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Pseudomonas aeruginosa cross-colonization and persistence in patients with cystic fibrosis. Use of a DNA probe

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SUMMARY

To investigate cross-colonization with and persistence of *Pseudomonas aeruginosa* in cystic fibrosis (CF), 181 isolates from 76 CF patients were typed using a *P. aeruginosa*-specific DNA probe. Whereas sibling pairs predominantly harboured genotypically identical *P. aeruginosa* strains, all of the other patients harboured different strains. Seventy-nine per cent (22/31) of the infected CF patients harboured the same strains at the beginning and the end of a summer camp. A change of strains was seen in 10% (3/31) of the patients at the end of the camp. Forty-six per cent (6/13) of the patients who were apparently initially uninfected, acquired *P. aeruginosa* by the end of the period. Genotyping proved that strain change or acquisition was due to cross-colonization in four of nine cases. Very little *P. aeruginosa* was isolated from the inanimate environment. Persistence of *P. aeruginosa* after a temporary loss due to antibiotic therapy was seen in 12/16 paired patient strains before and after antibiotic therapy. Thus, suppression followed a flare-up seemed to occur in these patients rather than eradication and a new infection. When 35 patients were followed over a period of 6 months, 7 (20%) changed the strain in their sputum. Only one of 43 patients harboured two different *P. aeruginosa* strains simultaneously over a long period.

INTRODUCTION

In patients with cystic fibrosis (CF) *Pseudomonas aeruginosa* is the predominant bacterium associated with pulmonary infection (Høiby, 1982). This infection is thought to be crucial to patient life expectancies since specific antibiotic therapy normally fails to eradicate the organism from the airways and since a close relationship exists between chronic *P. aeruginosa* infection and poor clinical status and prognosis (Høiby, Döring & Schiøtz, 1986). The sources from which CF patients acquire this opportunistic pathogen and the routes of infection are largely a matter of speculation. Previous epidemiological studies yielded conflicting

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results concerning nosocomial cross-colonization and cross-infection between CF patients and the role of the inanimate environment in this process (Høiby & Rosendal, 1980; Kelly *et al.* 1982; Speert & Campbell, 1987; Thomassen *et al.* 1985; Zimakoff *et al.* 1983; Speert, Lawton & Damm, 1982; Laraya-Cuasay, Cundy & Huang, 1976; Bergan & Høiby, 1975; Seale *et al.* 1979; Zierdt & Williams, 1975; Pedersen *et al.* 1986).

Until recently, epidemiological studies on *P. aeruginosa* in CF have been hampered by the lack of a stable strain specific marker, since the classic typing methods such as pyocin, bacteriophage, and serotyping have considerable limitations for *P. aeruginosa* isolates. The development of a DNA probe consisting of the upstream region of the 'exotoxin A' (*ExoA*) structural gene of *P. aeruginosa* has greatly facilitated investigations in this field (Vasil, Chamberlain & Grant, 1986). Using this probe in Southern blot analysis, more than 100 *P. aeruginosa* strains have been differentiated and isolates which were indistinguishable by serotyping, biotyping and antibiograms were easily distinguished (Ogle *et al.* 1987).

In the present study a similar DNA probe containing the ExoA structural gene and the adjacent upstream region was used to investigate several open questions in the epidemiology of *P. aeruginosa* in CF. Is cross-infection likely to occur between CF patients in a clinic or at home? Does the environment represent a major source for acquiring *P. aeruginosa* lung infections? Is a temporary 'loss' of *P. aeruginosa* after antibiotic therapy the result of bacterial eradication or suppression? How many strains are harboured by a CF patient at the same time in the sputum? How long can a given strain be detected in the sputum? We present evidence that (1) cross-infection in a CF clinic as well as between siblings at home may occur, (2) *P. aeruginosa* is rarely found in the inanimate environment, (3) *P. aeruginosa* is not eradicated but only suppressed after successful antibiotic therapy, (4) CF patients normally harbour only one *P. aeruginosa* strain in their sputa at a time, and (5) after 6 months 26% of the infected patients had changed their *P. aeruginosa* sputum genotype.

