

MOLECULAR EPIDEMIOLOGY

Molecular epidemiology of *Pseudomonas aeruginosa* infections in a cystic fibrosis outpatient clinic

LUIZ V.F. DA SILVA FILHO, JOSÉ E. LEVI*, CHRISTINA N.O. BENTO†, JOAQUIM C. RODRIGUES and SÔNIA R.T. DA SILVA RAMOS

*Instituto da Criança, Hospital das Clínicas, *Laboratory of Virology, Instituto de Medicina Tropical de São Paulo and †Microbiology Section of the Central Laboratory, Hospital das Clínicas, University of São Paulo Medical School (FMUSP), São Paulo, SP, Brazil*

Chronic respiratory infection by *Pseudomonas aeruginosa* is a significant determinant in the prognosis of cystic fibrosis patients. Cross-infection between cystic fibrosis patients and the prevalence of *P. aeruginosa* among them were investigated by microbiological surveillance and RAPD typing of the isolates. A total of 748 samples was cultured, including specimens from the respiratory tract (sputum or throat swabs) and hands of patients and medical staff, resulting in the collection of 86 isolates of *P. aeruginosa* from 65 samples. Prevalence of *P. aeruginosa* was 39.3% in respiratory samples, 0.2% on patients' hands and none in the medical staff's hand samples. RAPD typing characterised 51 genotypes and clonal persistence was observed in the majority of patients. These results suggest that cross-infection is not common in the outpatient clinic studied and a common source of acquisition is unlikely.

Introduction

Cystic fibrosis (CF) is the most common lethal genetic disease in Caucasians and is characterised by chronic and recurrent lung infections, pancreatic insufficiency and high chloride levels in the sweat. Chronic respiratory infections in these patients are usually due to *Pseudomonas aeruginosa*; its persistence is associated with the production of alginate, a polymer that protects the micro-organisms against host defences and amplifies the inflammatory response in the airways [1]. Therefore, lung disease is manifested as chronic endobronchitis related to the presence of mucoid *P. aeruginosa* strains that are seldom eradicated once established in the airways of these patients, even with aggressive antibiotic treatment [1, 2].

The acquisition of chronic *P. aeruginosa* infection has a major impact on prognosis because it is usually followed by progressive deterioration of lung function [3]. Considering the complexity of this disease, treatment in specialised centres is required and there has been concern about cross-infection between pa-

tients in the last two decades, particularly with the emergence of multiresistant *P. aeruginosa* and *Burkholderia cepacia* strains [4–8]. With the objective of preventing the dissemination of these organisms, several CF centres have adopted infection control measures, which include outpatient and inpatient cohorting by colonisation status, infection control measures at the pulmonary function test laboratories and patient education [9, 10].

Infection control measures have also led to the need to develop adequate techniques for strain typing, because antibiotic susceptibility and other phenotyping methods such as serotyping are not reliable for epidemiological studies. Molecular typing methods, such as DNA macro-restriction or PCR-derived techniques, have emerged as the most efficient tools for strain discrimination [11–13]. Random amplified polymorphic DNA (RAPD) typing [14] is a PCR-based method with high discriminatory power used previously for strain typing [13, 15, 16].

In Brazil, CF is an underdiagnosed condition that is primarily treated at specialised centres, usually university hospitals. The Paediatric Pulmonology Outpatient Clinic of the Instituto da Criança (Children's Institute of the Clinics Hospital – University of São Paulo Medical School) is a general paediatric pulmo-

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Corresponding author: Dr L.V.F. da Silva Filho (e-mail: vicres@usp.br).

nology clinic that treats c. 120 CF patients. Sporadic cases of infection with multiresistant *P. aeruginosa* strains have been identified, but usually in unrelated patients. Considering the substantial role of *P. aeruginosa* in the lung disease of these patients, the possibility of cross-infection between CF patients was assessed and the prevalence of *P. aeruginosa* among them was determined by microbiological surveillance and RAPD typing of isolates.

