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ORIGINAL RESEARCH

Genotypic characterization of *Pseudomonas aeruginosa* isolates from Turkish children with cystic fibrosis

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Dicle Sener Okur[1]
Caner Yuruyen[2]
Ozge Gungor[2]
Zerrin Aktas[2]
Zayre Erturan[2]
Necla Akcakaya[3]
Yildiz Camcioglu[3]
Haluk Cokugras[3]
Kaya Koksalan[4]

[1]Istanbul University Cerrahpasa, Faculty of Medicine, Department of Pediatrics, Division of Pediatric Infectious Diseases, 34098 Kocamustafapasa, Istanbul, Turkey; [2]Istanbul University Istanbul, Faculty of Medicine, Department of Microbiology and Clinical Microbiology, 34390 Capa, Istanbul, Turkey; [3]Istanbul University Cerrahpasa, Faculty of Medicine, Department of Pediatrics, Division of Pediatric Infectious Diseases, Clinical Immunology and Allergy, 34098 Kocamustafapasa, Istanbul, Turkey; [4]Istanbul University, Institute for Medical Experimental Research (DETAE), 34390 Capa, Istanbul, Turkey

Objective: To identify epidemic and other transmissible *Pseudomonas aeruginosa* strains, genotypic analyses are required. The aim of this study was to assess the distribution of *P. aeruginosa* strains within the Turkish pediatric cystic fibrosis (CF) clinic population.

Methods: Eighteen patients attending the pediatric CF clinic of Cerrahpasa Medical Faculty were investigated in the study. Throat swab and/or sputum samples were taken from each patient at 3-month intervals. The isolates of patients were analyzed by pulsed-field gel electrophoresis (PFGE). The intra- and interpatient genotypic heterogeneity of isolates was examined to determine the clonal isolates of *P. aeruginosa* within the cohort.

Results: A total of 108 clinical isolates of *P. aeruginosa* were obtained from 18 patients between May 2013 and May 2014. The pulsotypes of the first patient's isolates could not be obtained by PFGE. From the remaining 17 patients and 101 isolates, 55 distinct pulsotypes were detected. The number of pulsotypes observed in more than one patient (minor clonal strains, cluster strains) was 8 (14.5%), and one of them colonized three patients. However, none of them was detected in more than three patients. These pulsotypes were composed of 20 isolates. In addition, with the PFGE analysis of 81 isolates, we detected 47 (85.6%) pulsotypes, which belonged to only one patient. Over different periods of this study, only 2 (11.8%) patients were colonized with the same pulsotype.

Conclusion: Our study indicates that there was considerable genomic diversity among the *P. aeruginosa* isolates in our clinic. The presence of shared pulsotypes supports cross-transmission between patients.

Keywords: *P. aeruginosa*, cystic fibrosis, PFGE, genotype, phenotype, epidemiology

Introduction

Pseudomonas aeruginosa is a ubiquitous Gram-negative opportunistic pathogen in cystic fibrosis (CF) patients. It often causes chronic lung infection associated with respiratory failure, clinic deterioration, increased morbidity and mortality.[1,2]

It has been accepted that CF patients are colonized for long periods with their own unique *P. aeruginosa* strains acquired from the environment, and person-to-person transmission occurs infrequently.[3–7] Recently, cross-infection and epidemic strains have been reported from the following different countries: United Kingdom, Australia, Norway, Germany, Canada and Denmark.[8–15]

In addition, the spread of an epidemic strain over several countries on two continents has been reported.[16] Moreover, some epidemic strains are reported to be

Correspondence: Dicle Sener Okur
Istanbul University Cerrahpasa, Faculty of Medicine, Department of Pediatrics, Division of Pediatric Infectious Diseases, 34098 Kocamustafapasa, Istanbul, Turkey
Email diclesen71@yahoo.com

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related to a poorer prognosis and the increased need for intravenous antibiotic treatment.[10]

To control and prevent *P. aeruginosa* infections among CF patients, it is necessary to determine if there is a persistence of the same strain or reinfection due to a new strain. Epidemic and other transmissible *P. aeruginosa* strains cannot be identified reliably by phenotypic markers and antibiotic susceptibility profiles. Therefore, genotypic analyses are required. Pulsed-field gel electrophoresis (PFGE) is the "gold standard" method for microbial typing.[15,17–20]

The epidemiological relatedness of *P. aeruginosa* isolates and the extent of cross-infection with these organisms among Turkish CF pediatric patients have not yet been investigated using PFGE. To our knowledge, this is the first published study on the genetic relatedness of *P. aeruginosa* isolates from CF pediatric patients in Turkey using the PFGE method.

The aim of this study was to assess the distribution of *P. aeruginosa* strains within the Turkish pediatric CF clinic population. The intra- and interpatient genotypic heterogeneity of isolates was examined to determine the clonal isolates of *P. aeruginosa* within the cohort.