MATERIAL AND METHODS

Subjects

For *P. aeruginosa* typing, sputa from 46 CF patients at the beginning (43 sputa) and the end (44 sputa) of a 6-week recreational stay at the CF clinic Satteldüne, Amrum, Federal Republic of Germany, and from 4–10 months later (35 sputa) were obtained. Patients 1–15, 16–35 and 36–46 were together at the same time in the clinic (mean age, 14·7 years; range, 5–32 years). Additionally, *P. aeruginosa* isolates or whole sputum was obtained from 12 European CF sibling pairs and from 11 CF patients before and after antibiotic therapy. Sera were obtained from 7 CF patients. Diagnosis of CF was based on accepted criteria including significantly increased sweat electrolyte levels (Wood, Boat & Doershuk, 1976). Informed consent was obtained from patients, parents or guardians.

Clinical score

To evaluate the patients' clinical state, a scoring system was developed based on the following lung function parameters: airway resistance, intra-thoracic gas volume, the ratio of residual volume to total lung capacity, forced expired volume in 1 s, and inspiratory vital capacity. Additionally, p_{O_2} values were incorporated. The patients' weight and height were evaluated using the somatogram developed by Vogt (1959). The divergence of the parameters from normal values were graded from 0–10 and the seven figures were added up. The patients were classified as belonging to group A (0–15, mild); B (16–35, evident) or C (> 36, severe).

Specimen collection

In order to isolate *P. aeruginosa* from inanimate surfaces in the CF clinic at Amrum and in the homes of three families with *P. aeruginosa*-infected CF children, Rodac plates (Greiner, Nürtingen, Federal Republic of Germany) with cetrimide agar (Difco, Detroit, USA) were used. A total of 290 plates was rolled over beds, tables, toys, washing basins, door-handles, toilet seats, swimming pools, examining couches, carpets and indoor plants. Air samples in the CF clinic and in family homes were obtained by exposing open Rodac plates for 1–8 h in various rooms and using a slit sampler with a rotating cetrimide agar plate (35 l/h) (Heinrich Burghart, Wedel, Federal Republic of Germany). If the patients produced sputum, it was solubilized in 5 ml of trypticase soy broth (TSB) (Difco), incubated at 37 °C overnight, and cultured on cetrimide agar plates for 24–28 h; 5 ml TSB supplemented with 25% glycerol (w/w) was added to the plates, all bacterial colonies were scraped from the agar using a pipette and the suspensions were stored at −70 °C until use. From three sputum specimens six single colonies with non-mucoid and mucoid morphologies were separately isolated. Rodac plates were incubated at 37 °C for 48 h. The *P. aeruginosa* isolates were characterized by colony morphology, biochemical reactions, growth at 42 °C, and genotyping.

Genotyping of P. aeruginosa

Genotyping of *P. aeruginosa* with the *ExoA* DNA probe was carried out as described previously with some modifications (Vasil, Chamberlain & Grant, 1986). Chromosomal DNA was isolated using standard methods (Marmur, 1960). The purified DNA (1–2 μg) was digested with the restriction endonucleases *Bgl* II, *Sal* I and *Xho* I, electrophoresed through a 0·6% agarose gel and transferred to a nylon membrane (Pall, Dreieich, Federal Republic of Germany) using the method of Southern (1975). The *Escherichia coli* plasmid pCMtox (Vasil, Chamberlain & Grant, 1986) (Figure 1) was isolated (Maniatis, Fritsch & Sambrock, 1982), and labeled with biotin-11-dUTP (Gibco, Bethesda, USA) by nick translation. In modificaton of the original method, the whole pCMtox plasmid was used for hybridization. The nylon membranes were prehybridized for 2 h, followed by hybridization at 42 °C for 18 h using a hybridization solution of 45% (vol/vol) formamide, 5 × SSC, 1 × Denhardt's solution, 20 mM sodium phosphate, pH 6.5, 5% dextran sulfate and 0.2 mg/ml sheared herring sperm DNA. Streptavidin/alkaline phosphatase reagent was added to the washed and blocked membranes. The membranes were then developed using BCIP and NBT (Gibco-BRL,