Patients and methods

Patients

Ninety-six CF patients (47 male and 49 female; aged 9 months–19 years, mean age 8.46 SD 3.01 years) receiving treatment at the CF Outpatient Clinic of the Instituto da Criança were studied from Jan. to July 1996. Diagnosis of CF was based on clinical symptoms and two positive sweat tests. The clinic is a general paediatric pulmonology clinic with four offices with a shared corridor and a large waiting room, and CF patients are seen 3 days a week during the morning.

Samples

Samples of sputum or throat swabs and hand swabs of patients and medical staff were collected by one of the investigators (L.V.F. da S.F.) at each patient visit. Sputum or throat swabs were collected before swabbing patients' hands. Sputum samples were collected directly by expectoration in to a sterile plastic receptacle and throat swabs were collected by direct friction of a sterile cotton swab on the posterior pharynx, if possible after coughing and with use of a tongue depressor. Swabbing of the hands of medical staff was always performed after physical examination of the patient, without guidance or information regarding hand washing. Each sample collected was identified with a number and a letter ('A' for sputum or throat swabs, 'B' for patients' hand swabs and 'C' for medical staff hand swabs) and sent to the microbiology laboratory for culture within 4 h. Written informed consent was obtained from the patients' parents, accompanied by responses to a questionnaire about social contact with other patients. The Ethical Committee of the Institution approved the study.

Previous colonisation data

Data on previous colonisation were obtained by retrospective analysis of medical records. Chronic colonisation was defined as at least three cultures with isolation of *P. aeruginosa* in the last 6 months; intermittent colonisation was defined as positive cultures in the last year, but not fulfilling the criteria for chronic colonisation outside of the chronic criteria, and not colonised was defined as negative cultures in the last year (with at least two cultures collected). Serum precipitins were not determined.

Cultures

Samples were initially inoculated in to Thioglycollate Broth (Difco), incubated at 35°–37°C for 24 h, then sub-cultured on selective medium (Bacto Cetrimide Agar Base; Difco) and incubated for 24 h at 35°–37°C. Suspected isolates of *P. aeruginosa* were identified by standard test procedures with the GNI card, VITEK system (bioMérieux Vitek, Hazelwood, MO, USA) and additional biochemical tests when necessary. Bacterial growth was not quantified. All *P. aeruginosa* isolates were tested for their susceptibilities to antibiotics by the disk diffusion agar method as standardised by the National Committee for Clinical Laboratory Standards [17]. The following antimicrobial disks (μg) were used: amikacin 30, carbenicillin 100, cefoperazone 30, cefotaxime 30, ceftazidime 30, ceftriaxone 30, gentamicin 10, imipenem 10, pefloxacin 5, ciprofloxacin 5, aztreonam 30.

Results were recorded after incubation for 24 h at 37°C. Strains resistant to all of the agents in two or more of the following antimicrobial categories were defined as multiresistant: β -lactam antibiotics including imipenem and aztreonam, aminoglycosides, and the fluoroquinolone ciprofloxacin, or all of these [18].

DNA extraction

DNA was extracted from *P. aeruginosa* isolates by the proteinase K-phenol-chloroform method [19] with bacterial colonies suspended in 500- μl mixtures consisting of proteinase K (Gibco-BRL Gaithersburg, FL, USA) 200 $\mu\text{g}/\text{ml}$; 50 mM Tris, pH 8.0 and SDS 0.5%. The mixture was incubated at 65°C for 2 h and then boiled for 10 min. This was followed by two steps of organic extraction with phenol-chloroform and DNA precipitation with 2.5 volumes of cold ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2). The pellet was dried and suspended in sterile water, and DNA was quantified in a UV spectrophotometer (GeneQuant, Pharmacia, Uppsala, Sweden).

RAPD reactions

RAPD was performed as described previously [13] in 25- μl mixtures consisting of 10 mM Tris HCl (pH 8.0), 3 mM MgCl_2 , 50 mM KCl, containing 40 ng of template DNA, 1.6 μM of primer 272 (5'-AGCGGGC CAA-3'), 250 μM of each dNTP and Taq DNA polymerase (Gibco-BRL) 1 U. Amplification was performed as follows: four cycles of 94°C for 5 min, 36°C for 5 min and 72°C for 5 min; 30 cycles of 94°C for 1 min, 36°C for 1 min, 72°C for 1 min; and 72°C for 10 min. RAPD products were visualised in agarose 1.5% gels stained with ethidium bromide 0.5 $\mu\text{g}/\text{ml}$ with a UV transilluminator. RAPD patterns were determined by visual inspection. RAPD products between 100 bp and 2 kb with intensities such that

they were reproducible in two or more reactions were used for comparison between isolates.

