Molecular epidemiology of chronic pulmonary colonisation by *Pseudomonas aeruginosa* in cystic fibrosis

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Summary. The epidemiology of pulmonary colonisation by *Pseudomonas aeruginosa* was studied in 21 patients with cystic fibrosis (CF) by field inversion gel electrophoresis. *DraI*-DNA restriction patterns were analysed for 187 *P. aeruginosa* isolates from these patients. The results revealed that the strains present in individual patients varied during the course of chronic colonisation; the emergence of new strains often was associated with periods of antibiotic therapy. Patients often were colonised by more than one strain (two or three strains were present in 54% of the patients) and the strains obtained from unrelated patients were highly heterogeneous, in contrast to those isolated from a pair of twins. These results demonstrate the heterogeneity and variability of *P. aeruginosa* isolates in the pulmonary flora of chronically infected CF patients.

Introduction

Pseudomonas aeruginosa is a major opportunist pathogen in patients with cystic fibrosis (CF).^{1,2} Pulmonary infection by *P. aeruginosa* in these patients is refractory to treatment with antibiotics and ultimately leads to clinical deterioration and death.¹ Surprisingly, little is known about the epidemiology and transmission of *P. aeruginosa* in CF patients, particularly as regards (i) the diversity of strains present among different chronic carriers followed at the same medical centre, (ii) the variability and the multiplicity of strains present in the individual patients, (iii) the influence of antibiotic therapy on the emergence of new strains, and (iv) the impact of person-to-person and environmental exposure on chronically infected patients. This lack of information stems from the limits of conventional typing methods based on serotype, phage type or pyocin production. These methods are unsuitable for many of the mucoid strains isolated from CF patients.³ New typing methods based on genome analysis, such as pulsed field electrophoresis, may be more appropriate for epidemiological studies on *P. aeruginosa* isolates from CF patients. Pulsed field electrophoresis allows the separation of large nucleic-acid fragments (50–9000 kb), produced by digestion of bacterial DNA with endonucleases that cut at infrequent restriction sites.^{4,5} The restriction patterns produced appear specific to individual strains and have already been

applied to the analysis of the epidemiology of bacterial infections,^{6,7} including those in the lungs of CF patients.^{8–10} In the present study this method was used to study the epidemiology of *P. aeruginosa* in chronically infected CF patients.

Patients and methods

Patients

Twenty-one patients, 11 female and 10 male, aged between 4 and 22 years, were studied. They comprised 19 unrelated individuals and one pair of twins. All were followed in the CF Centre of the Hôpital Necker-Enfants Malades. All these patients were colonised by *P. aeruginosa*, some for < 1 year, but most for several years (fig. 1). Exacerbations of their infections were treated by 2–4-week courses of various antibiotic combinations, generally ceftazidime-tobramycin, ciprofloxacin-tobramycin or imipenem-amikacin. Patients 12 and 20 died during the study, the former shortly after heart-lung transplant. Most patients included in this study were regularly hospitalised, usually for periods of c. 2 weeks, in a paediatric ward of our hospital. This unit comprised 10 single bedrooms, two double-bedrooms, and two four-bedded rooms, each equipped with sinks. Patients shared the same shower, bath and toilet facilities. Infected CF patients were not segregated from one another and shared many common activities including meals, games, toys and hospital school, thus allowing frequent and close person-to-person exposure.

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Fig. 1. Duration of pulmonary colonisation with *P. aeruginosa* in 21 CF patients: ○ male; ● female.

Bacteriological methods

Selective Cetrimide Medium (Diagnostics Pasteur, Marne la Coquette, France) was used for culture of respiratory specimens. Most of the *P. aeruginosa* isolates from the sputum of the CF patients initially produced mucoid colonies. Sometimes, non-mucoid clones segregated, which variously had "fried-egg", "smooth" or "dwarf" colony morphology. Bacteria were identified with the API-20NE system (API System, La Balmes-les-Grottes, France). Serotyping was performed with 16 monospecific sera directed against O antigens (Diagnostics Pasteur). More than half of the isolates could not be serotyped. Antibiotic susceptibility was studied by the agar diffusion method on Mueller-Hinton Medium (Diagnostics Pasteur). Separated colonies were grown and stored at -70°C in LabLenco Medium (Oxoid) until electrophoretic analysis.