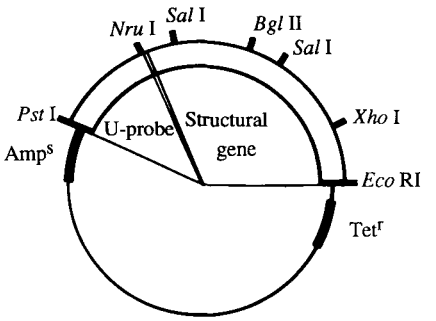

Fig. 1. pCMtox plasmid containing the *ExoA* structural gene and the variable upstream region of *P. aeruginosa* which is used for probing *P. aeruginosa* strains.

Bethesda, USA and Serva, Heidelberg, Federal Republic of Germany) according to the manufacturers' instructions. Isolates were compared visually for differences in probe-reactive fragments. Fragment size was determined by comparison with labelled lambda *Hin*dIII fragments. Two isolates were considered different strains if one or more of the three strain-specific, probe-reactive fragments differed in size. In cases of doubt, when strains were run on different gels and the size of respective fragments showed differences of less than 0·5 kb the strains were re-examined on one gel. Due to the reduced separation of DNA fragments > 10 kb in the 0·6% agarose gel, genotyping of strains with probe-reactive fragments of this size may be unprecise. However, the use of three restriction enzymes for DNA digestion may counteract this difficulty.

Radioimmunoassays

Detection of antibodies against the *ExoA*, elastase (Ela) and alkaline protease (AP) of *P. aeruginosa* in the sera from CF patients was performed by radioimmunoassay as described elsewhere (Döring *et al.* 1983; Döring *et al.* 1985).

RESULTS

The whole pCMtox plasmid was used for hybridization (Figure 1) for several reasons: first, the isolation of the *Pst* I–*Nru* I fragment resulted in low yield and reduced sensitivity. Second, the use of the whole pCMtox plasmid allows one to identify *P. aeruginosa* strains in cases where the variable region or the *ExoA* gene is missing. Hybridization patterns of *P. aeruginosa* DNA from 5 CF patients are seen in Fig. 2. Typically, there are two (*Bgl* II, *Xho* I) or three (*Sal* I) labeled fragments for each strain, revealing the variable region upstream of the *ExoA* gene and the constant region within and downstream of the *ExoA* gene.

One hundred and eighty-one isolates from 76 CF patients were typed using this DNA probe. Seven of 12 sibling pairs harboured genotypically identical *P. aeruginosa* strains (data not shown). All of the other patients harboured different strains revealing the discriminatory power of the method.

