the only available antibiotics for oral treatment of *P. aeruginosa* infections. However, *P. aeruginosa* easily becomes resistant to these drugs as we found in 31% among the CF population, followed by development of resistance to amikacin and gentamicin (each 24%). However, the carbapenems (imipenem and meropenem) remained very active against CF *P. aeruginosa* isolates, with susceptibility rates of 100%, whereas the majority of *P. aeruginosa* isolates from hospitalized non-CF patients were resistant to meropenem (69%) and were grouped in cluster 3; they were probably acquired from the hospital environment.

MRPA is a common respiratory pathogen found in CF patients, which routinely leads to chronic pulmonary infections.[31,32] Multiple antibiotic-resistant *P. aeruginosa* is more likely to be a marker of more severe disease and more intensive therapy and is less likely to be contributing independently to more rapid lung function decline.[33] In the present study, *P. aeruginosa* isolates from CF siblings with advanced lung disease and MRPA isolates had similar genetic relatedness on PFGE and were found within the same cluster, suggesting the transmission even of MRPA isolates among CF siblings. Clustering of *P. aeruginosa* isolate from another CF patient with advanced lung disease and MRPA isolates in different PFGE clusters 1, 2, and 4 suggests that the colonizing strain may occasionally be changed.

Polymyxin B sulfate and colistimethate, a prodrug of colistin, are administered to CF patients intravenously or by inhalation. Resistance to colistin has been rarely reported in CF patients, and there is potential for an emerging resistant strain.[34] In the present study, we found that *P. aeruginosa* from one CF patient with a non-*CFTR* I1234V mutation showed in vitro resistance to colistin although the patient did not receive either nebulized or intravenous colistin.

We concluded that cross-transmission is probably common among CF siblings even with MRPA isolates. In addition, the presence of several isolates in the same patient with distinct genotypes suggests that the colonizing strain may occasionally be changed. Different patient populations have different PFGE clusters and different drug susceptibilities.

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Disclosure

The authors report no conflicts of interest in this work.

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