Genetic relatedness of *Pseudomonas aeruginosa* isolates among a paediatric cystic fibrosis patient cohort in Ireland

Catriona Logan,1 Adele Habington,1 Gráinne Lennon,1 Juanita Grogan,1 Margaret Byrne,1 John O’Leary2,3 and Niamh O’Sullivan1,2

1Department of Microbiology, Our Lady’s Children’s Hospital, Crumlin, Dublin, Ireland
2Department of Pathology, Coombe Women and Infants University Hospital, Dublin, Ireland
3Trinity College Dublin, Dublin, Ireland

*Pseudomonas aeruginosa* is one of the primary pathogens in the cystic fibrosis (CF) lung and a significant cause of morbidity and mortality. Reports of the spread of epidemic or transmissible strains of *P. aeruginosa* within and across CF centres in Europe have raised concern regarding the possibility of clonal spread among and within CF centres in Ireland. *P. aeruginosa* isolates (313 isolates from 142 sputum samples and 53 throat swabs) from 68 CF patients were examined using PFGE to explore the diversity of *P. aeruginosa* isolates among CF patients in a Dublin paediatric hospital. Only 57 different *P. aeruginosa* genotypes were identified among the 313 isolates. Forty-three of the genotypes were observed only in individual patients (distinct genotypes) while 13 cluster strains (present in two to four patients) were observed. Typing of *P. aeruginosa* isolates identified one indistinguishable clonal isolate of *P. aeruginosa* present in 13 CF patients (13/68; 19.1 %) which displayed higher levels of antibiotic resistance than those displayed by *P. aeruginosa* isolates of distinct genotype.

**INTRODUCTION**

Chronic lung infection is one of the major clinical complications among cystic fibrosis (CF) patients. The progressive loss of pulmonary function associated with infection by pathogens such as *Pseudomonas aeruginosa* results in increased patient morbidity and mortality (Henry et al., 1992; Kosorok et al., 2001; Rabin et al., 2004). Patient age at *P. aeruginosa* acquisition has been identified as a significant determinant of patient prognosis (Nixon et al., 2001) as the establishment of mucoid *P. aeruginosa* variants results in chronic infection that cannot be successfully eradicated from the CF lung.

The environment was once considered to be the primary source of *P. aeruginosa* infection for CF patients, and the sharing of *P. aeruginosa* isolates among unrelated patients was considered unusual. In more recent years, however, the identification of epidemic strains of *P. aeruginosa* in countries including the UK, Australia, Canada and Denmark has highlighted the potential of patient-to-patient transmission of the organism (Jones et al., 2001; Smart et al., 2006; O’Carroll et al., 2004; Anthony et al., 2002; Speert et al., 2002; Jelsbak et al., 2007). Further investigation identified that some epidemic strains are capable of ‘superinfection’ (replacing infection with non-epidemic *P. aeruginosa* strains), spreading to non-CF relatives (McCallum et al., 2001, 2002), and result in increased treatment requirements (Jones et al., 2002) and increased morbidity among chronically infected patients (Al-Aloul et al., 2004). The Australian pulsotype-1 strain was specifically associated with more severe disease in young patients (Armstrong et al., 2002).

Many CF clinics do not routinely perform molecular typing of *P. aeruginosa* isolates, and the sharing of clonal strains may be even more common than currently acknowledged. Routine laboratory methodologies such as phenotypic markers and antibiotic susceptibility profiles cannot reliably identify epidemic strains and thus genotypic analysis of *P. aeruginosa* isolates is required to guide patient segregation strategies within CF clinics (Al-Aloul et al., 2004).

PFGE is a whole-genome, image-based DNA fingerprinting method based on the comparison of DNA fragments generated by restriction digestion using rare-cutting restriction endonucleases. PFGE has been demonstrated to be highly discriminatory for the typing of *P. aeruginosa* (O’Carroll et al., 2004; Scott & Pitt, 2004) and is considered the ‘gold-standard’ method for microbial typing.