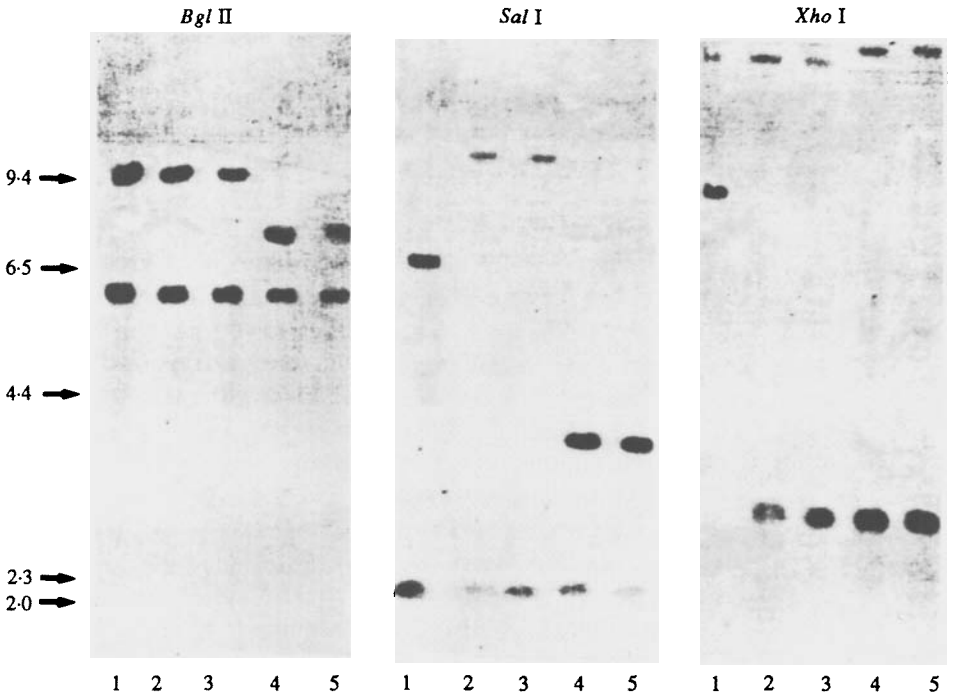

Fig. 2. DNA hybridization pattern of *P. aeruginosa* strains from CF patients from the beginning (*a*) and/or the end (*b*) of a summer camp. Lane 1, patient 40b; lane 2, patient 11b; lane 3, patient 12a; lane 4, patient 36a; lane 5, patient 36b. Purified *P. aeruginosa* DNA (1–2 μg) was digested with *Bgl* II, *Sal* I and *Xho* I, electrophoresed through a 0·6% agarose gel and transferred to a nylon membrane using the method developed by Southern. The isolated pCMtox plasmid was labeled with biotin-11-dUTP by nick-translation. The nylon membranes were prehybridized for 2 h, followed by hybridization at 42 °C for 18 h using a hybridization solution of 45% (vol/vol) formamide, 5 × SSC, 1 × Denhardt's solution, 20 mM sodium phosphate, pH 6.5, 5% dextran sulphate and 0.2 mg/ml sheared herring sperm DNA. Streptavidin/alkaline phosphatase reagent was added to the washed and blocked membrane. The membranes were then developed using BCIP and NBT. Isolates were compared visually for differences in probe-reactive fragments. Fragment size was determined by comparison with biotin-labelled lambda *Hin*dIII fragments. Two isolates were considered different strains if one or more of the three strain-specific, probe-reactive fragments differed in size. In cases of doubt, when strains were run on different gels and the size of respective fragments showed differences of less than 0.5 kb, the strains were reexamined on one gel.

Cross-colonization in a cystic fibrosis clinic.

Twenty-two patients (71%) of the 31 infected patients entering the CF clinic at Amrum carried the same *P. aeruginosa* strains at the beginning and end of the period, whereas a change of strains was seen in 10% of the patients (patients 25, 26, 40; Table 1). Genotyping revealed that strains 26b and 40b were identical to strains found in patients 17 and 41. Six (46%) of the 13 uninfected patients entering the clinic (patients 10, 11, 27, 31–33; Table 1) left the clinic harbouring *P. aeruginosa* in their sputa. Genotyping revealed that strains from patients 11 and 12 (Fig. 2) and from patients 33 and 34 were identical. Thus, cross-

Table 1. *Genotypes of* Pseudomonas aeruginosa *isolates from sputa of cystic fibrosis patients at the beginning* (a) *and end* (b) *of a summer camp* and later* (c)