Field inversion gel electrophoresis (FIGE)

Bacterial DNA was analysed by field inversion gel electrophoresis (FIGE). This pulsed field electrophoresis technique was based on that of Grothues *et al.*⁹ and allowed good separation of DNA fragments of 50–1000 kb. Briefly, bacteria from a single colony were grown in tryptone-water to logarithmic phase, harvested, and then resuspended to an optical density of 1.5 at a wavelength of 650 nm. The bacterial suspension was mixed with an equal volume of Incert Agarose (FMC BioProducts, Rockland, ME, USA), to form an agar insert, then incubated for 48 h at 55°C with 0.5 M EDTA (Sigma), pH 9, sarcosine (Sigma) 1% w/v and proteinase K (Appligene, Illkirch,

France) 1 g/L. Subsequently, the inserts were washed extensively: twice for 20 min each with TE buffer (0.1 M Tris, 0.01 M EDTA, pH 7.5) then with 0.01 M phenylmethyl sulphonyl fluoride (Sigma); next with TE buffer for 1 h at 37°C and finally with 1 mM Tris-HCl buffer, pH 7.5, overnight at 4°C . Since the GC mol % of *P. aeruginosa* is 67.2%,² restriction endonucleases recognising AT-rich restriction sites were used (*DraI*, *XbaI*, *SpeI*, *SspI*, *NheI*). The best results were obtained with *XbaI* and *DraI*, which cut TCTAGA and TTTAAA sites respectively. In this report, only the *DraI* restriction patterns are presented. Purified DNA was digested overnight by *DraI* (30 IU) (Appligene). The fragments were separated by FIGE in 0.025 M Tris-borate-EDTA buffer, pH 8.5, on a Rotaphore 10035 Apparatus (Biometra, Göttingen, Germany), run at 10°C and a field strength of 5 V/cm for 96 h. The forward:reverse ratio was 3:1. The gels were stained with ethidium bromide. Controls included DNA from λ phage (FMC BioProducts) and DNA from *P. aeruginosa* ATCC 27853, prepared by the same method as described above. Restriction profiles were considered to be identical even if they differed by one or two bands. Such minor differences most probably result from point mutations at restriction sites.⁹ As controls, we tested 43 wild-type non-mucoid *P. aeruginosa* isolates from opportunist infections in non CF-patients. These isolates all gave different *DraI* restriction patterns (data not shown), confirming the high genomic diversity amongst *P. aeruginosa* strains already observed by FIGE analysis.⁹

Results

Analysis of serial *P. aeruginosa* isolates from CF patients

The *P. aeruginosa* isolates cultured from 10 to 13 sequential sputum samples from each of three patients (nos. 12, 17 and 20) were examined. Each of these patients was between 15 and 20 years old and had been colonised for 4–10 years. With some samples, more than one colony type was seen on the culture plates. In such cases, each colony type was analysed separately and, in total, 68 isolates were examined by FIGE of *DraI*-digested DNA (fig. 2).

The first samples from Patient 20 contained a single strain. This strain was rapidly replaced by another strain, which remained dominant in the patient's sputum during the next 15 months, despite repeated antibiotic therapy. The other two patients frequently harboured more than one strain at a time (e.g., specimen 4 from Patient 12). Shortly after heart-lung transplantation, Patient 12 was colonised with a new strain, never previously isolated from this individual. The emergence of new strains was usually associated with periods of antibiotic therapy, which failed to eradicate *P. aeruginosa* strains from the sputum of these patients. No particular antibiotic therapy was

Fig. 2. *DraI*-restriction patterns of 68 mucoid strains of *P. aeruginosa* sequentially isolated from three CF patients. The specimens from each patient are numbered in order of collection, and the dates of collection are indicated in the upper part of the figure. One to four colonial phenotypes were studied for every specimen. T, heart-lung transplantation; †, death; R, reference strain ATCC 27853; λ , concatemer phage λ .

Fig. 3. FIGE restriction patterns of 14 *P. aeruginosa* isolates from twins with CF. Day 1: lane 11, twin No. 9; 12–14, twin No. 10. Day 41: lanes 1–3, twin No. 9; 4–6, twin No. 10. Day 69: lanes 7–8, twin No. 9; 9–10, twin No. 10.

associated with the emergence of new strains in these three patients.