Materials and methods

This study was approved by the Ethical Committee of Istanbul University Cerrahpasa Medical Faculty (date of approval 08/01/2013, approval number A-07) and conducted in accordance with the Declaration of Helsinki. Written informed consent to participate in the study was obtained from the patients enrolled or their parents.

Patients

Nineteen patients chronically infected with *P. aeruginosa* who attended the pediatric CF clinic of Cerrahpasa Medical Faculty were planned to be enrolled in the study. One patient left the study voluntarily, and the study was conducted with 18 patients. Their contact information was evaluated to explore possible routes of *P. aeruginosa* transmission among the infected patients. Half (female/male: 9/9) of our patients were female. The mean age of participants was calculated as 13±4.5 (1.83–22.08). Other clinical characteristics of patients' are summarized in Table 1.

Bacterial isolates

The isolates of *P. aeruginosa* from 18 children attending the pediatric CF clinic of Cerrahpasa Medical Faculty were collected over 1 year (May 2013 to May 2014).

Table 1 Clinical profiles of patients'

	n	%
ΔF508 homozygous	3	16.7
Baseline lung function (FEV1)	Severe lung disease: 5 Mild lung disease: 3	Severe lung disease: 27.8 Mild lung disease: 16.7
Pancreatic insufficiency	7	38.9
CF-related diabetes	1	5.6
Coinfections	*S. aureus*: 8 *H. influenza*: 5 Acinetobacter: 1 Aspergillus: 1	

Throat swab and/or sputum samples were taken from each patient at 3-month intervals (May, August and November). The sputum samples were homogenized by mixing with dithiothreitol . The samples were inoculated onto MacConkey agar (Becton Dickinson, Franklin Lakes, NJ USA) and tryptic soy agar. Oxidase-positive Gram-negative bacilli colonies in different morphologies were further identified by the API 20 NE system (bioMérieux, Marcy-l'Étoile, France, Vitek Inc.). The antibiotic susceptibilities of isolates were investigated in Müller–Hinton agar (Bio-Rad Laboratories Inc., Hercules, CA, USA) by the disc diffusion method according to the criteria of the Clinical and Laboratory Standards Institute (CLSI).[21]

The studied antibiotics were ofloxacin, ciprofloxacin, ceftazidime, imipenem, meropenem, ticarcillin-clavulanate, piperacillin, piperacillin-tazobactam, aztreonam, amikacin, gentamicin, netilmicin and tobramycin.

PFGE

PFGE was performed using a CHEF-DRIII drive module (Bio-Rad Laboratories Inc., Hercules, CA, USA). The isolates were grown overnight in tryptic soy agar at 37°C on a shaker. After standardization of the cell suspension by optical density measurement, the cells were embedded in low-melting agarose (Bio-Rad). Other steps, from the digestion (lysozyme and proteinase K) of bacteria to washing of the plugs and the subsequent restriction digestion of the bacterial DNA with SpeI (New England Biolabs, Ipswich, MA, USA) overnight, were performed in accordance with the kit manufacturer's instructions. Electrophoresis was performed in 1% agarose gel prepared in 0.5x TBE (Tris-borate-EDTA) buffer. The running temperature was 14°C. The

optimal run conditions for the separation of fragments were set as 18 hrs at 6 V/cm^2 with an initial switch time of 6 s, a final switch time of 22 s and a 120° angle.

Lambda Ladder PFGE marker (BioLabs, Ipswich, MA, USA) was used as a molecular size marker. The gel was stained with ethidium bromide (1 µg/mL) for 30 mins, visualized under UV light using a transilluminator and photographed. A TIFF image of each gel was taken. The band profiles were analyzed by the GelCompar II system (version 6.0, Applied Maths, Sint-Martens-Latern, Belgium). Dendrograms of the band profiles were produced using the unweighted pair group method with mathematical averaging. The relatedness of isolates was calculated using the Dice coefficient with a band position tolerance setting of 1–1.5%. Isolates were defined as the same PFGE type (clonal) if the Dice coefficient was ≥85%. Tenover criteria were also applied for visual analysis of the bands[22] (Figures 1–4).

Results

A total of 108 clinical isolates of *P. aeruginosa* were obtained from 18 patients between May 2013 and May 2014. According to the colonial morphology, the number of mucoid isolates (59%, 54.7%) was higher than the number of nonmucoid isolates (49%, 45.3%), and pigment production was observed in 21 (19.6%) isolates. The ratio of isolates sensitive to all antibiotics was 34.2% (37), and they were distributed among 15 patients (patients 1–4, 6, 8–15, 17 and 19). All the isolates of two patients (patients 10 and 19) were sensitive to all antibiotics. *P. aeruginosa* isolates were most susceptible to meropenem (98%, 90.7%), imipenem (96%, 88.8%), ciprofloxacin (95%, 87.9%) and piperacillin-tazobactam (95%, 87.9%). The numbers of mucoid isolates susceptible to all antibiotics (19%, 17.5) and nonmucoid isolates susceptible to all antibiotics (18%, 16.6%) were almost the same.

