Identification and characterization of transmissible *Pseudomonas aeruginosa* strains in cystic fibrosis patients in England and Wales

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Most past studies of cross-infection with *Pseudomonas aeruginosa* among cystic fibrosis (CF) patients in the UK suggest that it is a rare occurrence. However, two recent reports of highly transmissible strains in patients in regional centres in England (Liverpool and Manchester) have raised questions as to the extent of the problem and prompted a nationwide survey to establish the distribution of *P. aeruginosa* strain genotypes among these patients. Isolates of *P. aeruginosa* were requested from over 120 hospitals in England and Wales and a sample size of approximately 20% of the CF patient population in each centre was recommended. In total, 1225 isolates were received from 31 centres (range 1 to 330). Single patient isolates were typed by *SpeI* macrorestriction and PFGE. A panel of strains of the common genotypes including representatives of reported transmissible strains was assembled and further characterized by fluorescent amplified fragment length polymorphism (FAFLP) genotyping. At least 72% of all patients harboured strains with unique genotypes. Small clusters of related strains were evident in some centres, presumably indicating limited transmission of local strains. The most prevalent strain was indistinguishable from that previously described as the ‘Liverpool’ genotype, and accounted for approximately 11% of patient isolates from 15 centres in England and Wales. The second most common genotype (termed Midlands 1) was recovered from 86 patients in nine centres and the third genotype, which matched closely the PFGE profile of Clone C, a genotype originally described in Germany, was found in eight centres and was isolated from 15 patients. A fourth genotype, identical to the published Manchester strain, was found in three centres. FAFLP analysis revealed some microheterogeneity among strains of the Liverpool genotype but all isolates of this genotype were positive by PCR for a strain-specific marker. These data suggest that cross-infection with *P. aeruginosa* has occurred both within and widely between CF centres in England and Wales. The two most common genotypes accounted for more than one-fifth of patients’ isolates examined and transmissible genotypes were found in all but three centres studied. These results emphasize the need for continued surveillance of *P. aeruginosa* genotypes in CF patients to provide informed infection control policy in treatment centres.

INTRODUCTION

Cross-infection with *Pseudomonas aeruginosa* among cystic fibrosis (CF) patients remains a contentious issue. Up to 80% of CF patients are colonized in the lung with this species and its acquisition by patients is often associated with clinical deterioration and subsequent morbidity and mortality (Doring et al., 2000). *P. aeruginosa* is widespread in the natural environment and the latter is believed to be the most common source of infection for CF patients. An alternative source of infection for non-colonized patients is contact with respiratory secretions of other infected patients. Patients may occasionally be superinfected with highly transmissible strains, from other patients or the environment, which may replace a patient’s original strain.

Five to ten years ago, patients were thought to harbour their own unique strains, and cross-infection was considered to be rare, except between siblings and in group activities such as summer camps. However, more recently, cross-infection between unrelated individuals has been reported with increasing frequency. In the UK, two centres, Liverpool (Cheng et al., 1996) and Manchester (Jones et al., 2001), reported cross-infection within their centre between unrelated patients with antibiotic-multiresistant transmissible strain genotypes. The Liverpool genotype, which was originally found in paediatric patients, was subsequently identified in...
adult CF patients, where it occasionally ‘superinfected’ individuals and eventually replaced their original strains (McCallum et al., 2001). The occurrence of these transmissible strains in UK CF patients and other reports of similar strains worldwide, e.g. Melbourne (Armstrong et al., 2002), Germany and other parts of Europe (Römling et al., 1994), has provoked debate on infection control issues and the management of these patients. However, some surveys have found no evidence of cross-infection within their clinics (Speert et al., 2002).

Currently, many centres segregate patients on the basis of their colonization status with *Burkholderia cepacia* and it remains controversial whether this should be extended to include transmissible strains of *P. aeruginosa*. Moreover, it has been suggested recently that patients colonized with a transmissible genotype of *P. aeruginosa* have a poorer prognosis and an increased need for treatment with intravenous antibiotics (Armstrong et al., 2002; Jones et al., 2002).

