Epidemiologic characterization of *Pseudomonas aeruginosa* in patients with cystic fibrosis

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**Objective** To determine persistence and variability of colonization with *Pseudomonas aeruginosa* in cystic fibrosis patients over long time periods, and to look for possible cross-colonization.

**Methods** In total, 469 *Pseudomonas aeruginosa* isolates were obtained from 30 patients during the period from April 1994 to April 1996. The sources were mainly sputum and a few deep throat swabs. All grown strains dissimilar in macromorphology were processed separately. Typing with PFGE was carried out by contour-clamped homogeneous electric field electrophoresis. Genomic DNA was subjected to the rare-cutting restriction enzyme *SpeI*. For pyocin typing, the procedure described by Fyfe was applied.

**Results** After typing with PFGE, we observed 40 restriction profiles. Eighteen different pyocin types were found. The most frequent pyocin type was type 3, followed by types 1 and 5. Twenty-two patients were persistently colonized by one clone specific and different for each patient, and four were co-colonized by a second clone also different for each of these patients. Cross-colonization had apparently been rare in the cystic fibrosis center of Leipzig.

**Conclusions** Typing with PFGE is well suited for detailed investigations of colonization with *Pseudomonas aeruginosa* in cystic fibrosis patients. Pyocin typing can provide additional information for epidemiologic purposes.

**Keywords** *Pseudomonas aeruginosa*, pulsed-field gel electrophoresis, pyocin typing, cystic fibrosis

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**INTRODUCTION**

The acquisition of *Pseudomonas aeruginosa* is of great concern among patients with cystic fibrosis (CF), as colonization with _P. aeruginosa_ is responsible for increased morbidity, deterioration of lung function, and reduced life-expectancy [1].

The means by which this organism is acquired and transmitted are not completely clear. Nevertheless, once _P. aeruginosa_ has taken up residence in the CF respiratory tract, it is virtually impossible to eradicate it by any regimen of antimicrobial chemotherapy [1].

Infection epidemiology has been investigated by means of several phenotypic [2–6] and, more recently, genetic typing methods [7–18]. Though the new typing methods are very helpful tools, some epidemiologic information is still contradictory. For example, the heterogeneity of strains among different chronic carriers and their phenotypic and/or genotypic variability within one individual patient, as well as the roles of antibiotic therapy and patient-to-patient contact in strain exchange, are not yet completely understood.

Therefore, in this study this issue was addressed by typing _P. aeruginosa_ with macrorestriction analysis by PFGE and by pyocin typing in order to determine the persistence and variability of the pulmonary colonization and the patient-to-patient transmission of _P. aeruginosa_ in CF patients of the Leipzig area.

**MATERIALS AND METHODS**

**Patients**

The patient population consisted of 30 children, 17 girls and 13 boys, who attended the CF center at the Department of
Pediatrics at the University of Leipzig between April 1994 and April 1996. Per patient, 2–53 samples (sputum or deep throat swab specimens) were analyzed (mean value 11.5). The youngest patient was 4 years old, and the oldest 19 (mean age 13.9 years). The patient group comprised 26 unrelated children and two pairs of siblings. Most of the patients were hospitalized for regular 2-week courses of intravenous chemotherapy or for treatment of acute exacerbations and were followed up in the outpatient clinic.

One patient (patient 6) died in this period, and another one (patient 13) moved away. The patient population comprised 28 patients colonized chronically with *P. aeruginosa*; that is, *P. aeruginosa* was detectable in several specimens for at least 6 months. Furthermore, two patients with less than three strains isolated in the investigation period were included in this study, as they were siblings of two other patients. A synopsis of patients and isolates is shown in Table 1.

**Bacteriologic methods**

Sputum samples or deep throat swab specimens were collected on admission and before discharge from our CF ward and on outpatient appointments. Isolated strains were assessed by morphologic features, including pigment production, and identified as *P. aeruginosa* by a positive oxidase reaction and appropriate results with the API-20 NE system (BioMérieux, Nütttingen, Germany). The strains were stored in special vials (Microbank, Mast, UK) at −20 °C. Susceptibility testing was performed using the disk diffusion method according to published standards [19].