The pulsotypes of the first patient's isolates could not be obtained by PFGE. From the remaining 17 patients and 101 isolates, 55 distinct pulsotypes were detected. The number of pulsotypes observed in more than one patient (minor clonal strains and cluster strains) was 8 (14.5%), and one of them colonized three patients. However, none of them was detected from more than three patients. These pulsotypes were composed of 20 isolates. From the remaining 81 isolates, 47 (85.6%) pulsotypes were detected, and each of them belonged to only one patient. (Table 2)

Only 2 (11.8%) patients (patients 2 and 15) were colonized with the same pulsotype (persistent pulsotype) over different periods of this study. Two (11.8%) patients (patients 14 and 17) harbored the same pulsotypes at the beginning of the study (the first two periods), and 5 (29.3%) patients (patients 6, 8, 9, 16 and 19) harbored the same pulsotypes in the last two periods. The other 8 (47.1%) patients' (patients 3–5, 7 and 10–13) *P. aeruginosa* isolates all belonged to different pulsotypes in each study period. When the hospital records of these patients were examined, it was found that patient 15 had never been hospitalized and patient 2 had been hospitalized once in the past 5 years. Patients 6, 8 and 9 were hospitalized once in the study period between the second and third sample collection sessions. (Table 3)

Our study indicates that there was considerable genomic diversity among the *P. aeruginosa* isolates in our clinic. The presence of shared pulsotypes supports cross-transmission between patients. In addition, we could not find a relationship between the genotype and phenotypic characteristics of *P. aeruginosa* isolates. Their antibiotic susceptibilities varied independently from their pulsotypes as well.

When we examined the patients' contact information retrospectively from their hospital records, it was found that patient 10, a 13-month-old boy, had been hospitalized with patient 11 on the same dates several times. This finding was also observed in patients 3 and 4, as well as patients 12–14. However, there was no contact between patients 4 and 16, 4 and 14 or 16 and 19. Indeed, patients 16 and 19 were from different cities in Turkey. Infection prevention and control strategies in our CF center were carried out according to the 2003 North American guidelines.[23] However, we experienced some difficulties fulfilling the recommendations. Health care personnel give great attention to hand hygiene, and although children and families are educated about respiratory hygiene practices, we observe that they sometimes do not comply with recommendations. Guidelines recommend that people with CF be placed in single-patient rooms, so we often hospitalize them in single-patient rooms. However, they must infrequently be placed in double-patient rooms due to our inpatient ward's limited capacity. As a result, in our study, hospitalization was important for the transmission of strains. The transmission and acquisition routes of the pulsotype between patients 16 and 19 remain unclear. The source of this common pulsotype could be the environment. Furthermore, it could be a widespread strain. Inpatient rooms are cleaned and disinfected between patients, but unfortunately, this is not always possible for outpatient exam rooms. Generally, children with CF resist avoiding social and physical contact with other CF