The extent of cross-infection with *P. aeruginosa* among CF patients in UK centres was unknown. Therefore, a 15-month surveillance project of the genotypes of isolates was undertaken. CF centres in England and Wales were invited to submit *P. aeruginosa* isolates for molecular identification. These isolates were characterized by the gold-standard molecular typing technique, PFGE, which generates DNA profiles based on restriction fragment patterns, allowing comparison between isolates and designation of genotypes. The relatedness of representatives of the predominant genotypes was investigated further by fluorescent amplified fragment length polymorphism (FAFLP) analysis, a PCR-based molecular typing technique that randomly samples a small fraction of the entire genome and has been used successfully in the past to type *P. aeruginosa* (Speijer et al., 1999). Isolates were also tested in a PCR assay designed to identify a marker highly associated with the Liverpool genotype (Parsons et al., 2002).

**METHODS**

**Participants.** The strategy to determine the sample size for the survey was as follows. There are approximately 7500 CF patients in the UK, about 70 % (5250) of whom are assumed to be colonized with *P. aeruginosa*. The required sample size was calculated assuming a range of values for the proportion of patients colonized by a transmissible strain genotype and for different levels of precision in a sample estimate. A satisfactory level of precision (±2 %) was found to result if 993 isolates were surveyed and the proportion of the population colonized by a transmissible strain was less than 15 %. Therefore, the intended sample size was chosen to be greater than 993. Less satisfactory levels of precision would be expected if the proportion of the CF population colonized by transmissible genotypes was greater than 15 %.

Microbiologists from 120 laboratories in England and Wales were invited to participate in the survey. Single patient isolates of *P. aeruginosa* from at least 20 % of out- and in-patient attendees over a 1 month period were requested. Sending laboratories were asked to pick the predominant colonial form from primary culture plates or, where necessary, to submit different colonial variants from the same specimen. In order to optimize compliance, centres were asked to submit the isolates blinded, so isolates were received on agar slopes with numerical identifiers only. Representative isolates of the Liverpool and Manchester genotypes were received from John Govan, Cystic Fibrosis Microbiology Laboratory, Edinburgh, UK and isolates of the Melbourne genotype were received from Craig Winstanley, Department of Medical Microbiology and Genitourinary Medicine, University of Liverpool, UK. Isolates of Clone C were received from U. Römling, Microbiology and Tumor Biology Center, Karolinska Institute, Stockholm, Sweden.

The study was performed blinded and epidemiological information was not requested or examined until after completion of data analysis.

**Genotyping and strain characterization.** To determine the size and range of predicted fragments, the genome sequence of *P. aeruginosa* strain PA01 was digested in silico with *Spe*I on the TIGR CMR restriction digest tool website (http://www.tigr.org/tigr-scripts/CMR2/Restrict_display.pl). The Chef Mapper (Bio-Rad) auto algorithm was used to determine switching times for the optimal separation of fragments by PFGE. Isolates were cultured on nutrient agar at 37 °C overnight and stored on beads (TCS) at −70 °C and on nutrient agar stab at room temperature. PFGE was performed as described previously (Kaufmann, 1998) with the following modifications: a Gram-positive bacteria lysis step (using lysis buffer containing 500 μl lysozyme ml⁻¹) was included prior to the addition of Gram-negative lysis buffer. DNA was digested with 30 U *Spe*I (Helena BioScience) in the accompanying Y¹/Tango buffer with BSA. Gels were run using the theoretically determined switching times: initial and final, 1 and 50 s, respectively. Running conditions were: temperature 12 °C, gradient 120 % and run time 30 h.