**PFGE**

PFGE was carried out by contour-clamped homogeneous electric field electrophoresis (CHEF-DR III apparatus, BioRad, Munich) using the Genepath Group 3 Reagent Kit and the Genepath Gel Kit. The test strains were grown in nutrient broth overnight at 37 °C to a density of 10⁷ cells/mL. Cells were centrifuged at 11,000 rev/min for 2 min, and the pellet was resuspended, mixed with 1.2% agarose and pipetted into plug molds. The embedded bacteria were incubated with 1mg of lysozyme for 1h at 37 °C and 1mg of proteinase K at 50 °C for 16h. After four washing steps, restriction digestion was performed with the rare-cutting restriction endonuclease SpeI (25 U per plug) for 16 h at 25 °C. The DNA digest with SpeI generated between 14 and 25 bands per strain. Forty different restriction profiles were found and were assigned a number (1–40). Of these clones, 21 had between two and four clonal variants. Strains were defined as belonging to the same clone if their restriction patterns were identical or differed in six or less bands [22]. Identical restriction patterns in different patients were assigned the same number. Four strains, all from patient 1, were not typable by macrorestriction fragment analysis, probably because of high contents of endogenous nucleases [15].

**Pyocin typing**

Pyocin typing was done by the spotting method as described by Fyfe et al [21]. This method is based on the inhibition of 13 *P. aeruginosa* indicator strains by pyocin produced by the patients’ strains to be typed. The inhibition patterns of eight indicator strains (set 1–8) describe the main type, and the other five strains (set A–E) code for the subtype. Thus, 108 main types and 31 subtypes can be identified.

**RESULTS**

In total, 469 strains from 30 patients were examined. One to 42 strains per patient (mean 15.6) were isolated. The data are summarized in Table 1, together with information on how long the airways of the individuals had already been colonized with *P. aeruginosa*.

**PFGE**

The DNA digest with SpeI generated between 14 and 25 bands per strain. Forty different restriction profiles were found and were assigned a number (1–40). Of these clones, 21 had between two and four clonal variants. Strains were defined as belonging to the same clone if their restriction patterns were identical or differed in six or less bands [22]. Identical restriction patterns in different patients were assigned the same number. Four strains, all from patient 1, were not typable by macrorestriction fragment analysis, probably because of high contents of endogenous nucleases [15].

**Pyocin typing**

Eighteen different pyocin types were determined. The most common pyocin type was type 3 (14 patients), followed by
Table 1 Results of typing of 469 \textit{Pseudomonas aeruginosa} strains isolated from 30 patients with cystic fibrosis at the Department of Pediatrics, University of Leipzig, Germany (sampling period April 1994 to April 1996)

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Colonized since</th>
<th>Total no. of isolates</th>
<th>PFGE pattern</th>
<th>No. of isolates</th>
<th>No. of clonal variants</th>
<th>Pyocin type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>11</td>
<td>01/1993</td>
<td>12</td>
<td>1</td>
<td>8</td>
<td>1</td>
<td>6/e(S4,B,E)</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>14</td>
<td>06/1992</td>
<td>28</td>
<td>NT</td>
<td>4</td>
<td>–</td>
<td>6/e(S4,B,E)</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>11</td>
<td>before 1994</td>
<td>26</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>2/v</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>16</td>
<td>1984</td>
<td>31</td>
<td>6</td>
<td>24</td>
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<td>3/e</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>17</td>
<td>before 1994</td>
<td>16</td>
<td>9</td>
<td>16</td>
<td>2</td>
<td>10/c(S6,7)</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>17</td>
<td>1993</td>
<td>27</td>
<td>3</td>
<td>18</td>
<td>2</td>
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<tr>
<td>10</td>
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<td>16</td>
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<td>M</td>
<td>16</td>
<td>1990</td>
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<td>02/1993</td>
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<td>9</td>
<td>1992</td>
<td>30</td>
<td>29</td>
<td>30</td>
<td>2</td>
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<td>4</td>
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<td>13</td>
<td>04/1995</td>
<td>6</td>
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<td>6</td>
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<td>1/g</td>
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<td>18</td>
<td>1978</td>
<td>5</td>
<td>7</td>
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<td>01/1996</td>
<td>2</td>
<td>7</td>
<td>1</td>
<td>1</td>
<td>5/x</td>
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</tbody>
</table>

F, female; M, male; NT, not typable with PFGE; nt/nt, no reaction with indicator set 1–8/A-E.
Designation of pyocin typing according to Fyfe et al. [25].
types 1 (six patients) and 5 (five patients). They were found in 57% of the patients. There was no pyocin activity found with the indicator set 1–8 in five patients and with the set A–E in eight patients. All strains with pyocin deficiency were typable by PFGE.