Statistical analysis

Mucoid and non-mucoid strain susceptibility to antibiotics was compared with χ^2 Yates corrected or Fisher's two-tailed exact tests. Comparisons between age groups and *P. aeruginosa* isolation in respiratory samples were performed by the χ^2 test.

Results

A total of 748 samples (135 sputum, 119 throat swabs, 254 swabs of patients' hands, 240 hand swabs from medical staff) was cultured. *P. aeruginosa* was isolated from 65 samples (Table 1). *P. aeruginosa* was isolated from at least one sample from 38 patients (39.5%). The majority of the patients with at least one *P. aeruginosa* positive culture (28 of 38, 58%) were previously characterised as chronically colonised (Table 2). The age distribution of the patients and culture results are presented in Table 3, and a surprisingly higher prevalence of *P. aeruginosa* was observed for the age group 5–10 years. *P. aeruginosa* was isolated from more than one sample from the same patient in 17 instances, but concomitant positive cultures from

different sites were not observed. The two patients with positive hand swabs were infants, and *P. aeruginosa* was not isolated from respiratory samples from these patients. Hand samples from the medical staff were all negative for *P. aeruginosa*.

A total of 86 *P. aeruginosa* strains was identified in the 65 positive samples. Mucoid strains were detected in 23 samples. The susceptibility of *P. aeruginosa* isolates to the antibiotics tested is presented in Table 4. Resistance to the amikacin, carbenicillin and ciprofloxacin was significantly greater for mucoid strains ($p < 0.05$). Resistance to ceftazidime was similar for mucoid and non-mucoid strains (20%). Three multiresistant strains (3.4%) were isolated, two of them from the same patient on different occasions, with the same susceptibility pattern except for susceptibility to ceftazidime.

There were ten CF sibling pairs among the 96 patients studied, but *P. aeruginosa* was isolated from both children in only one sibling pair (two sisters). Five different strains were isolated on different occasions from these girls, each one with a particular pattern of susceptibility to antibiotics.

RAPD typing was performed on 83 isolates of *P. aeruginosa* and 51 distinct patterns were obtained (Figs. 1 and 2). *P. aeruginosa* isolates from the same patient usually had the same RAPD pattern; this was also observed with the isolates from the same sample with different morphology (mucoid, non-mucoid) or antimicrobial susceptibility. Some patients had more than one genotype during the period, e.g., patient LR (Fig. 2), who had four different genotypes and patient HY (Fig. 1), who apparently acquired a new strain as shown by the RAPD pattern of the last isolate (lane 14). RAPD results on the isolates from the two sisters revealed that they were colonised with different strains, but the isolates from each one had the same RAPD pattern (Fig. 2; NE and NA). There were two occasions when two patients gave organisms with the same RAPD pattern; they did not report social contact and the samples were collected on different days. RAPD typing of the three multiresistant strains showed different genotypes, even for the two isolates from the same patient.

Table 1. Distribution of samples and *P. aeruginosa* isolates

Sample	Number collected	<i>P. aeruginosa</i> -positive (%)
Sputum	135	53 (39.3)
Throat swabs	119	10 (8.4)
Swabs of patients' hands	254	2 (0.8)
Swabs of medical staff hands	240	0
Total	748	65 (8.6)

Table 2. Previous bacterial colonisation and *P. aeruginosa* isolation from respiratory samples

Previous colonisation	Number of patients (%)	<i>P. aeruginosa</i> -positive
Chronic	48 (50)	28
Intermittent	30 (31)	8
Not colonised	18 (19)	2
Total	96	38

Table 3. Age distribution and *P. aeruginosa* isolation from respiratory samples

Age (years)	Number of patients	<i>P. aeruginosa</i> -positive (%)
0–2	10	2 (20.0)
2–5	19	7 (36.8)
5–10	33	16 (48.4)
10–15	21	8 (38.1)
>15	13	5 (38.4)
Total	96	38

(χ^2 ; 4 degrees of freedom; p value $\cong 0.59$).