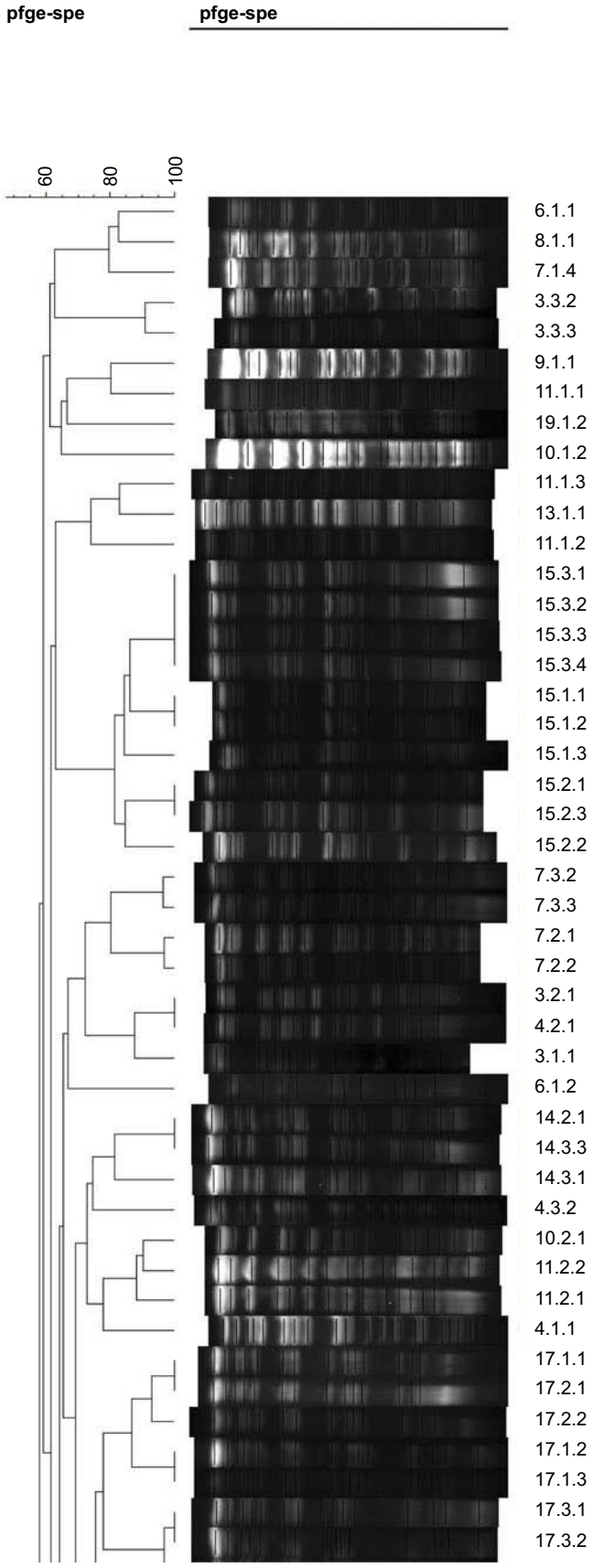

Figure 1 Dendograms of the band profiles (first page).

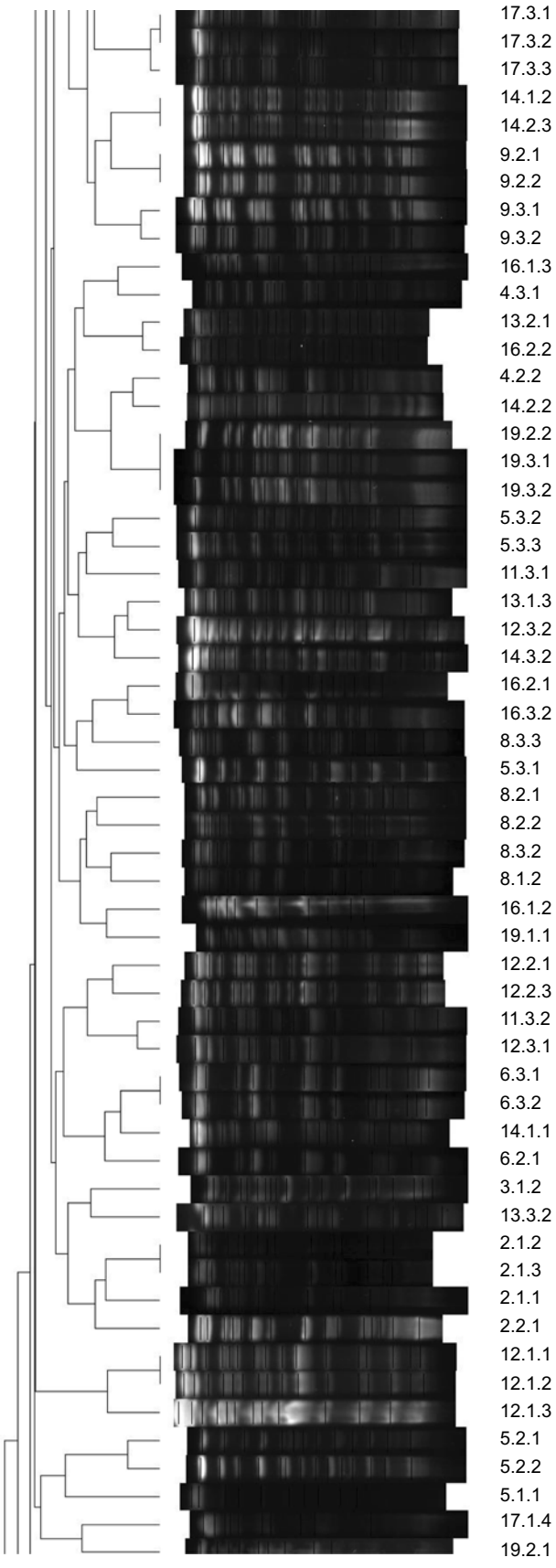

Figure 2 Dendograms of the band profiles (second page).

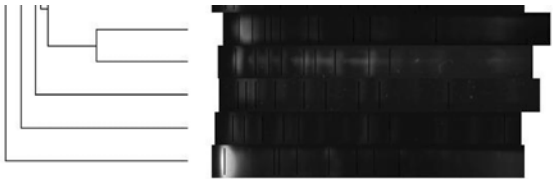

Figure 3 Dendograms of the band profiles (third page).

patients. Therefore, social or physical contact in outpatient waiting rooms may be another transmission route.

Discussion

This study explores the genotypic characterization of *P. aeruginosa* isolates collected from 18 patients at the pediatric CF center in Cerrahpasa Medical Faculty between 2013 and 2014. This is the first known published study exploring the genetic relatedness of *P. aeruginosa* isolates among CF children in Turkey. The identification of clonal, dominant or transmissible strains based on phenotypic features is not possible and instead requires molecular genotyping analyses.