Patients

Twenty-two patients were persistently colonized by one clone specific and different for each patient; in 18 patients, only one clone was found with PFGE. Four patients (patients 4, 6, 11 and 22; see Table 1, Figs 1–3) had two co-colonizing clones, also different for each of these patients. In seven patients, other transiently colonizing clones (i.e. a clone detected only once or twice in those patients’ sputa) were found; patient 29 harbored two transient clones. Only three clones were isolated from more than one patient. Clone 3 was repeatedly found in patient 6 and isolated once from patient 2 (Table 1). Clone 7 was found in patients 4, 29 and 30. Clone 10 was chronically colonizing patients 6 and 14. Patients sharing identical clones were also found to have identical pyocin types and antibiograms for the respective strains. In isolates of individual patients with a stable pyocin type, PFGE confirmed the identity of the isolates, apart from patients 4 and 11. Figure 1 demonstrates the course of colonization in patient 4. This patient constantly harbored clone 6 (24 isolates) and, between July 1994 and April 1995, clone 7 (four isolates). In August 1995, another strain emerged (three isolates) with a PFGE pattern differing in more than six bands from clone 7, and was assigned a separate PFGE number (clone 8). The antibiotic susceptibility changed from sensitive (clone 7) to resistant to azlocillin, piperacillin, and ceftazidime (clone 8). However, colony morphology and pyocin type were iden-

![Figure 1](Image)

**Figure 1** PFGE patterns of 31 strains from patient 4. The patient harbors three clones: clone 7 (lanes 2, 6, 9, and 16), clone 8 (lanes 2, 3, 28, and 31), and clone 6 with two clonal variants (the remaining lanes). Strains 23, 28 and 31 differ in more than six bands from strains 2, 6, 9 and 16, and therefore were assigned a separate PFGE number. Clones 7 and 8 show the same pyocintype 5/ x(S5), and clone 6 has pyocintype 1. λ is the molecular size marker (PFG Marker, New England Biolabs, 50–100 kb). See also Figure 2.
tical for both clones. In patient 11, altogether 14 strains were found with the similar pyocin types 94/β and 101/β, 13 of which belonged to clone 17. One strain, however, gave a different pattern (clone 18) and a different antibiogram. This patient was colonized by two unrelated clones with several clonal variants. Besides the above mentioned clones 17 and 18, 13 strains belonged to clone 16 (Table 2).

We also typed strains isolated from two pairs of siblings. Patients 29 (aged 18) and 30 (aged 4) are brothers. Both attended our CF center until 1992 and again at the end of 1995 and had no contact with the other patients in between. Among the five isolates of patient 29, three different clones were identified: clones 7, 39 and 40. Patient 30 harbored clones 7 and 40 (Figure 3 and Table 3). However, this patient was only sampled twice, and thus a conclusion about the persistence of these strains cannot be drawn. Patients 16 and 17 are sisters. Patient 17 carried five strains of clone 24. In patient 16, chronic colonization had not yet occurred and only one strain was isolated, giving a fragment pattern different from that of her sister (clone 23). Identical isolates of one clone often displayed a variable pyocin type (e.g. patients 6, 9, 18, 19 and 22). Usually, there was no relationship between minor changes in pyocin type and the appearance of clonal variants. Also, the appearance of clonal variants with alterations in only a few bands had as a rule no influence on pyocin pattern, antibiotic susceptibility, or colony morphology. For the majority of strains with similar pyocin type, PFGE revealed the genetic diversity of the strains (Table 4).