Discussion

In this study, *P. aeruginosa* was isolated at least once from 39% of the patients during the 6-month period of surveillance, while retrospective analysis of medical records showed 50% of patients with chronic and 31% with intermittent colonisation. Although the proportion of colonised patients in the retrospective analysis is similar to that observed in many CF centres around the world [10, 20, 21], a lower prevalence of *P. aeruginosa* was observed in airway samples from the patients in

Table 4. Antimicrobial susceptibility of *P. aeruginosa* isolates

Antibiotic	Number susceptibility/number tested (%)			p value
	Mucoid strains	Non-mucoid strains	Total	
Amikacin*	15/23 (65.2)	59/63 (93.6)	74/86 (86.0)	<0.01
Carbenicillin*	18/23 (78.26)	60/63 (95.2)	78/86 (90.6)	0.03
Cefoperazone	11/15 (73.3)	40/48 (83.3)	51/63 (80.9)	0.46
Cefotaxime	15/23 (65.2)	43/63 (68.2)	58/86 (67.4)	1.00
Ceftazidime	18/23 (78.2)	52/63 (82.5)	70/86 (81.3)	0.76
Ceftriaxone	8/23 (34.8)	19/62 (30.6)	27/85 (31.8)	0.92
Gentamicin	14/23 (60.9)	51/63 (80.9)	65/86 (75.5)	0.10
Imipenem	19/23 (82.6)	59/63 (93.6)	78/86 (90.6)	0.20
Pefloxacin	2/5 (40.0)	6/14 (42.8)	8/19 (42.1)	1.00
Ciprofloxacin*	15/23 (65.2)	56/63 (88.9)	71/86 (82.5)	0.02
Aztreonam	20/23 (86.9)	59/63 (93.6)	79/86 (91.8)	0.38

*p < 0.05 (χ^2 Yates corrected or Fisher's two-tailed exact tests).