One of the objectives was to establish if there was patient-to-patient transmission. Through PFGE analysis, we identified eight minor clones that included isolates from nine patients. The pulsotypes of the first patient's isolates could not be obtained by PFGE. The endogenous endonucleases of the isolates could be the cause of this problem.

The presence of shared pulsotypes supports patient-to-patient transmission was possible. In the previous studies, which analyzed the PFGE patterns of *P. aeruginosa* isolates, the risk of transmission rates was extremely low.[6,14,24,25]

Similarly, the risk of transmission was low at our center. However, it cannot be ignored in some cases. It has been reported that different management of pulmonary exacerbations and infections due to *P. aeruginosa* is probably responsible for differences in epidemiology.[26]

Antimicrobial agents administered in the presence of pulmonary exacerbations, infection-control strategies in our center and efforts to eradicate *P. aeruginosa* might have prevented outbreaks of epidemic strains at our center. According to our infection-control program, for CF patients, we often hospitalized them in single-patient rooms. However, they must infrequently be placed in double-patient rooms due to our inpatient ward's limited capacity. In this respect, our study is a guide for improving our infection-control program for children with CF. In addition, it is clear that we have to update our IPAC practices according to more recent guidelines published in 2013.[27]

The ability of *P. aeruginosa* to adapt and survive host immune responses, the extensive use of antibiotic therapy and the heterogeneity of the deteriorating lungs of CF patients cause clonal pathoadaptive variants with different phenotypic features.[28]

As this is the first known study that investigated the link between *P. aeruginosa* pulsotypes and phenotypic features, our phenotypic analysis showed that there is phenotypic heterogeneity within the pulsotype and between the minor clones as well. In addition, we observed that the isolates of shared pulsotypes did not have similar antimicrobial susceptibility patterns. Consistent with the previous studies, this study also found no relationship between the *P. aeruginosa* phenotype and genotype.[29,30]

Previous authors have reported that the majority of CF patients are colonized only with a unique genotype.[14,30,31] However, coinfection with multiple *P. aeruginosa* isolates has also been observed in these patients.[32–34]

In our study, only two patients were determined to harbor just one *P. aeruginosa* genotype over the study period. Even though the design of the study was longitudinal, the results were different from those of previous studies, demonstrating that most patients were colonized for long periods with a single clone. More importantly, detecting several isolates with different genotypes in the same patient indicates that the colonizing strain may occasionally be replaced. Hospital admission and antimicrobial usage could also be responsible for these findings.

The main limitation of our study is the small sample size, but fewer children in our country are diagnosed with CF than in other countries. This may be because of differences in genetic predisposition between populations or the recent performance of newborn screening tests in our country. We are one of the main centers following children with CF in our country. More children with CF are followed by our center than most other CF centers in our country. Therefore, the number of patients we have followed is also low. In addition, we explored whether there is cross-transmission between patients by collecting samples over a year from each patient at 3-month intervals. We thought that taking three samples rather than one from each patient would increase the chance of detecting transmissible isolates.

One limitation of this study is that our investigation was a single-center study and the number of patients in our cohort was small. This study helped us recognize the need to organize a multicentre study. This study will also encourage other CF centers to investigate their *P. aeruginosa*