**DISCUSSION**

Recent research has shown that the initial colonizing strain remains dominant in the CF lung. Römling et al [15] studied three sisters and nine unrelated patients over a period of 8 years, using macrorestriction analysis with PFGE. The initially acquired strain persisted over the whole study period; only two patients lost their clone after 3 years. Horrevorts et al [3] monitored 15 CF patients over periods up to 5 years, applying serotyping, phage typing, and pyocin typing. They found that, generally, one serotype predominated and that the colonization with *P. aeruginosa* was quite constant. Mahenthiralingam et al [13] typed 385 isolates from 20 CF patients by...
random amplified polymorphic DNA (RAPD)-PCR. The observation period varied from 1 to over 10 years per patient. The authors found a strain replacement in four patients, and four other patients were co-infected with two or more clones. The replacing strains showed new RAPD fingerprints. Our results basically support these findings. In this study, macrorestriction analysis reliably revealed the identity or high similarity of isolates from a given patient as well as distinct differences in the case of co-colonization by two or more clones. In contrast to other studies [1,18], however, we could not show a strain loss or turnover. It should be kept in mind that the situation in CF patients who receive regular antimicrobial treatment over many years may not reflect the natural course of infection in the CF respiratory tract [9]. Intensive antimicrobial therapy might well lead to a significant selection of optimally adapted \textit{P. aeruginosa} strains.

In patient 4, we observed a loss of more than four restriction fragments in a strain chronically colonizing this patient. The emergence of this altered strain was accompanied by an obvious change in the antibiotic susceptibility pattern. Interestingly, the pyocin type and colony morphology remained stable. Further investigations are necessary to make clear whether an association between the loss of restriction fragments and change in antibiotic susceptibility exists. The assumption of a genetic relatedness rather than a strain replacement was supported by the unchanged pyocin type. Here, PFGE together with pyocin typing has provided valuable epidemiologic information, which would be not possible using either a genetic or a phenotypic method.

Several epidemiologic studies have addressed the question of cross-infection between unrelated patients [2,11,15,17,18] and siblings [2,11,15]. It has been shown that \textit{P. aeruginosa}

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|c|}
\hline
Strain & Date of isolation & Colony & Pyocin type & PFGE pattern & Antibiogram \\
& & & & & PIP & CAZ & IMP & GEN & CIP & AZL \\
\hline
1 & 26/04/94 & nm & 1/k & 16A & r & r & s & r & s & r \\
3 & 12/08/94 & nm & 1/p & 16A & r & s & s & r & s & r \\
5 & 29/08/94 & nm & 1/u & 16C & r & r & s & r & s & r \\
6 & 08/09/94 & nm & 1/p & 16A & r & i & s & r & i & r \\
8 & 16/09/94 & nm & 1/nt & 16B & r & r & s & r & s & r \\
9 & 07/10/94 & nm & 1/p & 16A & r & i & s & r & s & r \\
10 & 23/11/94 & nm & 1/nt & 16B & r & s & s & r & s & r \\
12 & 17/01/95 & nm & 1/nt & 16C & r & i & s & r & r & r \\
13 & 07/02/95 & nm & 1/nt & 16A & r & s & s & r & s & r \\
16 & 28/03/95 & nm & 1/nt & 16C & r & s & s & r & s & r \\
23 & 17/11/95 & nm & 1/nt & 16A & r & i & i & r & s & r \\
25 & 24/01/96 & nm & 1/g & 16D & r & r & r & s & r & r \\
27 & 06/02/96 & nm & 1/g & 16D & r & r & s & r & r & r \\
11 & 17/01/95 & m & 94/(\text{S_A,E}) & 17A & s & s & nd & s & nd & s \\
14 & 15/02/95 & m & 94/(\text{S_A,E}) & 17A & s & s & s & s & s & s \\
15 & 28/03/95 & m & 94/(\text{S_A,E}) & 17A & s & s & s & s & s & s \\
17 & 20/06/95 & m & 94/(\text{S_A,E}) & 17A & s & s & s & s & s & s \\
18 & 20/07/95 & m & 94/(\text{S_A,E}) & 17A & s & s & s & s & s & s \\
19 & 05/10/95 & m & 94/(\text{S_A,E}) & 17A & s & s & s & s & s & s \\
21 & 06/11/95 & m & 94/(\text{S_A,B}) & 17A & s & s & s & s & s & s \\
24 & 24/01/96 & m & 94/\beta & 17C & r & s & s & r & s & r \\
26 & 06/02/96 & m & 94/(\text{S_A}) & 17A & s & s & i & s & s & s \\
2 & 12/07/94 & nm & 94/(\text{S_A,B,E}) & 18 & r & r & s & r & s & r \\
4 & 12/08/94 & m & 101/(\text{S_A,B,E}) & 17A & ND & ND & ND & ND & ND & ND \\
7 & 16/09/94 & nm & 101/(\text{S_B}) & 17D & s & s & r & s & s & s \\
20 & 05/10/95 & m & 101/(\text{S_A,B}) & 17B & s & s & s & s & s & s \\
22 & 17/11/95 & m & 101/(\text{S_A,B}) & 17B & s & s & s & s & s & s \\
\hline
\end{tabular}
\caption{Comparison of pyocin types and fragment patterns from 27 \textit{P. aeruginosa} strains isolated from patient 11}
\end{table}
was isolated which persistently colonized patient 6. On the day
environmental source remains unclear. From patient 2, a clone
to the other or whether it was contracted from a common
study period. Whether the strain was passed from one patient
Patients 6 and 14 harbored an identical clone over the whole
close and/or prolonged. We included a male and a female sib-
spreads among affected children of a family, as the contact is
only three clones were found in more than one patient. Patients 6 and 14 harbored an identical clone over the whole
or/and prolonged. We included a male and a female sib-
Only three clones were found in more than one patient. Patients 6 and 14 harbored an identical clone over the whole
other during the study period. Whether the strain was passed from one patient
to the other or whether it was contracted from a common
environmental source remains unclear. From patient 2, a clone
was isolated which persistently colonized patient 6. On the day
of isolation, both patients were hospitalized on different
wards. Patient 6 was critically ill and admitted to the ICU, so
cross-colonization could have taken place only during a visit
by patient 2. Patients 4, 29 and 30 attended our center in differ-
ent months and thus were unable to directly co-infect each
other during the study period.