To date, there is limited information regarding the genotypic variability of *P. aeruginosa* isolates within CF patients in Ireland. Kenna et al. (2007) examined the
hypermutilability of \textit{P. aeruginosa} strains from a number of sources. The paper listed four epidemic strains from Ireland (Dublin 1–4) that were examined as part of the study. However, no information on the strains was provided regarding their source, epidemiology or method of identification with the authors simply referring to the strains as ‘unpublished’. A study of \textit{P. aeruginosa} isolates among adult CF patients in Northern Ireland identified a high degree of genetic heterogeneity among isolates with no evidence for the existence of a clonal strain within the cohort examined (Clarke \textit{et al.}, 2008). More recently, however, Kakinuma \textit{et al.} (2010) have reported the isolation of the Liverpool epidemic strain (LES) from the sputum of adult patients attending a CF centre in Northern Ireland. A study exploring the diagnostic potential of real-time PCR for the early detection of \textit{P. aeruginosa} found no evidence of either the LES or the Manchester epidemic strain (MES) among the patient cohort that is the subject of this current manuscript (Logan \textit{et al.}, 2010).

To the authors’ knowledge, this study is the first published report detailing an examination of the genetic relatedness of \textit{P. aeruginosa} isolates from CF patients in Ireland. The current authors aimed to explore the distribution of \textit{P. aeruginosa} strains within the CF clinic population of a Dublin paediatric hospital. This study utilized PFGE to examine the inter- and intra-patient variability of isolates, and to determine whether a clonal isolate(s) of \textit{P. aeruginosa} is harboured by multiple patients within the cohort.

**METHODS**

**Collection of bacterial isolates.** Isolates of \textit{P. aeruginosa} (single colonies representative of each morphological type) from CF patient clinical samples (sputum and throat swabs) were collected over a 32-month time period (March 2007–October 2009) in the course of routine clinical care of the patients, including attendance at the hospital CF clinic, at hospital admission and during inpatient stay. The bacterial isolates were retained on cryo-beads at 80°C as part of a longitudinal study that also investigated the efficacy of real-time PCR for the early detection of \textit{P. aeruginosa} in CF patients (Logan \textit{et al.}, 2010).

**Microbiological methods.** Throat swabs were initially inoculated into 1 ml PBS, mixed well and one drop of this suspension was placed on each of the culture plates. Sputum samples were inoculated directly onto the culture plates and a swab was dipped in sputum and inoculated into 1 ml PBS. PBS samples were frozen at –20°C and tested by real-time PCR for \textit{P. aeruginosa} (Logan \textit{et al.}, 2010). Samples were cultured on Columbia Chocolate agar, Columbia Blood agar, MacConkey agar (Oxoid) and a range of selective media to maximize the isolation of all potential pathogens and were processed according to routine laboratory procedures (Kiska & Gilligan, 2003). Blood, Chocolate and MacConkey agar plates were examined at 24 and 48 h incubation for \textit{P. aeruginosa} colonies. Gram-negative bacilli that were oxidase-positive, non-fermenters and produced pyocyanin and pyoverdin (bright green), pyrorubin (red) or pyomelanin (brown-black) on Pseudomonas cetrimide agar were identified as \textit{P. aeruginosa} (Kiska & Gilligan, 2003). Isolates that failed to produce pigment or only produced pyoverdin were further identified using the API 20NE (bioMérieux Vitek) identification system. For all \textit{P. aeruginosa} isolates, sensitivity to the following antibiotics was determined by disc diffusion susceptibility testing as described in the Clinical and Laboratory Standards Institute documentation (CLSI, 2009): amikacin, gentamicin, tobramycin, colistin, ciprofloxacin, ofloxacin, ceftazidime, imipenem, meropenem, aztreonam and piperacillin/tazobactam.