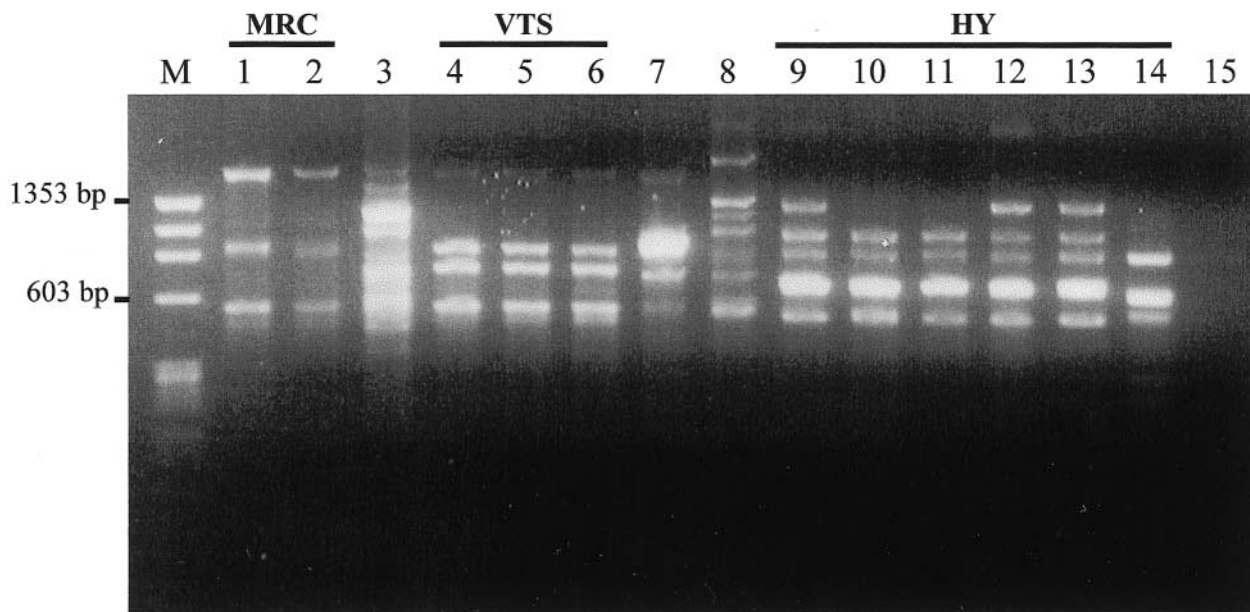


Fig. 1. Agarose 1.5% gel electrophoresis stained with ethidium bromide of RAPD products from *P. aeruginosa* isolates of CF patients. Lanes: **M**, mol. wt markers (PhiX174 RF DNA, *Hae*III digest; Pharmacia, Uppsala, Sweden); upper case letters indicate patients' initials, randomly re-arranged; **15**, negative control (no DNA added).

the present study. These results probably reflect the short period of observation, because the majority of patients were seen only once or twice in that period, and negative cultures can be obtained from patients with intermittent or even chronic colonisation.

The bacteriology of respiratory infections in CF has been well studied by several authors [22, 23] and some agree that there is good correlation between upper and lower respiratory tract colonisation [24, 25]. Indeed, sputum and throat cultures have been used world-wide to monitor and guide treatment of respiratory infections in CF patients. However, recent published data comparing oropharyngeal and quantitative broncho-alveolar lavage (BAL) cultures have concluded that oropharyngeal cultures do not reliably reflect lower respiratory tract infection [26, 27]. Nevertheless, most of the studies that evaluated the impact of bacterial colonisation on prognosis used oropharyngeal and sputum cultures for investigation [3], and the BAL criteria for the diagnosis of lower respiratory tract

infection in CF were established only in 1994 [28]. Therefore, so far there is no information as to whether the BAL criteria have the same correlation with prognosis.

The isolation of *P. aeruginosa* from throat swabs (8.4%) was significantly inferior to that obtained from sputum (39%). This is not surprising and a lower prevalence of *P. aeruginosa* was expected in patients with less suppuration or younger age, but this probably also reflects the reduced sensitivity of the method for detection of *P. aeruginosa*. The low sensitivity of throat swab sampling is problematic, because early diagnosis and treatment of *P. aeruginosa* colonisation seems to delay chronic colonisation and to result in a better prognosis, and BAL samples are not simple to obtain routinely from these patients. These technical aspects of airway sampling have led some authors to investigate more sensitive techniques for *P. aeruginosa* detection, such as PCR [29–31], but this approach has never been tested on clinical grounds as a routine test.