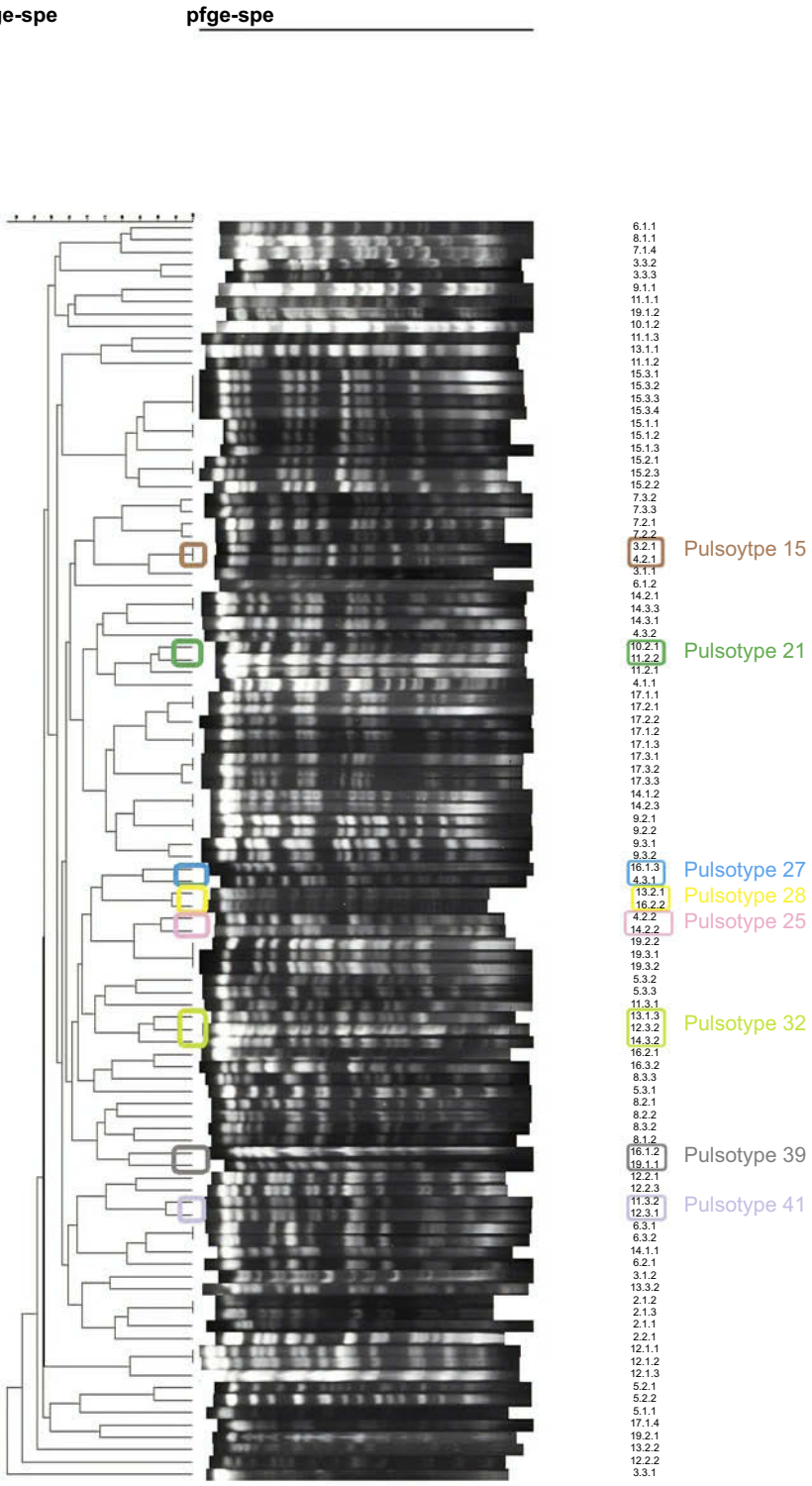

Figure 4 Dendograms of the band profiles all in one page, with minor clonal strains coloured.

isolates' genetic relatedness and transmission properties and to collaborate with other researchers in this field.

Other two limitations of our study are that we did not perform environmental microbiological sampling, so we cannot comment on whether the transmissible pulsotypes were acquired from an environmental source and acquisition from social contact cannot be excluded.

Table 2 Distribution of *Pseudomonas aeruginosa* isolates from 18 patients according to phenotypic feature and genotype (pulsotype)

Patient	No of Isolates	Mucoid/ Nonmucoid	Pigment	Genotype (Pulsotype)	No of isolates with the same genotype	Resistance or Intermediate Resistance to
1	111	Nm	-	-	-	OFX(R)
	112	Nm	+	-		OFX(I)
	121	Nm	+	-		
	122	Nm	-	-		
	131	Nm	-	-		
	132	M	-	-		
	133	Nm	-	-		
2	211	M	+	46*	4	-
	212	Nm	+	46*		OFX(IR)
	213	Nm	+	46*		-
	221	M	+	46*		-
3	311	Nm	-	16	2	-
	312	Nm	-	44		-
	321	M	-	15		OFX(R), CP(I)
	331	Nm	+	55		-
	332	Nm	+	4*		PIP(I)
	333	M	-	4*		OFX(I)
4	411	Nm	+	22	0	TIM(I)
	421	M	-	15		CAZ(R)
	422	Nm	-	25		-
	431	M	-	27		TZP(I), ATM(I), TIM(I)
	432	Nm	-	20		CAZ(I), PIP(R), AN(R), GM(I), ATM(R), TIM(R)
5	511	M	-	50	4	AN(I), NET(I), GM(I), OFX(I)
	521	M	-	49*		CAZ(I), PIP(I), AN(R), NET(R), GM(R), TIM(I), NN(R)
	522	NM	-	49*		CAZ(R), PIP(I), AN(R), NET(I), GM(R), NN(I)
	531	NM	-	35		AN(R), NET(R), GM(R), TIM(R), NN(R)
	532	M	-	30*		CAZ(I), AN(R), GM(R), NN(R)
	533	NM	+	30*		CAZ(R), PIP(R), AN(R), NET(R), GM(R), OFX(R), CP(I), TZP(I), TIM(I), NN(R)
6	611	NM	+	1	3	-
	612	NM	-	17		MPM(R), IPM(I), CAZ(I), PIP(R), OFX(R), CP(I)
	621	M	-	42*		IPM(R)
	631	M	-	42*		IPM(R), PIP(I), OFX(I), TZP(I)
	632	NM	-	42*		OFX(R), CP(I)
7	714	NM	-	3	4	CAZ(I), PIP(R), AN(R), GM(R), OFX(I), TIM(I)
	721	M	-	14*		MPM(R), IPM(R), CAZ(R), PIP(R), OFX (R), CP(R), TZP(I), ATM(I), TIM(R)
	722	M	-	14*		MPM(R), IPM(R), AN(R), NET(R), GM(R), OFX(I), CP(I), NN(R)
	732	M	-	13*		-
	733	NM	-	13*		MPM(R), IPM(R), CAZ(R), PIP(R), AN(I), OFX(R), CP(I), TZP(R), ATM(I), TIM(R)