Patient		Restriction enzymes‡			Patient		Restriction enzymes‡			Patient		Restriction enzymes‡			Patient		Restriction enzymes‡		
No.	S†	*Bgl* II	*Sal* I	*Xho* I	No.	S†	*Bgl* II	*Sal* I	*Xho* I	No.	S†	*Bgl* II	*Sal* I	*Xho* I	No.	S†	*Bgl* II	*Sal* I	*Xho* I
1a	A	3·9	N.D.§	2·5	13a	C	5·1	9·0	2·6	24c		N.D.	N.D.	N.D.	35c		N		
1b		3·9	N.D.	2·5	13b		N			25a	A	10·0	11·0	8·4	36a	C	7·5	3·5	2·5
1c		N.D.	N.D.	N.D.	13c		5·1	9·0	2·6	25b		10·0	11·0	8·4	36b		7·5	3·5	2·5
2a	A	4·2	N.D.	2·6	14a	A	N			25b		8·3	3·8	2·5	36c		7·5	3·5	2·5
2b		4·2	N.D.	2·6	14b		N			25c		10·0	11·0	8·4	37a	B	4·9	9·4	5·9
2c		N			14c		8·0	10·0	N.D.	25c		8·3	3·8	2·5	37b		4·9	9·4	5·9
3a	A	7·3	2·4	14	15a	C	N			26a	B	3·8	N.D.	2·5	37c		ND	ND	ND
3b		7·3	2·4	14	15b		N			26b		9·8	9·8	14·0	38a	B	9.0	11.8	7·2
3c		7·3	2·4	14	15c		7·2	3·6	5·2	26c		4·7	10·2	13·0	38b		9·0	11·8	7·2
4a	C	7·5	3·6	3·7						27a	A	N			38c		8·6	10·1	7·0
4b		7·5	3·6	3·7	16a	B	4·6	9·0	12·0	27b		4·7	6·5	N.D.	39a	B	9·3	9·0	15·0
4c		N.D.	N.D.	N.D.	16b		4·6	9·0	12·0	27c		N.D.	N.D.	N.D.	39b		9·3	9·0	15·0
5a	B	7·4	3·8	2·7	16c		4·6	9·0	12·0	28a	B	N.D.	N.D.	N.D.	39c		ND	ND	ND
5b		7·4	3·8	2·7	17a	A	9·8	9·8	14·0	28b		5·0	9·5	6·0	40a	B	4·6	10·5	14·2
5c		4·0	N.D.	2·5	17b		9·8	9·8	14·0	28c		3·8	N.D.	2·4	40b		9·4	6·5	9·0
6a	C	6·6	3·6	2·8	17c		9·8	9·8	14·0	29a	A	N.D.	N.D.	N.D.	40c		9·4	6·5	9·0
6b		6·6	3·6	2·8	18a	B	4·8	10·2	12·2	29b		6·3	7·3	2·4	41a	C	9·4	6·5	9·0
6c		N.D.	N.D.	N.D.	18b		4·8	10·2	12·2	29c		6·3	7·3	2·4	41b		N		
7a	B	7·4	3·7	3·0	18c		4·8	10·2	12·2	30a	B	N.D.	N.D.	N.D.	41c		9·4	6·5	9·0
7b		7·4	3·7	3·0	19a	C	9·4	9·8	14·2	30b		8·3	3·9	5·6	41c		8·1	4·6	ND
7c		N.D.	N.D.	N.D.	19b		9·4	9·8	14·2	30c		8·3	3·9	5·6	42a	B	7·5	4·0	2·7
8a	B	4·7	10·0	11·5	19c		N.D.	N.D.	N.D.	31a	B	N			42b		ND	ND	ND
8b		4·7	10·0	11·5	20a	B	7·5	3·7	2·7	31b		9·8	10·5	14	42c		7·5	4·0	2·7
8c		N.D.	N.D.	N.D.	20b		7·5	3·7	2·7	31c		8·5	10·1	13·0	43a	A	N		
9a	B	7·2	3·7	2·5	20c		7·5	3·7	2·7	32a	B	N			43b		N		
9b		N.D.	N.D.	N.D.	21a	C	8·5	9·8	12·5	32b		9·8	10·5	14	43c		8·8	8·0	11·5
9c		5·0	9·4	5·9	21b		8·5	9·2	12·5	32c		9·8	10·5	14	44a	B	N		
10a	C	N			21c		8·5	9·2	12·5	33a	B	N			44b		N		
10b		10·2	11·2	9·2	22a	C	7·0	3·4	4·8	33b		7·6	3·6	2·5	44c		N		
10c		7·2	3·5	5·2	22b		7·0	3·4	4·8	33c		7·6	3·6	2·5	45a	B	N		
11a	C	N			22c		7·0	3·4	4·8	34a	A	7·6	3·6	2·5	45b		N		
11b		9·4	10·2	2·5	23a	C	7·0	3·6	2·5	34b		N			45c		N.D.	N.D.	N.D.
11c		N			23b		7·0	3·6	2·5	34c		N			46a	B	N		
12a	A	9·4	10·2	2·5	23c		7·0	3·6	2·5	35a	A	4·0	0·0	2·3	46b		N		
12b		N			24a	C	9·0	11·5	10·2	35b		N			46c		N.D.	N.D.	N.D.
12c		9·4	10·2	2·4	24b		9.0	11·5	10·2										

* 1, patients 1–15, 16–35, and 36–46 were together at the same time at the summer camp on the island of Amrum for 6 weeks. † 2, CF clinical score: mild (A), evident (B), severe (C) disease state. ‡ 3, fragment sizes in kilobases. § 4, not determined. ¶ 5, sputum negative for *P. aeruginosa*

colonization was evident in 4 patients (patients 26, 40, 11, 33). In total, 9 patients were either newly colonized or changed their strains.

The strains of the newly colonized patients 10, 25, 27, 31, and 32 were not identical to any of the other patient strains. In order to investigate whether these strains were derived from the inanimate environment in the clinic, 230 samples from various clinic sites were taken during the third recreation course (patients 36–46). Only two identical *P. aeruginosa* isolates were recovered from these sites which could not be traced back to a patient strain. Thus, the spreading of *P. aeruginosa* from infected patients in the clinic was observed but contamination of the inanimate sites in the clinic was minimal.

In order to rule out that the newly colonized patients already carried the strains when entering the clinic, antibody titers of these patients to the *P. aeruginosa* antigens alkaline protease, elastase and *ExoA* were determined. No significant titers ($\leqslant 10$) were found in sera at the beginning of the clinic stay. At the end of the clinic stay two patients were antibody-positive to the three antigens (data not shown). This result supports the theory that a new colonization took place during the stay. Furthermore, no history of *P. aeruginosa* infection was reported in the patients' clinical sheets. However, it cannot entirely be ruled out that the patients were already colonized with *P. aeruginosa* before entering the clinic, since antibody titers to the investigated antigens become positive an average of 6–15 months after the onset of colonization (Döring & Høiby, 1983).

Persistence of P. aeruginosa

In order to investigate whether the temporary 'loss' of *P. aeruginosa* after antibiotic therapy in some patients resulted from bacterial eradication or suppression, *P. aeruginosa* isolates were genotyped before and after the period in which they were undetectable in the sputum. Genotypically identical 'before-and-after' isolate pairs were obtained from 3 of 5 patients who were treated with antibiotics at the CF clinic Satteldüne and 'lost' their strains (patients 12, 13, 41; Table 1) and from 9 of the 11 CF patients treated at the Inselhospital in Bern, Switzerland (data not shown) (Schaad *et al.* 1987). Thus, the reappearance of *P. aeruginosa* after a temporary loss due to intensive antibiotic therapy is not caused by reinfection with a new strain but is probably due to the effective suppression of large numbers of *P. aeruginosa* in the sputa.

The persistence of the *P. aeruginosa* genotypes in CF patients was further assessed by investigating the sputum samples of 35 patients 4–10 months after the recreation period at the CF clinic. Twenty (57%) of the patients harboured strains identical to those they carried before, including 4 (patients 25, 32, 33, 40) of the 9 newly colonized patients In the remaining 12 patients a change of the *P. aeruginosa* genotype or a strain loss was seen (Table 1). Strain change or loss was independent of any change in the clinical state of the patients (Table 1). The analysis of six single colonies of *P. aeruginosa* with non-mucoid and mucoid morphology from three patients revealed that in these patients only one strain was present at a time. Similarly, when all *P. aeruginosa* sputum isolates were used for genotyping, the majority of patients revealed two DNA hybridization bands on Southern blots indicating the existence of only one *P. aeruginosa* strain. Only one patient (25; Table 1) was colonized with two different strains simultaneously over a long period.

DISCUSSION

CF patients suffering from chronic *P. aeruginosa* lung infections may spread the bacteria to the environment and thus may be a risk for other infected or uninfected CF patients who live in close contact with them. Several previous studies using classical typing methods for *P. aeruginosa* addressed the question of where CF patients acquire *P. aeruginosa* and whether cross-infection between patients occurs. Although cross-infection does not seem to be a major problem in some hospitals (Hardy *et al.* 1986), transient cross-colonization (Speert & Campbell, 1987) and an epidemic spread of multiresistant *P. aeruginosa* between CF patients was observed in two hospital settings (Høiby & Rosendal, 1980; Zimakoff *et al.* 1983; Bergan & Høiby, 1975; Zierdt & Williams, 1975; Pedersen *et al.* 1986). However, the inaccuracies of the older typing methods made an interpretion of the data difficult. Thus, loss of high molcular weight lipopolysaccharide in mucoid *P. aeruginosa* isolates from CF patients render strains non-typable with O-specific antibodies. Furthermore, CF isolates are less sensitive to lysis by typing phages than are those from other patients (Pitt, 1988). Lastly, different pyocin patterns occur with changes in bacterial metabolism and antibiotic therapy may alter pyocin production patterns (Thomassen, Demko & Doershuk, 1987). The present study corroborates previous results concerning the usefulness of the *ExoA*–DNA probe for epidemiological studies on P. *aeruginosa* (Ogle *et al.* 1987). Although genotyping is generally much more time-consuming than classical typing methods such as phage typing, pyocin typing or serotyping, the discriminatory ability of the method is higher. Nevertheless, a comparative study of all major typing methods for CF and non-CF *P. aeruginosa* strains seems to be valuable.

Previous studies suggested that siblings may harbour the same strain (Laraya-Cuasay, Cundy & Huang, 1976; Pedersen *et al.* 1986; Ogle *et al.* 1987). However, the numbers of sibling pairs investigated were small or the results based on the serotyping and/or pyocin typing of *P. aeruginosa* strains. The results of the present study on 12 sibling pairs show that the majority of CF siblings share the same *P. aeruginosa* strain. The question still arises whether this reflects cross-infection from patient to patient or whether the sibling strain is derived from a common source in the environment of the patients.

In contrast to the siblings, all of the infected CF patients living together at the Satteldüne clinic harboured different strains before entering the clinic. However at the end of the period four patients became colonized with other patients' strains. In view of the finding that 46% of the uninfected patients harboured *P. aeruginosa* in their sputa at the end of the period, the results support previous studies (Høiby & Rosendal, 1980; Zimakoff *et al.* 1983; Zierdt & Williams, 1975; Pedersen *et al.* 1986), suggesting cross-colonization between CF patients not only at home but also in the clinic. The routes by which such a high percentage of patients had been newly colonized during the clinic stay remain to be clarified.

One source for contamination with *P. aeruginosa* may be the inanimate environment. In previous studies *P. aeruginosa* (Speert & Campbell, 1987; Pedersen *et al.* 1986) and *P. cepacia* (Hardy *et al.* 1986) were rarely recovered from the inanimate environment in CF clinics. A similar result was obtained in the

present study revealing little contamination on dry surfaces. Also in the CF families, not a single *P. aeruginosa* isolate was found. Several facts may be responsible for this surprising result: first, the selective *P. aeruginosa* isolation medium cetrimide used in the present study may suppress *P. aeruginosa* strains from CF (Fonseca, MacDougall & Pitt, 1986); second, the survival rate of *P. aeruginosa* on dry surfaces may be short; third, other places than those investigated may represent important sources for *P. aeruginosa* in the environment.

One interesting finding of the present study is that CF patients harboured only one *P. aeruginosa* strain in their sputum at a time and that this strain persisted in the majority of patients over 6 months both with and without antibiotic therapy. Larger and longer studies are needed to explain the *P. aeruginosa* genotype change in 20% of the CF patients and correlate it with bacterial virulence or bacterial growth in the CF respiratory tract.

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