Heterogeneity of *P. aeruginosa* isolates from CF patients

FIGE was used to analyse a total of 187 mucoid *P. aeruginosa* isolates from the 21 chronically infected

Table. Colonisation with multiple *P. aeruginosa* strains in CF patients

Duration of colonisation by <i>P. aeruginosa</i>	Patient* Nos.	Number of samples	Number of <i>P. aeruginosa</i> strains detected†		
			1	2	3
< 1 year	1, 2	2	2	0	0
3–5 years	7, 9–13 16, 17, 19	15	5	8	2
> 10 years	20, 21	4	3	1	0
Total	13	21	10	9	2

* See reference numbers in fig. 1.

† Three to six isolated colonies of *P. aeruginosa* were tested by FIGE for each specimen, except for Patient 1 from whom 14 clones were tested. In total 83 isolates were examined.

CF patients. The strains isolated from 19 unrelated patients all appeared different to one another (not shown). In contrast, the restriction patterns of 14 serial isolates cultured from a pair of CF twins were identical during a 2-month period (fig. 3). The twins (Patients 9 and 10), aged 12, had been colonised at the same time, 3 years before the study. At the start of the study, both carried the same single strain. After a 2-week period of antibiotic therapy (day 41), they were found to be colonised by two or three different strains, still including the pre-therapy strain, which again predominated by day 67 of the study (fig. 3).

Multiple *P. aeruginosa* isolates from individual patients

The existence of multiple *P. aeruginosa* strains in sputum specimens from CF patients was demonstrated by analysing, with FIGE, between three and six colonies from each of 21 samples obtained from 13

colonised CF patients. These comprised two individuals colonised for < 1 year, nine infected for 3–5 years, and two colonised for > 10 years (table); 83 clones were analysed. In seven (54%) of the 13 patients, two or three strains dominated, two patients (15%) being infected by three distinct strains. The two recently colonised patients (< 1 year), probably harboured only single strains; six to 14 colonies examined were identical within each of these individuals.

Discussion

When FIGE was used to study the epidemiology of chronic pseudomonal colonisation in CF patients, it was found that the *P. aeruginosa* strains isolated from sputum varied during chronic infection. Analysis of 68 serial isolates from three patients over periods of 15–18 months revealed that the dominant strain could vary, that new strains could emerge and sometimes previous strains could re-emerge after periods in which they were not apparent in the flora (fig. 2). Administration of antibiotics, which always failed to eradicate *P. aeruginosa* infection, often led to changes to the strains isolated, probably caused by the selection of strains that were previously present in low numbers. However, we did not find any change to the strain present in Patient 20, regardless of treatment. A similar result was reported for another patient, followed over a 2-year period by Pasloske *et al.*¹¹ by a DNA probing method.

Previous studies, based on examination of colonial phenotypes or classical markers, have disagreed on whether pulmonary colonisation by *P. aeruginosa* in CF patients is homogeneous, or heterogeneous.^{11–15} In

this study, 83 strains from 21 sputum samples obtained from 13 patients were examined. Seven of 13 (54%) patients were colonised by more than one strain. Grothues *et al.*⁸ also used FIGE, and found that 37% of patients were infected by more than one strain. Since we analysed only three to six colonies from the sputum of each patient, it is probable that we failed to detect the presence of multiple strains in many more patients.

Based on *DraI*-restriction patterns, the strains isolated from 19 unrelated patients were highly heterogeneous, in contrast to strains isolated from a pair of twins. Similar results were reported by Grothues *et al.*⁸ The diversity of strains isolated from unrelated patients who had been followed with periodic hospitalisation, for several years in the same medical centre with repeated contacts, argues against cross-infection. This is in contrast to the behaviour of *P. cepacia*, with which cross-infection has been reported.¹⁶ It has been reported that non-infected CF patients are resistant to colonisation by mucoid strains of *P. aeruginosa*.¹⁷ Although the environmental source of primary colonisation by *P. aeruginosa* remains poorly understood,^{18,19} our data suggest that it is most likely from the wide variety of environmental sources where *P. aeruginosa* can occur, thus explaining the heterogeneity of strains.

In conclusion, in addition to plasmid fingerprinting^{20,21} and to DNA probing,^{11,22} pulsed field electrophoresis is a powerful epidemiological tool for the study of colonisation by *P. aeruginosa* in CF patients.

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