(*Continued*)

Table 2 (Continued).

Patient	No of Isolates	Mucoid/ Nonmucoid	Pigment	Genotype (Pulsotype)	No of isolates with the same genotype	Resistance or Intermediate Resistance to
8	811	NM	-	2	2	-
	812	M	-	38		AN(R), NET(I), GM(I), TIM(R)
	821	M	-	36		M(I), OFX(I)
	822	M	-	37*		NET(I), TZP(I), TIM(R)
	832	NM	-	37*		AN(R), GM(I), OFX(I), CP(I)
	833	M	-	34		CAZ(R), PIP(I), OFX(R), CP(I), TZP(I), ATM(R), TIM(R)
9	911	NM	-	5	4	-
	921	M	-	26*		-
	922	NM	-	26*		-
	931	M	-	26*		PIP(R), AN(R), NET(I), GM(R), OFX(I), TIM(I), NN(I)
	932	M	-	26*		AN(R), NET(R), GM(R), OFX(I), NN(R)
10	1012	NM	-	8	0	-
	1021	NM	-	21		-
11	1111	M	-	6	2	CAZ(R), ATM(I), TIM(R)
11	1112	M	+	11		CAZ(R), OFX(I), ATM(I), TIM(I)
11	1113	M	-	9		CAZ(R), PIP(I), ATM(I), TIM(I)
11	1121	M	-	21*		-
11	1122	NM	+	21*		-
11	1131	M	-	31		TIM(I)
11	1132	NM	-	41		PIP(I), AN(I), GM(R), OFX(R), CP(I)
12	1211	NM	-	47*	4	AN(I), GM(I)
	1212	M	-	47*		AN(I), GM(I)
	1213	M	+	48		-
	1221	M	-	40*		AN(R), GM(I), NN(I)
	1222	M	-	54		GM(I)
	1223	NM	-	40*		-
	1231	M	+	41		AN(R), NET(R), GM(R), NN(I)
	1232	M	+	32		GM(I)
13	1311	NM	-	10	0	CAZ(I), TIM(I)
	1313	M	-	32		-
	1321	M	-	28		MPM(I), IPM(R), CAZ(R), PIP(I), AN(I), OFX(I), TZP(I), ATM(R), TIM(R)
	1322	NM	-	53		CIP(I), NN(R)
	1332	NM	-	45		PIP(I), TZP(I), TIM(R)
14	1411	M	-	43	5	-
	1412	NM	-	25*		-
	1421	M	-	18*		-
	1422	M	-	25*		-
	1423	NM	-	25*		AN(R), NET(I), GM(R)
	1431	M	-	19		AN(I)
	1432	M	-	32		GM(I)
	1433	NM	-	18*		PIP(I), AN(I), GM(I), OFX(I)

(*Continued*)

Table 2 (Continued).

Patient	No of Isolates	Mucoid/ Nonmucoid	Pigment	Genotype (Pulsotype)	No of isolates with the same genotype	Resistance or Intermediate Resistance to
15	1511	NM	+	12*	9	PIP(I), AN(R), GM(R)
	1512	M	-	12*		AN(R), GM(I)
	1513	M	-	12*		-
	1521	M	-	12*		M(R)
	1522	M	-	12*		-
	1523	NM	+	12*		AN(R), NET(R), GM(R), NN(I)
	1531	NM	-	12*		CAZ(R), PIP(I), AN(R), NET(R), GM(R), NN(I)
	1532	NM	-	12*		MPM(R), IPM(R), PIP(R), ATM(I)
	1533	M	-	12*		MPM(R), IPM(R), PIP(R), AN(I), GM(R), OFX(I), TIM(I)
16	1612	M	-	39	2	TIM(I),ATM(I)
	1613	M	-	27		TIM(I)
	1621	NM	-	33*		TIM(I)
	1622	M	-	28		TIM(I)
	1632	M	-	33*		PIP(I), TZP(I), TIM(I), ATM(I)
17	1711	NM	+	23*	8	AN(R), NET(R), GM(R), NN(R)
	1712	M	-	23*		-
	1713	M	-	23*		OFX(R), TIM(I)
	1714	NM	+	51		AN(R), NET(R), GM(R), NN(R)
	1721	M	-	23*		-
	1722	NM	-	23*		OFX(R), TIM(R)
	1731	NM	-	24*		GM(I), OFX(R), TIM(I)
	1732	NM	+	24*		CAZ(R), PIP(I), AN(R), NET(R), GM(R), TIM(I)
	1733	M	-	24*		PIP(I)
19	1911	M	-	39	2	-
	1912	M	-	7		-
	1921	M	-	52		-
	1922	M	-	29*		-
	1931	M	-	29*		-

Notes: Number of isolates are defined according to patient number, period of isolation, number of isolates in the mentioned period (eg, 213; 2 is patient number, 1 is period of isolation, 3 is the third isolate in the first period). *Persistent pulsotypes detected in each patient.

Abbreviations: M, mucoid; NM, nonmucoid; R, resistance; I, intermediate resistance; MPM, meropenem; IPM, imipenem; CAZ, ceftazidime; PIP, piperacillin; AN, amikacin; NET, netilmicin; GM, gentamicin; OFX, ofloxacin; CP, ciprofloxacin; TZP, piperacillin-tazobactam; ATM, aztreonam; TIM, ticarcillin-clavulanate; NN, tobramycine.

Conclusions

This study suggested patient-to-patient transmission at our center by detection of shared clones (minor clones). To prevent the dissemination of this pathogen, infection-control strategies, such as the separation of patients and careful hygiene practices, should be followed.

Epidemiological and surveillance studies using molecular genotyping methods, although complex and expensive, help to monitor the emergence of epidemic clones and implement infection-control strategies within CF centers.

Further molecular epidemiological studies are needed to identify contaminated environmental sources and routes of patient-to-patient transmission and to improve infection-control and therapeutic measures. Longitudinal and multicentre studies are needed to explore the epidemic and widespread clones of other CF centers in Turkey.

Acknowledgments

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Ethics approval and consent to participate

This study was approved by the Ethical Committee of Istanbul University Cerrahpasa Medical Faculty (date of

Table 3 Genotypes of *Pseudomonas aeruginosa* isolates from 18 patients

Genotype	No of Patient	No of Isolates*
1	6	6.1.1
2	8	8.1.1
3	7	7.1.4
4	3	3.3.2, 3.3.3
5	9	9.1.1
6	11	11.1.1
7	19	19.1.2
8	10	10.1.2
9	11	11.1.3
10	13	13.1.1
11	11	11.1.2
12	15	15.3.1, 15.3.2, 15.3.3, 15.3.4, 15.1.1, 15.1.2, 15.1.3, 15.2.1, 15.2.3, 15.2.2
13	7	7.3.2, 7.3.3
14	7	7.2.1, 7.2.2
15	3, 4	3.2.1, 4.2.1
16	3	3.1.1
17	6	6.1.2
18	14	14.2.1, 14.3.3
19	14	14.3.1
20	4	4.3.2
21	10, 11	10.2.1, 11.2.2, 11.2.1
22	4	4.1.1
23	17	17.1.1, 17.2.1, 17.2.2, 17.1.2, 17.1.3
24	17	17.3.1, 17.3.2, 17.3.3
25	4, 14	4.2.2, 14.1.2, 14.2.2, 14.2.3
26	9	9.2.1, 9.2.2, 9.3.1, 9.3.2
27	4,16	4.3.1, 16.1.3
28	13, 16	13.2.1, 16.2.2
29	19	19.2.1, 19.3.1
30	5	5.3.2, 5.3.3
31	11	11.3.1
32	12, 13, 14	12.3.2, 13.1.3, 14.3.2
33	16	16.2.1, 16.3.2
34	8	8.3.3
35	5	5.3.1
36	8	8.2.1
37	8	8.2.2, 8.3.2
38	8	8.1.2
39	16, 19	16.1.2, 19.1.1
40	12	12.2.1, 12.2.3
41	11, 12	11.3.2, 12.3.1
42	6	6.2.1, 6.3.1, 6.3.2
43	14	14.1.1
44	3	3.1.2
45	13	13.3.2
46	2	2.1.1, 2.1.2, 2.1.3, 2.2.1
47	12	12.1.1, 12.1.2
48	12	12.1.3
49	5	5.2.1, 5.2.2
50	5	5.1.1
51	17	17.1.4
52	19	19.2.1
53	13	13.2.2
54	12	12.2.2
55	3	3.3.1

Notes: *Number of isolates are defined according to patient number, period of isolation, number of isolate in the mentioned period (eg, 213; 2 is patient number, 1 is period of isolation, 3 is the third isolate in the first period).

approval 08/01/2013, approval number A-07) and conducted in accordance with the Declaration of Helsinki. Written informed consent to participate in the study was obtained from the patients enrolled or their parents.

Disclosure

The authors report no conflicts of interest in this work.

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