FAFLP analysis was performed and interpreted as described previously (Scott et al., 2002) using the selective primer combination Eco+0 Mse+ A. Briefly, genomic DNA was digested with MseI and EcoRI and specific double-stranded oligonucleotide adapters were ligated to the restricted fragments. PCR was then performed using a fluorescently labelled Eco primer and a one-base-pair-selective Mse primer. The resulting fragments were separated and detected using an ABI377 sequencer. The data were analysed using GenoTyper software. Dice coefficients of similarity were determined with in-house software and UPGMA clustering was performed using the PHYLIP program (Felsenstein, 1993).

PS21 PCR was performed as described by Parsons et al. (2002). Strains were tested for susceptibility, based on MICs, to nine antimicrobial agents according to British Society for Antimicrobial Chemotherapy guidelines (MacGowan & Wise, 2001).

**Data analysis.** DNA fragment analysis was performed with BioNumerics version 3.00 (Applied Maths) and clusters were defined using the Dice coefficient of similarity. UPGMA dendrograms were drawn with a position tolerance of 1-00 % and optimization of 1-00 %, Centres were analysed individually by calculating the ratio of the number of isolates that clustered together (at greater than 80 % similarity) to the number of unique isolates, to give a crude measure of the heterogeneity of strain populations within a centre. When all the isolates were combined in a single analysis, a cluster of isolates was defined as more than ten isolates clustering at greater than 80 % similarity in DNA profile.

**RESULTS**

**Transmission within centres**

In total, 1225 isolates were received from 31 centres with a mean of 27 isolates per centre (range 1 to 330); 376 duplicate or multiple isolates of the same genotype from the same patient were excluded from the final analysis, leaving 849 individual patient isolates. Initially, the PFGE patterns of
isolates from patients within single centres were compared with each other and the ratio of the number of isolates that shared similar DNA patterns (≥80% similarity) to those with unique patterns was used as a crude measure of the diversity of patient strain populations within each centre. The degree of clustering of isolates varied markedly between centres. In some hospitals there was an equivalent number of strains in each category, but in five centres the clustered isolates outnumbered the unique genotypes, suggesting that cross-infection between patients was more prevalent in these centres (data not shown).

Transmission between centres

The PFGE fragment patterns of the 849 patient isolates were compared across all centres and six clusters containing 233 isolates were identified (Fig. 1). The patterns of each of these clusters were matched with the DNA patterns of the representative strains from published outbreaks (Liverpool, Manchester, Melbourne and Clone C). Ninety-three isolates from 15 centres (Table 1 and Fig. 2) clustered with the representatives of the Liverpool genotype (cluster 1), 11 isolates from three centres clustered with the Manchester genotype (cluster 3) and 15 isolates from eight centres with the Clone C genotype (cluster 2). Three novel clusters (clusters 4, 5 and 6) were identified. Cluster 5 (Midlands 1) contained 86 isolates, of which 66 were from hospital 1 (Midlands), five were from hospital 8 (Trent), five from hospital 12 (Midlands) and three from hospital 16 (London). Five further centres each had isolates of this genotype (Table 1). Cluster 4 contained 12 isolates from four geographically distinct hospitals. Ten isolates from hospital 8 (Trent) formed cluster 6, designated Trent. None of the UK isolates clustered with the Melbourne genotype.

Additional typing markers

FAFLP of the transmissible panel strains revealed more heterogeneity among the Liverpool strains than shown by PFGE (Fig. 3), but these isolates were still grouped together as a single genotype. Their relatedness was further confirmed by the fact that only isolates of this genotype were positive for the PS21 marker (Table 2). In contrast, isolates of the Midlands 1 genotype exhibited tight clustering in FAFLP analysis.

There was considerable variability in the antimicrobial susceptibility of different isolates of the Liverpool genotype (Table 2), with some isolates showing susceptibility to all of the nine agents tested while others expressed resistance to all antimicrobials but colistin. Variation was also evident in the susceptibility of the two Manchester genotype isolates from different centres and for representatives of the Midlands 1 genotype, one of which expressed resistance to colistin.