**Typing of \textit{P. aeruginosa} isolates by PFGE.** PFGE of \textit{P. aeruginosa} isolates was performed using the CHEF MAPPER apparatus and the CHEF Bacterial Genomic DNA Plug kit (Bio-Rad). \textit{P. aeruginosa} isolates were grown overnight in Brain Heart Infusion Broth at 37°C on a shaker. Cells (0.5 ml) were harvested by centrifugation of culture broth at 3500 g for 15 min. The cell pellet was resuspended in cell suspension buffer (CHEF Mammalian Genomic DNA Plug kit; Bio-Rad) to give a final concentration of 1 × 109 c.f.u. ml⁻¹, mixed with an equal volume of 2% agarose which had been equilibrated to 50°C and transferred to plug moulds and allowed to solidify. All resuspensions, mixing and transfer steps were carried out using wide-bore pipette tips to minimize cell damage. The digestion (lysozyme and proteinase K) of bacteria, washing of the plugs, and subsequent restriction digestion of the bacterial DNA with SpeI (New England Biolabs) overnight was performed as recommended by the kit manufacturer. Electrophoresis was performed in a 1.0% agarose (SeaKem) gel prepared in 0.5 × TBE buffer with a buffer (0.5 × TBE, 50 µM thio urea) flow rate of 1 min⁻¹ and at a running temperature of 14°C. The optimal run conditions for the separation of samples were determined to be 22.5 h at 6.0 V cm⁻¹ with initial switch time of 1 s, final switch time of 50 s, and included angle of 120 degrees. A PFGE Lambda DNA ladder was included on each gel as a molecular size marker (New England BioLabs). The gel was stained in 3 × Gel Red solution (Cambridge Bioscience) for 30 min and visualized under UV light.

A total of 11 \textit{P. aeruginosa} isolates, nine isolates (from four patients) and two isolates of the clonal strain, were analysed further by PFGE analysis (as described) following overnight Dral digestion.

**Analysis of PFGE profiles.** Relatedness among PFGE profiles was evaluated using Fingerprinting II software (Bio-Rad). A band-based dendrogram was produced using the Dice coefficient and an unweighted pair group method using arithmetic averages, band optimization set to 1.5% and band position tolerance set to 1.5%. The criterion for defining genetic relatedness was determined following repeated subculture and analysis of \textit{P. aeruginosa} PAO1, which identified a minimum inter-run similarity of 93.77%. Banding patterns were also compared visually and the Tenover criteria were applied to describe strain relatedness (Tenover \textit{et al.}, 1995).

Consistent with the recommendations of O’Carroll \textit{et al.} (2004), the term ‘clonal strain’ was used in this study to refer to a \textit{P. aeruginosa} genotype (>90% similar bands by PFGE) identified in more than four patients as there was no definitive epidemiological evidence of the transmission of \textit{P. aeruginosa} from patient to patient or from environment to patient. Minor clonal strains occurring in two to four patients were termed a ‘cluster strain’.

**Statistical analysis.** Chi-square with Yates’ correction or Fisher’s two-tailed exact tests (if there were fewer than five observations) were employed to compare antimicrobial resistance rates.

**RESULTS**

**PFGE analysis of \textit{P. aeruginosa} isolates**

\textit{P. aeruginosa} PFGE profiles typically contained 10–17 bands in the 50–490 kbp range used for analysis (Fig. 1). The reproducibility of PFGE profiles was confirmed by the
repeated analysis of *P. aeruginosa* PAO1 with each gel run (*n*=46), while stability of the PFGE profiles was confirmed by multiple passages of the isolates and PFGE analysis.

**Analysis of patient isolates of *P. aeruginosa***

*P. aeruginosa* isolates from 195 clinical specimens (142 sputa, 53 throat swabs) from 68 patients (including nine sibling pairs) were analysed by PFGE following digestion with SpeI producing a total of 313 *P. aeruginosa* PFGE profiles. The mean number of clinical samples examined per patient was 2.9 (range 1–8) while the mean number of isolates examined per patient was 4.6 (range 1–14). The mean patient age was 12.98 years at the end of the study (range 1.70–20.72 years) and 35 of the 68 patients (51.5 %) were male.

**Individual patient profiles**

To examine inter-patient variability of isolate genotypes, efforts were made to examine bacterial isolates from a number of clinical specimens per patient over the 32-month time period of the study. Thirteen patients had isolates from just one clinical specimen available for analysis. However, the isolates from two clinical specimens from 23 patients were examined, while the isolates from three clinical specimens from 16 patients were analysed. The remaining 16 patients had isolates from four–eight clinical specimens examined by PFGE. The maximum number of morphologically distinct isolates observed in an individual clinical specimen was five.