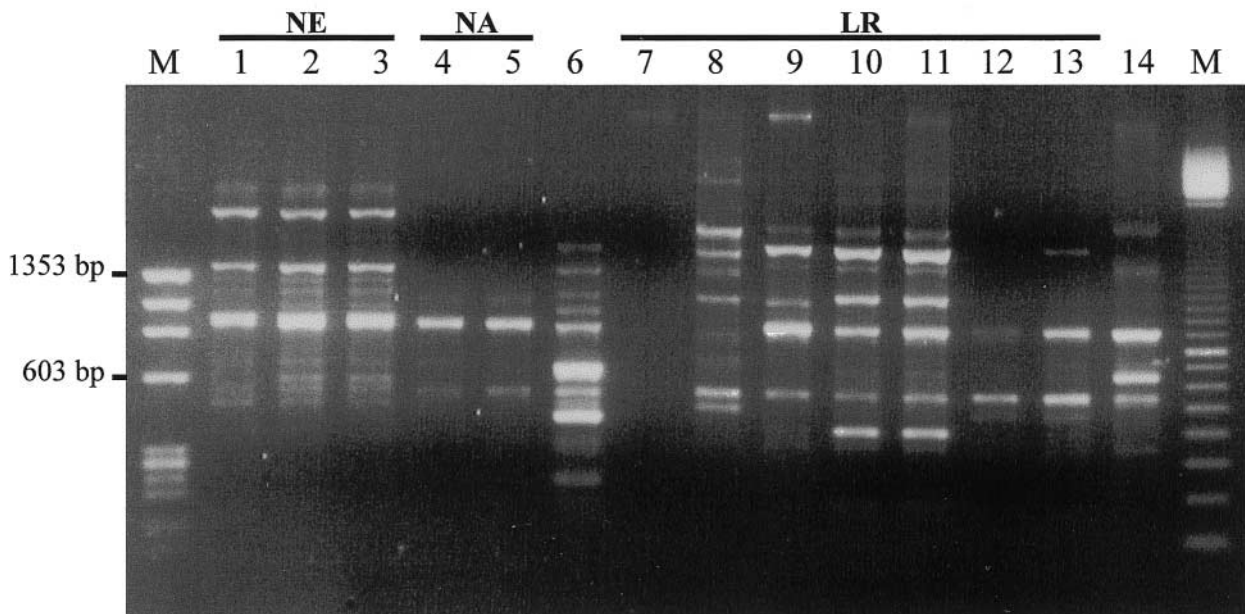


Fig. 2. Agarose 1.5% gel electrophoresis stained with ethidium bromide of RAPD products from *P. aeruginosa* isolates of CF patients. Lanes: **M**, mol. wt markers (PhiX174 RF DNA, *Hae* III digest and 100-bp ladder; Pharmacia); upper case letters indicate patients' initials, randomly re-arranged; 7, an isolate that did not result in RAPD products, classified as non-typable (NT); 15, negative control (no DNA added).

A trend was observed (although it was not statistically significant) of a higher prevalence of *P. aeruginosa* among younger patients, 5–10 years of age. This finding may be a consequence of delayed diagnosis in Brazil, with many patients referred to this hospital after the age of 5 years, when chronic *P. aeruginosa* colonisation had started in the first years of life. Delayed diagnosis has a major impact on prognosis and can also be the cause of premature death among patients with more severe disease (and a higher proportion of *P. aeruginosa* colonisation) causing this 'preponderance' of *P. aeruginosa* colonisation at younger ages. The fact that the prevalence of *P. aeruginosa* colonisation remained stable after the age of 10 years supports this hypothesis, because an increasing prevalence of *P. aeruginosa* would be expected with age. This result can also be viewed as an alert, because this clinic does not routinely employ aggressive treatment for the first colonisation of *P. aeruginosa* as suggested by some authors [32, 33], and that may explain *P. aeruginosa* colonisation in younger children.

With regard to antibiotic susceptibility, a relatively low proportion of resistance to ceftazidime (19%) was found, although this drug is our first line drug for the treatment of pulmonary exacerbation in *P. aeruginosa* colonised patients, always in addition to aminoglycosides. This result is similar to observations made at other CF centres [34], except for a British CF centre that recently reported epidemic spread of a β -lactamase-producing *P. aeruginosa* strain and, therefore, detected 70% resistance to ceftazidime [8]. However, the latter centre used to prescribe ceftazidime as monotherapy, and this could induce or select β -

lactamase-producing *P. aeruginosa* strains. Mucoid isolates from the patients in the present study showed higher resistance to several antibiotics than non-mucoid strains, which can be attributed to the presence of large amounts of alginate acting as a physical barrier to antibiotic diffusion, or to a colonisation of longer duration and greater exposure to antibiotics. Multi-resistant *P. aeruginosa* strains were found on only three occasions (3.4%), a rate lower than that reported by Saiman *et al.* [34], who identified 13.3% of multi-resistant strains among 1296 isolates from 67 CF centres.

RAPD typing characterised 51 genotypes among 83 isolates of *P. aeruginosa*. Isolates with different genotypes were not observed in the same sample even when different morphological appearances or susceptibility to antibiotics were present. These data suggest that antibiotic susceptibility and morphology of the colonies are not reliable means for typing or discriminating among *P. aeruginosa* strains, and genotyping is warranted for such purposes. *P. aeruginosa* strains from CF patients are particularly difficult to type; they are usually lipopolysaccharide-deficient and tend to lose the phage and pyocin typing responses due to phenotypic conversion [11, 12].