DISCUSSION

The genetic population structure of P. aeruginosa from various clinical and environmental sources is characterized by the presence of unique strain lineages intermixed with major clonal complexes. These complexes may be widespread and contain strains of diverse geographical and ecological origin with no demonstrable direct link or association between them (Pirnay et al., 2002). The results obtained here suggest that there is a similar population structure among CF P. aeruginosa strains. The relative concordance of isolates within a genotype cluster may be interpreted as indicative of transmission of a single strain between patients, or it may reflect independent acquisition of this strain from diverse sources. Variability of DNA fingerprints derived from macrorestriction analysis is often the consequence of insertions/deletions rather than single nucleotide polymorphisms (Kiewitz & Tümmler, 2000).
### Table 1. Distribution of clusters of *P. aeruginosa* isolates from CF patients attending different hospitals, and prevalence of predominant genotypes

<table>
<thead>
<tr>
<th>Hospital</th>
<th>Region</th>
<th>No. isolates</th>
<th>No. clusters within centre*</th>
<th>No. isolates of predominant genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Midlands</td>
<td>249</td>
<td>26</td>
<td>Liverpool: 20</td>
</tr>
<tr>
<td>2</td>
<td>North West</td>
<td>35</td>
<td>6</td>
<td>Liverpool: 6</td>
</tr>
<tr>
<td>3</td>
<td>East</td>
<td>5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>North West</td>
<td>12</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>North</td>
<td>12</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>South East</td>
<td>19</td>
<td>2</td>
<td>Liverpool: 1</td>
</tr>
<tr>
<td>7</td>
<td>East</td>
<td>37</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Trent</td>
<td>36</td>
<td>3</td>
<td>Liverpool: 16</td>
</tr>
<tr>
<td>9</td>
<td>South West</td>
<td>42</td>
<td>5</td>
<td>Liverpool: 7</td>
</tr>
<tr>
<td>10</td>
<td>South East</td>
<td>13</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>London</td>
<td>35</td>
<td>4</td>
<td>Liverpool: 5</td>
</tr>
<tr>
<td>12</td>
<td>Midlands</td>
<td>24</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Midlands</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Trent</td>
<td>19</td>
<td>1</td>
<td>Liverpool: 12</td>
</tr>
<tr>
<td>15</td>
<td>London</td>
<td>71</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>London</td>
<td>167</td>
<td>26</td>
<td>Liverpool: 11</td>
</tr>
<tr>
<td>17</td>
<td>South West</td>
<td>9</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Wales</td>
<td>23</td>
<td>4</td>
<td>Liverpool: 1</td>
</tr>
<tr>
<td>19</td>
<td>South West</td>
<td>6</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Wales</td>
<td>9</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Combined hospitals (21–31)</td>
<td>21 (11)*</td>
<td>4 (3)</td>
<td>1 (1)</td>
<td>Liverpool: 11</td>
</tr>
<tr>
<td>Reference strains</td>
<td>10</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>849</strong></td>
<td><strong>93</strong></td>
<td><strong>11</strong></td>
<td><strong>15</strong></td>
</tr>
</tbody>
</table>

*Individual centre analysis, clusters defined as two or more isolates showing greater than 80% similarity in DNA profile.
†Number of centres.

**Fig. 2.** Uniformity of the PFGE profile of the Liverpool genotype in 15 CF centres. *, Shown as combined hospitals in Table 1.
Nevertheless, strains can acquire diverse genomic islands and plasmids without the disruption of the overall genetic architecture of the clone, as reflected by their PFGE profile (Dinesh et al., 2003). In this study we have used the term genotype to describe isolates with PFGE patterns that fall within an 80% similarity level and so constitute a phylogenetic cluster. This cut-off value was derived from an earlier study of the capacity of SpeI macrorestriction patterns resolved by PFGE to distinguish between geographically and temporally unrelated strains of \textit{P. aeruginosa} (Grundmann et al., 1995) and equates to four or more band differences between isolates of different genotype.