Forty-seven patients (69.1 %) were observed to harbour just one *P. aeruginosa* genotype (range one to eight clinical samples examined per patient) as isolates from the same patient with different morphology and/or antibiotic susceptibilities were most commonly observed to be genotypically indistinguishable. Nine patients (13.2 %) were observed to harbour two genotypes (range two to eight clinical samples examined per patient), and six patients (8.8 %) were observed to harbour three *P. aeruginosa* isolate genotypes (range two to seven clinical samples examined per patient). Six patients (8.8 %) had only one bacterial isolate examined as part of this study.

**Sibling pairs**

Analysis of the profiles of the nine individual sibling pairs identified that eight of the pairs harboured indistinguishable genotypes. In the case of two sets of sibling pairs, one of the siblings was observed to harbour two additional *P. aeruginosa* genotypes.

**Clonal analysis**

A dendrogram comparing each of the PFGE banding patterns with all other banding patterns was constructed using Fingerprinting II software and demonstrated the rates of genomic similarity (Fig. 2). Algorithmic analysis correlated well with visual inspection of the banding patterns using the criteria of Tenover *et al.* (1995). Only 57 different *P. aeruginosa* genotypes were identified by PFGE among the 313 isolates examined. Forty-three of the genotypes were observed only in individual patients (distinct genotypes, corresponding to 150 isolates). Thirteen cluster strains and one clonal strain of *P. aeruginosa* were observed.

Twenty-seven patients (39.7 %) were observed to harbour one or more distinct genotypes. Thirty-four patients (50.0 %) harboured one or more cluster strains, and seven patients (10.3 %) were observed to carry both distinct and cluster *P. aeruginosa* strains. Cluster strains were identified in two groups of four patients (one group of which consisted of two sibling pairs), two groups of three
patients, and nine groups of two patients (three of which were sibling pairs). One group of 13 patients (including three sibling pairs) harboured the same genotype, i.e. a clonal strain. The clonal strain identified in 13 of 68 (19.1%) patients was observed in 40 of the 313 isolates examined. PFGE profiles of the 40 isolates were assessed by the software as having 92.43% similarity (Fig. 2; Dice coefficient, Fingerprinting II software). A visual inspection of the PFGE profiles identified that 37 of the isolates demonstrated an indistinguishable SpeI PFGE banding pattern. One isolate contained a single band difference and the remaining two isolates displayed two band differences from the banding pattern displayed by the 37 isolates. Two of the patients harbouring the clonal P. aeruginosa strain were observed to harbour one additional cluster strain to the clonal strain identified, while one of the patients harbouring the clonal strain was observed to harbour another cluster strain and a distinct P. aeruginosa strain.

Nine isolates (from four patients) observed to cluster at 84.03% similarity with isolates of the clonal strain following SpeI digestion were examined by PFGE analysis following Dral macrorestriction (Fig. 2). Visual analysis of the 11 isolate profiles following Dral digestion determined that the nine isolates were not genotypically identical to isolates of the clonal strain.

The age profiles of patients harbouring the clonal strain are detailed in Table 1. Three of the five patients 12 years of age or under that were observed to harbour the clonal strain had older siblings also harbouring the clonal strain. Six (46.2%) of the 13 patients harbouring the clonal strain were male, while 29 of 55 (52.7%) patients from whom the clonal strain was not detected were male. Patients harbouring the clonal strain (n=13) had a mean age of 12.43 years at the end of the 32-month study period (range 1.70–18.51 years) as compared to a mean age of 13.11 years (range 2.20–20.72 years) for those patients from whom the clonal strain was not detected (n=55).

**Genotype and clinical specimen type**

Nineteen patients had isolates derived from both throat swabs and sputa (range of 2–8 clinical specimens and 2–14 isolates per patient). Fourteen of these 19 patients were observed to harbour just one P. aeruginosa strain demonstrating agreement between the P. aeruginosa genotypes derived from each sample type. The remaining five patients harboured multiple P. aeruginosa strains: four patients harboured three strains and one patient harboured two strains. Analysis identified that genotypically indistinguishable isolates were cultured from the throat swab and sputum specimens of all four individual patients. