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Genotyping of *Pseudomonas aeruginosa* sputum and stool isolates from cystic fibrosis patients: evidence for intestinal colonization and spreading into toilets

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SUMMARY

Three hundred and fifty-eight stool and 131 sputum specimens from 40 cystic fibrosis (CF) patients and 100 toilet sinks were investigated for occurrence of *Pseudomonas aeruginosa*; 67% (21/31) of the patients with chronic *P. aeruginosa* lung infections carried the organism repeatedly in the stool but the organism was found only once in the stools of nine uninfected patients. *P. aeruginosa* stool carriage was correlated to high *P. aeruginosa* numbers in patients' sputa. Typing of *P. aeruginosa* with a DNA probe showed identity of sputum and stool strains. Seven patients repeatedly carried additional stool strains, not found in the sputum, suggesting intestinal colonization. No differences were seen in the clinical state of patients with *P. aeruginosa*-negative stool samples and patients with positive stool samples. Toilets in households of *P. aeruginosa*-infected CF patients were significantly more often contaminated with *P. aeruginosa* (42%) than toilets in households of non-infected CF patients (20%; $P < 0{\cdot}03$). The study shows that *P. aeruginosa*-infected CF patients may harbour the organisms also in the intestinal tract, and may spread the bacteria into toilets.

INTRODUCTION

Chronic *Pseudomonas aeruginosa* lung infection is a well-known phenomenon affecting more than 90% of all cystic fibrosis (CF) patients (1). Surprisingly little is known about stool carriage of *P. aeruginosa* in CF patients but, if this occurs, the questions arise, whether the intestine is colonized with this Gram-negative organism, and whether this has clinical implications for the patients. In an earlier study (2) the incidence of *P. aeruginosa* in CF stool samples was no higher than in stool samples of normal healthy individuals (3); however, three other studies demonstrated elevated *P. aeruginosa* stool carriage in CF patients [4–6]. Unfortunately, these studies do not differentiate between CF patients with and without pulmonary *P. aeruginosa* infection (4, 5) or lack clinical data on the patients (6).

Thus, the objectives of the present study were to determine the incidence of *P. aeruginosa* in stool specimens of CF patients with and without pulmonary *P. aeruginosa* infection. In order to investigate whether *P. aeruginosa* stool isolates

are derived from swallowed sputum or from other sources, *P. aeruginosa* stool and sputum isolates were typed using a *P. aeruginosa*-specific DNA probe. Additionally, data about antibiotic treatment and the patients' clinical state were included. Finally, the incidence of *P. aeruginosa* in CF household toilets was investigated, since *P. aeruginosa*-infected CF patients may spread the bacteria into this environment — a possible source for reinfection and cross-infection (7–9).

We present evidence that (*a*) a high percentage of CF patients with *P. aeruginosa* lung infections who are not treated with antibiotics active against *P. aeruginosa* carry the organism repeatedly in the stool, that (*b*) in some cases *P. aeruginosa* stool strains differ from sputum strains, suggesting intestinal colonization, that (*c*) patients with or without *P. aeruginosa* stool carriage do not reveal different clinical states, and that (*d*) toilets in households of *P. aeruginosa*-infected CF patients are significantly more highly contaminated with *P. aeruginosa* than toilets in households of non-infected CF patients.

MATERIALS AND METHODS

Patients

From each of 40 German CF out-patients (mean age 14·7 years, range: 5–32 years) 7–10 stool specimens and 3–4 sputum specimens were sent to the Hygiene Institute, University of Tübingen, Tübingen, Federal Republic of Germany, over a period of 4 weeks. Diagnosis of CF was based on accepted criteria including significantly increased sweat electrolyte levels (10). None of the patients was hospitalized during the study or at least 1 month before the study onset. Nine patients did not yield *P. aeruginosa* in triplicate sputum cultures and were considered non-infected with *P. aeruginosa* (group B). These patients were positive for *Staphylococcus aureus* in their sputa. All other patients were repeatedly positive for *P. aeruginosa* in the sputum cultures (group A). Antibiotic therapy against *S. aureus* and *P. aeruginosa* within 1 month before onset of the study and during the study, and the patients' clinical score were assessed. Antibiotics against *P. aeruginosa* included cephalosporins, aminoglycosides and quinolones and antibiotics against *S. aureus* included penicillins, tetracyclines, macrolides and trimethoprim plus sulfamethoxazole. All patients who were treated with antibiotics active against *P. aeruginosa* were treated at home. Two patients received quinolones orally, and five patients received aminoglycosides i.v. or by inhalation. All patients regularly took pancreatic enzyme substitution drugs. Informed consent was obtained from patients, parents or guardians.