Indeed, genotyping has been used as the main tool for epidemiological studies of *P. aeruginosa* colonisation in CF patients. Techniques of DNA macro-restriction and pulsed-field or field inversion gel electrophoresis have been used in many studies to identify cross-infection between CF patients [35–37]. Although these techniques have high discriminatory power, they are expensive, difficult, and generally require a hybridisa-

tion step. As a result, RAPD has emerged as a fast and simple PCR-derived technique with high discriminatory power, suitable for application in epidemiological studies [13, 16, 38, 39]. However, because of the low stringency conditions and the random features of the method, DNA extraction and quantification must be very well controlled to avoid amplification of spurious DNA and to maintain its reproducibility and reliability [40].

The results obtained with RAPD typing of the *P. aeruginosa* isolates were in keeping with the results described by other investigators [11, 13], as the persistence of one strain in each patient was observed throughout the study. Despite that, some patients had more than one genotype during the period studied, and one patient had four different genotypes. This clonal persistence of *P. aeruginosa* strains in CF patients was described previously during an 8-year follow-up by Römling *et al.* [11] and confirmed later by Mahenthiralingam *et al.* [13]. Although the typing method applied was different, both studies also reported few temporal changes in genotype patterns, which were attributed to re-arrangements in DNA structure or mutations, and perhaps these changes represent the genetic basis for conversion of *P. aeruginosa* strains to a common phenotype.

The method used by Mahenthiralingam *et al.* [13] was reproduced in this laboratory with similar results, although with fewer changes in RAPD profiles, perhaps because of the shorter period of observation.

Cross-infection between CF patients was investigated by several authors and identified between sibling pairs [24, 35] and patients with close social contact, such as those who attend CF summer camps [24, 36, 38, 41]. Multiresistant *P. aeruginosa* [5] and *B. cepacia* [7, 42] cross-infections were also identified. The implications of these findings were significant, and patient cohorting by colonisation status ensued in several CF centres world-wide [9, 10]. Furthermore, in a recent publication Mahadeva *et al.* [43] reported earlier colonisation with *P. aeruginosa* among referred patients who were formerly cared for at specialised CF centres compared with those cared for by general practitioners, which could be attributed to a higher risk of cross-colonisation in CF centres. These findings were contested by Spencer [44], who supports the belief that the earlier colonisation reflects a major need for early detection of *P. aeruginosa* in CF centres, and that cross-colonisation is unusual in paediatric CF centres. In the present study, indistinguishable isolates were identified in different patients on only two occasions; these four patients did not report social contact and the samples were collected on different occasions. Furthermore, neither pair of patients ever shared a common room during hospital admissions, and these CF patients do not have common social activities like summer camps or physiotherapy sessions, suggesting that cross-colonisation, if it occurs, is rare in this outpatient clinic.

This idea was reinforced by the small number of isolates on the hands of patients (2 of 254, 0.8%), the absence of *P. aeruginosa* on the hands of medical staff, and the finding of different genotypes among the three multiresistant strains.

The role of the inanimate environment as a route of *P. aeruginosa* transmission in CF patients is a matter of debate. Previous studies [45–47] could not identify *P. aeruginosa* reservoirs in hospital wards or found a small number of strains that were different from CF patients' isolates. However, Döring *et al.* [48] identified transmission of *P. aeruginosa* between CF patients and the inanimate environment (sink drains), in addition to contamination of the hands of patients and personnel in a paediatric ward. In the present study, a common source of *P. aeruginosa* acquisition seems unlikely, because a large number of genotypes was identified among the patients, but it cannot be ruled out because many of them were admitted to the hospital at least once. However, the small number of isolates from the hands of patients may be a consequence of the method of sampling (cotton swabs), because the method of hand immersion in saline solution used by Döring *et al.* [48] seems to be more sensitive.

In conclusion, the prevalence of *P. aeruginosa* in respiratory samples from these CF patients was 39%. No *P. aeruginosa* cross-infection between patients was observed and no common source of acquisition was identified. RAPD typing characterised 51 genotypes among the 83 isolates of *P. aeruginosa*, and clonal persistence was observed in the majority of patients with more than one isolate. However, these results are the outcome of a short period of observation, and longer periods of surveillance are required to confirm them.

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