The results of the survey indicate that, although the majority of CF patients harboured unique strains, about one-fifth of those sampled were infected by one of two transmissible genotypes. A third transmissible genotype, which was first identified in Germany, and subsequently in other parts of Europe (Dinesh et al., 2003), was also widespread in the centres studied. The Liverpool genotype accounted for 11% of isolates analysed and was present in 48% of the centres. Midlands 1 was the second most common genotype, representing 10% of isolates and found in 29% of centres. In contrast, the Manchester genotype was present in only three centres and accounted for 1% of all isolates; although widespread, clone C was represented by only 2% of isolates. The most likely explanation for the distribution of these genotypes is that cross-infection with \textit{P. aeruginosa} occurred in patients both within and between centres in England and Wales.

\begin{table}
\centering
\caption{Antimicrobial susceptibility of representative predominant genotypes of \textit{P. aeruginosa} from CF patients}
\begin{tabular}{|c|c|c|c|c|c|c|c|c|c|c|c|c|}
\hline
Isolate & Hospital & Genotype* & PS21 PCR & \multicolumn{12}{c|}{Susceptibility to:} \\
& & & & Amk$^\dagger$ & Ctz & Cip & Gen & Imp & Mer & Pip & Pip/Taz & Col \\
\hline
08599 & 19 & L & + & R & S & S & R & S & S & S & S & S \\
08794 & 16 & L & + & I & S & S & R & S & S & S & S & S \\
08811 & 18 & L & + & R & R & R & R & R & R & R & R & S \\
08959 & Reference strain & L & + & S & R & S & S & R & S & R & R & S & S \\
08981 & 14 & L & + & R & S & S & R & S & S & S & S & S \\
09346 & Reference strain & L & + & R & R & R & I & R & R & S & S & S \\
09876 & Reference strain & L & + & R & S & I & I & S & S & R & S & S \\
08799 & 16 & M & – & I & S & I & I & R & S & S & S & S \\
08960 & Reference strain & M & – & I & R & I & I & R & R & S & S & S \\
08916 & 1 & Md1 & – & S & R & S & S & R & R & R & R & S \\
09245 & 1 & Md1 & – & I & S & I & R & S & S & S & S & R \\
09231 & 14 & Md1 & – & I & S & S & I & S & S & S & S & S \\
09608 & 9 & Md1 & – & I & S & I & I & R & S & S & S & S \\
\hline
\end{tabular}
\begin{flushleft}
*L, Liverpool genotype; M, Manchester; Md1, Midlands 1.
$^\dagger$Amk, Amikacin; Ctz, ceftazidime; Cip, ciprofloxacin; Gen, gentamicin; Imp, imipenem; Mer, meropenem; Pip, piperacillin; Taz, tazobactam; Col, colistin. S, Sensitive; I, intermediate; R, resistant.
\end{flushleft}
\end{table}
The widespread distribution of the Liverpool and other transmissible genotypes was not expected, as previous studies have not reported cross-infection between centres (Jones et al., 2001; McCallum et al., 2001). Indeed, it has been suggested that the presence of transmissible genotypes in adult CF centres may be due to colonization with these strains whilst attending paediatric clinics from where they are imported into the adult CF population. We are unable to comment on this owing to the lack of patient identifiers and clinical data accompanying the isolates. However, we found no evidence of the Manchester genotype, which was originally described in adult patients, in samples from the paediatric clinics in this city.

For many centres we failed to reach the statistically recommended sample size of 20% of patients, as the response was very variable across centres. The use of single patient samples and the absence of follow-up samples in clinics to establish whether further acquisition and accumulation of transmissible genotypes was occurring are obvious limitations of this type of snapshot study. Nevertheless, the evidence of widespread cross-infection has significant implications for clinical practice and infection control. The original studies of the Liverpool and Manchester genotypes (Cheng et al., 1996; Jones et al., 2001) suggest that acquisition of these strains is strongly associated with an in-patient stay in hospital. Despite this, the Manchester genotype was not found in extensive samples of the ward environment or on the hands of attendant staff, although it was detected in a small number of room air samples following airway clearance, spirometry and nebulization procedures (Jones et al., 2003).

The results of FAFLP analysis suggested that there was greater microheterogeneity within the Liverpool genotype than within the Midlands 1 genotype. FAFLP randomly samples multiple sites of the bacterial genome and the variation seen with the Liverpool genotype may reflect the number of years since the emergence of the strain (Cheng et al., 1996). Over this time, genetic rearrangements due to insertions, deletions and substitutions along with acquisition of foreign DNA, such as phage, and recombinational events have all probably contributed to the diversity of the clone, although the overall genomic structure as revealed by PFGE remains remarkably conserved (Fig. 2). The Midlands 1 genotype, in contrast, is probably quite recent in origin and this is supported by the lack of heterogeneity revealed by FAFLP. The availability of the PS21 PCR as a specific tool for the Liverpool genotype should be of great benefit to centres wishing to explore its prevalence and distribution in their patient population and environment as it offers a rapid and sensitive means of confirming the strain without the need for labour-intensive DNA fingerprinting. This fragment was one of a number of gene sequences revealed by suppressive subtractive hybridization to select a strain-specific marker (Parsons et al., 2002) and further work in this area is indicated to provide similar test methods for other transmissible strains.

Some centres currently segregate patients based on their microbiological status, as is the case for B. cepacia. There is now an ongoing debate over whether segregation should be extended to patients with P. aeruginosa or even to extend this to include transmissible genotypes. This is of particular importance, as recent work suggests that patients colonized with the Manchester genotype have an increased treatment requirement, and need more intravenous antibiotics and resources (Jones et al., 2002). This raises the issue as to the number of separate out-patient clinics that can be realistically implemented by a treatment centre, and the effectiveness of such segregation in containing the spread of these transmissible strains is questionable given the interaction of patients resident in the hospital ward and/or those who meet socially.

It is clear, and probably reassuring for the CF community, that transmissibility of certain strains does not appear to be linked with antimicrobial resistance, as isolates of the predominant genotypes showed variable susceptibility to most of the antibiotics tested. Colistin resistance was restricted to the Midlands 1 genotype, but this was not uniform for all members of the genotype. It is noteworthy that the Liverpool genotype showed variable susceptibility to ceftazidime, although ceftazidime resistance was the defining marker for this strain when it was first described in 1996 (Cheng et al., 1996). However, the use of ceftazidime monotherapy was linked to the appearance of resistance to this agent and one might assume that susceptible and resistant populations may have coexisted and spread independently over the years as a consequence of an undefined “transmissibility marker”. An alternative explanation for the variability of modern isolates of this strain may lie in mutational events in either the ampC porin or the efflux pump apparatus leading to instability of resistance to the antibiotic (Livermore, 2002).

There has been some discussion regarding the population structure of P. aeruginosa. Early work, whilst cautioning against inferring a population structure from a single class of genetic marker, suggested a panmictic structure (Denamur et al., 1993; Picard et al., 1994). However, current thinking based on a polyphasic approach involving outer-membrane protein gene sequences, serotype and pyoverdin type suggests an epidemic structure (Pirnay et al., 2002) similar to organisms such as Neisseria meningitidis, where, although the population is sexual in the long term, epidemic clones arise and proliferate (Feil et al., 2001). This model of population structure would partially explain the number and diffusion of transmissible clones (e.g. Clone C) that have been described worldwide, which have no particular environmental niche (Kiewitz & Tümmler, 2000) and are found both in the environment and within different patient groups. The transmissible genotypes described here, however, appear to be unusual in that they predominate in CF patients and, as mentioned previously, have yet to be isolated from the hospital environment.

This survey has clearly shown that cross-infection has taken place within and, for the first time, between CF centres
nationally, which highlights the need for continued surveillance to inform infection control policies for this group of patients.

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