Processing of sputum, faecal and toilet samples

Sputum samples were processed by diluting the sputum volume 1:1 with physiological saline. The sputum was homogenized for 1–2 min on a vortex mixer and 200 μl were plated on cetrimide agar (Difco) plates. Five millilitres 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. For quantitative bacterial determinations the homogenized sputum was serially diluted 10-fold with physiological saline and aliquots were plated on cetrimide agar. Faecal samples were processed as

described previously (2, 11, 12) with some modifications: an aliquot of the specimens was weighed, and 0·5 g were suspended in 1 ml physiological saline and homogenized for 1 min on a vortex mixer. The samples were further homogenized by dilution with 20 ml physiological saline, incubated for 2 h at 37 °C, and homogenized on a vortex mixer. 200 μl of the suspension were plated on cetrimide agar and incubated for 48–96 h at 37 °C. Five millilitre toilet samples were collected from toilet sinks using 10 ml sterile glass pipettes. The samples were transferred to sterile glass vials, and then inoculated on cetrimide agar. *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 (13) with some modifications. Briefly, *P. aeruginosa* colonies growing on cetrimide agar were used for isolation of chromosomal DNA (14). 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 Southern method (15). The *Escherichia coli* plasmid pCMtox (16) was isolated (17) and labelled with biotin-11-dUTP (Gibco, Bethesda, USA) by nick translation. In modifications of the original method (13), 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 × Denhard'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 membranes. The membranes were then developed using BCIP and NBT (Gibco-BRL, Bethesda, USA; Serva, Heidelberg, Federal Republic of Germany) according to the manufacturers' instructions. Isolates were compared visually for differences in probe-reactive fragments (Fig 1). Fragment size was determined by comparison with labelled lambda *Hind* III fragments. Typically there are two (*Bgl* II, *Xho* I) or three (*Sal* I) labelled fragments for each strain, revealing the variable region upstream of the ExoA gene and the constant region downstream of the ExoA gene. 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.

RESULTS

Incidence of P. aeruginosa *in CF faecal specimens*

A bacteriological analysis of 358 CF faecal samples for the incidence of *P. aeruginosa* revealed a significantly higher percentage of positive samples (39%) (Table 1) than has been reported for the normal, healthy population (2·3%) (3). This comparison between our CF patient population and the normal healthy population is valid, since none of the CF patients was hospitalized during or before the study period. When data from groups of CF patients with (A) and without (B)

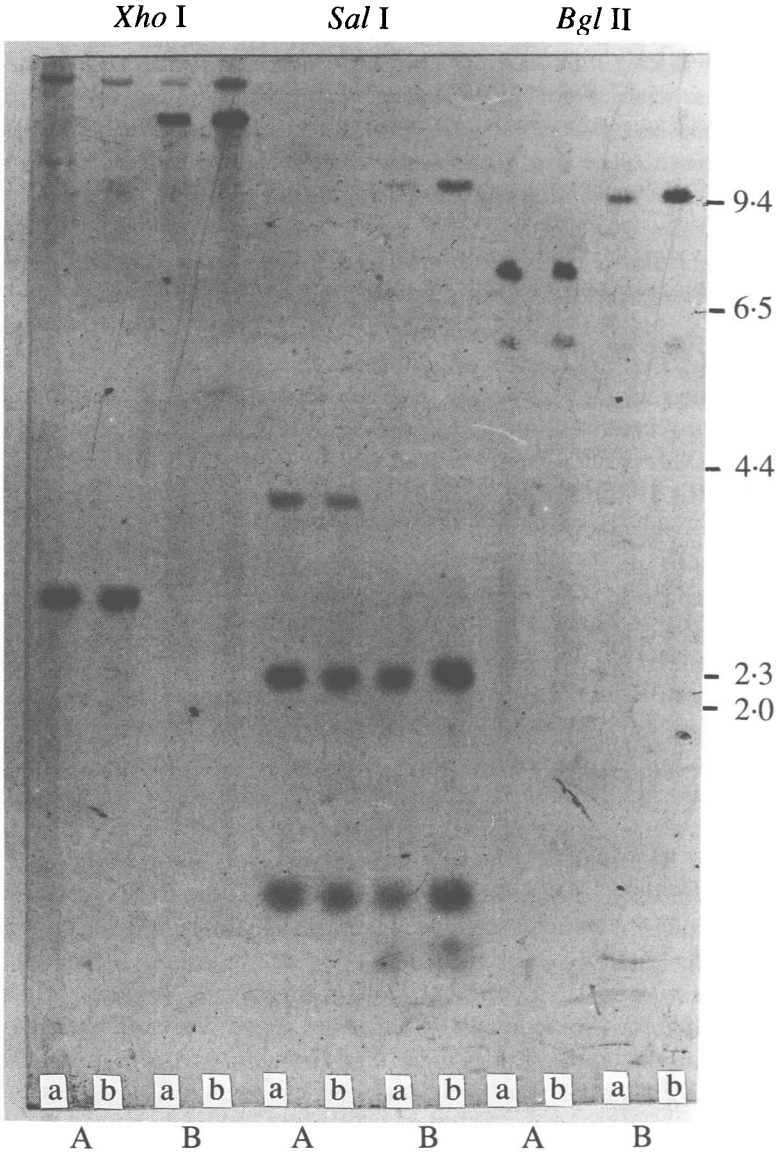

Fig. 1. DNA hybridization pattern of *Pseudomonas aeruginosa* strains from sputum (a) and stools (b) of two CF patients (A, B). Purified *P. aeruginosa* DNA was digested with *Bgl* II, *Sal* I and *Xho* I, electrophoresed through a 0·6% agarose gel and hybridized with the biotin-labelled pCMtox probe. For details see Materials and Methods. Isolates were compared visually for differences in probe-reactive fragments. DNA digestion with the restriction enzymes yields constant band(s) (*Xho* I: 14 kb; *Sal* I: 0·1 kb and 1·95 kb; *Bgl* II: 5·9 kb) and variable bands. The variable bands were used for strain differentiation. Two isolates were considered different strains if one or more of the three strain-specific, probe-reactive fragments differed in size.

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Table 1. *Incidence of* Pseudomonas aeruginosa *in stool samples of cystic fibrosis patients with (A) and without (B)* P. aeruginosa *lung infection*

Group	Cystic fibrosis patients: Positive/total no. (%)	Stool samples: positive/total no. (%)
A	21/31 (68)	140/276 (51)
B	1/9 (11)	1/82 (1)
A+B	22/40 (55)	141/358 (39)

P. aeruginosa lung infection were compared, a clear correlation between *P. aeruginosa* lung infection and the incidence of the organism in the patients' stool was seen. In patients with *P. aeruginosa* lung infection (Group A), the percentage of *P. aeruginosa*-positive stool samples was 68%, whereas in uninfected patients (Group B) only one of nine patients (11%) had a *P. aeruginosa*-positive stool culture.

A quantitative determination of *P. aeruginosa* colony-forming units (CFU) in the sputa indicated that CF patients with positive *P. aeruginosa* stool samples (patients 1–21, Table 2) had significantly higher numbers of CFU/ml than the 19 patients with negative stool samples (patients 22–40; Table 3; Chi square test: $\alpha = 0{\cdot}05$). When *P. aeruginosa* was quantitated in serial sputa obtained from single patients, CFU below 10^6/ml correlated with negative faecal samples and CFU above 10^6/ml correlated to positive faecal samples (data not shown). These results suggest that the presence of *P. aeruginosa* in CF stool samples is most likely caused by patients swallowing sputum highly contaminated with *P. aeruginosa*. Therefore, *P. aeruginosa* sputum and stool isolates were typed using the pCMtox plasmid.

Intestinal colonization with P. aeruginosa

The comparison of the *P. aeruginosa* genotypes of sputum and faeces isolates revealed identity in most cases (Table 2, Fig. 1). All 21 patients in whom *P. aeruginosa* was present in sputum and stool carried strain(s) in the stool which were also found in the sputum, corroborating the above hypothesis that the patients were repeatedly swallowing *P. aeruginosa*-contaminated sputum. However, seven patients (patients, 1, 2, 3, 5, 9, 17, 20) repeatedly carried additional *P. aeruginosa* strains in their stools which were not found simultaneously in the sputum. Since all *P. aeruginosa* colonies from the sputum which grew on cetrimide agar were used for genotyping, we do not believe that we have missed the additional stool strains in the sputum. In three of the seven patients (patients 3, 17, 20), the additional *P. aeruginosa* strain had been isolated 6 months earlier from the patients' sputum, strongly suggesting that it was colonizing the patient in a part of the body other than the respiratory tract. The intestinal tract is the most likely candidate for such colonization.

Since it is unknown whether intestinal colonization with *P. aeruginosa* in CF patients may influence the patients' clinical state, we compared patients with and without positive *P. aeruginosa* stool samples using a modified Kraemer score. The

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Table 2. *Genotypes of* Pseudomonas aeruginosa *strains isolated from sputum and stool specimens of patients with cystic fibrosis and* P. aeruginosa *lung infection*

	P. aeruginosa genotypes from		
	Sputum samples*		Stool samples*
Patient No.	July–Aug. 1988	Jan.–Feb. 1988	Jan.–Feb. 1988
1†	1a§	1a	1a, 1b, 1c
2†	2a	2a, 2b	2a, 2c, 2d
3†	3a, 3b	3c, 3d	3b, 3c, 3d, 3e
4	4a	4a	4a
5†	5a	5b	5b, 5c
6	6a	6a	6a
7	7a	7a	7a
8	8a	8a	8a
9†	9a	9a, 9b	9b, 9c
10	10a	10a, 10b	10b
11	11a	11a, 11b	11a, 11b
12	12a	12a	12a
13	13a	13a	13a
14	14a	14a	14a
15	15a	15b	15b
16	ND‡	16a	16a
17†	17a, 17b	17a	17a, 17b
18	18a, 18b	18a, 18b	18a, 18b
19	19a	19a	19a
20†	20a	20b	20a, 20b
21	ND	21a	21a
22	22a	22a	—¶
23	23a, 23b	23c	—
24	ND	24a	—
25	ND	25a	—
26	26a	26b	—
27	ND	27a	—
28	ND	28a	—
29	29a	29a, 29b	—
30	ND	30a	—
31	ND	31a	—
32–40	—	—	—

* From every cystic fibrosis patient, 7–10 stool specimens and 4–5 sputum samples were obtained.

† Patients with additional strains in the stool, which were not found at the same time in their sputa.

‡ ND, not determined.

§ *P. aeruginosa* genotype determined by bacterial DNA isolation, digestion with three different restriction enzymes and hybridization using a DNA probe consisting of the pCMtox plasmid (for details see *Materials and Methods*. Each letter represents a distinct genotype, specific for a certain patient. Identical letters in different patients represent different *P. aeruginosa* genotypes.

¶ —, negative.

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Table 3. *Incidence of* Pseudomonas aeruginosa (*PA*) *in sputum and stool of cystic fibrosis patients with* P. aeruginosa *lung infection*

Sputum	Stool	
PA+ CFU*	PA− % (+/Total)	PA+ % (+/Total)
$< 10^6$	60 (6/10)	0 (0/21)
10^6–10^8	20 (4/10)	48 (10/21)
$> 10^8$	0 (0/10)	52 (11/21)
PA-antibiotics	70 (7/10)	5 (1/21)

* Colony forming units.

following lung function parameters were included: airway resistance, intrathoratic gas volume, the ratio of residual volume to total lung capacity, forced expired volume in 1 sec, and inspiratory vital capacity. Additionally, pO_2 values and the patients' weight and height using the somatogram developed by Vogt (18) were incorporated. No differences were seen in the clinical state of patients with *P. aeruginosa*-negative stool samples and patients with positive stool samples.

Use of antibiotics

Negative *P. aeruginosa* stool cultures in patients suffering from *P. aeruginosa* lung infection may be due to treatment with antibiotics active against *P. aeruginosa*. Therefore we investigated whether there was a difference in the use of these antibiotics before and during the study period between patients with *P. aeruginosa*-positive and negative stool samples (Table 3). Indeed 7 of 10 patients with negative *P. aeruginosa* stool cultures (70%) took antibiotics active against *P. aeruginosa*, whereas only 1 of 21 patients with positive stool cultures (5%) took those antibiotics (Fischer-test; $P < 0{\cdot}01$). The data from the quantitative determination of *P. aeruginosa* organisms in CF sputa and from the uptake of antibiotics active against *P. aeruginosa* suggest that effective antibiotic therapy against *P. aeruginosa* reduces the bacterial load in the airways and leads to *P. aeruginosa*-negative stool cultures.

The frequent use of antibiotics against *S. aureus* may disrupt the normal gut flora (19), increasing the local adherence of *P. aeruginosa* (20). However, *P. aeruginosa* was found only in 1 of 81 stools (1%) of the 9 patients without *P. aeruginosa* pulmonary infection who took antibiotics active against *S. aureus*.