The results indicate that cross-colonization is rare in our
CF center, which might be explained by the segregation of
infected and non-infected patients and patients with multiply
resistant strains. Furthermore, the results correlate well with
the findings of other investigators, indicating remarkable
genetic heterogeneity among \textit{P. aeruginosa} strains colonizing
the patient population of the CF center of Leipzig
[13,14,16,18].

Summarizing our results, the typing of restriction fragment
patterns of \textit{P. aeruginosa} strains with PFGE combined high typ-
ability and discriminatory capacity. Under selected condi-
tions, pyocintyping can provide interesting additional
information. The surveillance of patients regularly attending a
CF center may form a valuable part of a hygienic quality con-
control program considering measures against cross-coloniza-

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mon pyocin types of \textit{Pseudomonas aeruginosa} from patients with

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Strain} & \textbf{Date of} & \textbf{Colony form} & \textbf{Pyocin type} & \textbf{PFGE pattern} \\
\hline
1 & 19/10/95 & nm & 5/x & 7 \\
2 & 19/10/95 & nm & 19/k & 39 \\
3 & 19/1095 & m & 70/nt & 40 \\
4 & 23/01/96 & nm & 5/x & 7 \\
5 & 12/02/96 & nm & 5/x(S4) & 7 \\
6 & 23/01/96 & nm & 5/x & 7 \\
7 & 02/04/96 & m & 70/nt & 40 \\
\hline
\end{tabular}
\caption{Pheno- and genotyping results of seven \textit{P. aeruginosa} strains isolated from the brothers, patient 29 (strains 1–5) and patient 30 (strains 6 and 7). Both brothers share the clones 7 and 40 with the pyocin types 5/x and 70/nt, respectively.}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Group} & \textbf{Pyocin type} & \textbf{Patient} & \textbf{PFGE type} & \textbf{Colony form} \\
\hline
1 & 3/e & 3 & 4 & m \\
2 & 3/e & 10 & 15 & nm \\
3 & 3/e & 19 & 26 & m \\
4 & 3/e & 23 & 32 & m \\
5 & 3/e(S4) & 9 & 13 & nm/m \\
6 & 3/e(S4) & 8 & 12 & m \\
7 & 3/e(S4) & 15 & 21 & m \\
8 & 3/e(S4) & 21 & 29 & nm \\
9 & 3/e(S4) & 24 & 33 & m \\
10 & 3/e(S4) & 2 & 2 & nm \\
\hline
\end{tabular}
\caption{Comparison of strains from 10 patients with the most common pyocin type 3/e with the restriction fragment patterns}
\end{table}

All strains were susceptible to the antibiotics tested. Strain
numbers correspond with lanes in Figure 3. m, mucoid; nm, not
mucoid; nt, no reaction with indicator set A–E.