Spreading of P. aeruginosa *into toilets*

P. aeruginosa-infected CF patients may spread the bacteria into toilets via sputum or stool. Contaminated toilets represent a possible hazard for reinfection and cross-infection (9–11). Therefore we investigated the incidence of *P. aeruginosa* in toilets of a CF hospital ward, as well as in CF and normal household toilets. Toilets in households of *P. aeruginosa*-infected CF patients were significantly more often contaminated with *P. aeruginosa* (42%) than toilets in households of non-infected CF patients (20%; Chi square test with Yates correction: $P < 0{\cdot}03$).

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DISCUSSION

P. aeruginosa is rarely found in the faeces of normal healthy individuals (1·2–2·3%) (3, 21, 22), although the ingestion of food contaminated with this opportunistic pathogen is common (23). Apparently, gastric acidity and substances produced by a variety of anaerobic and facultative anaerobic bacteria, particularly the volatile fatty acids from *Bacteroides* spp., are involved in protection against intestinal *P. aeruginosa* colonization (19, 20).

CF patients may be at a higher risk of developing intestinal *P. aeruginosa* infections because of a variety of predisposing factors. First, CF patients may harbour large numbers of *P. aeruginosa* in their sputum (24) and swallowed sputum may lead to intestinal colonization with the organisms (25). Second, the frequent use of antibiotics may disrupt the normal gut flora (19), increasing the local adherence of *P. aeruginosa* (20). Third, the abnormal mucus (26) which is thought to contribute to *P. aeruginosa* adherence to the lung mucosa may also be present in the CF intestinal tract (27). Indeed, *P. aeruginosa*-positive stool samples were consistently found in 68% of the CF patients with *P. aeruginosa* lung infections. The known ability of *P. aeruginosa* to grow anaerobically (28), is a prerequisite for intestinal colonization.

Three lines of evidence show that the *P. aeruginosa* present in patients' stool specimens are in part derived from already contaminated sputum. First, *P. aeruginosa* was rarely found in CF patients without *P. aeruginosa* lung infections. Secondly, genotyping of sputum and stool strains revealed identity in all cases. Third, a strong correlation exists between sputum *P. aeruginosa* numbers greater than 10^6/ml and positive stool samples. The latter correlation corroborates previous investigations of the fate of ingested *P. aeruginosa* in normal individuals (25) showing that at ingestion doses above 10^6/ml, organisms were regularly recovered, in contrast to lower doses.

A striking difference between CF patients with and those without positive *P. aeruginosa* stool carriage but all suffering from chronic *P. aeruginosa* lung infection was related to the use of antibiotics. Negative stool specimens were seen in the patients who took a high percentage of antibiotics active against *P. aeruginosa* within 1 month before onset of the study and during the study. Apparently, the antibiotics reduced *P. aeruginosa* numbers in the sputa and/or the stool.

The use of *S. aureus*-specific antibiotics did not seem to increase the risk for the establishment and proliferation of *P. aeruginosa* in the human intestinal tract (20). Whether these antibiotics could disrupt the natural protective flora, as has been shown in animal models (20), remains to be investigated in humans. The present findings that *P. aeruginosa* was recovered only once in 81 stool samples of 9 CF patients treated against *S. aureus* makes this explanation rather unlikely.

Interestingly, some of the patients carried *P. aeruginosa* strains in their stool which were not detectable in the sputum at the time of the investigation. Since these additional strains were isolated from a number of stool specimens from each patient over a period of 4 weeks it seems improbable that the strains were derived from the ingestion of contaminated food. Furthermore, it seems unlikely that the patients were exposed to an environmental *P. aeruginosa* source, since ingestion doses must exceed 10^6 organisms to get positive stool cultures (25) as shown above.

The results strongly suggest that the additional *P. aeruginosa* strains were derived from intestinal colonization. In some of the patients two or more strains were isolated from the stools. Thus, it cannot be excluded that more than one *P. aeruginosa* strain may colonize the intestinal tract.

Our findings indicate the need for regular therapy with antibiotics active against *P. aeruginosa* in CF patients which will lead to a decreased number of *P. aeruginosa* organisms in the respiratory tract and also in the stools. Besides the beneficial clinical effects of a regular antibiotic therapy against *P. aeruginosa* for CF patients, the incidence of *P. aeruginosa*, spread via the stool in household or community toilets may thus be diminished. As was shown previously [8, 9], there is a possibility that a person may acquire an infection from an aerosol produced by flushing toilets. The peculiar affinity of CF patients for *P. aeruginosa* may put this patient group at a special risk in this respect. A continuing prospective study in our laboratory investigates this hypothesis.

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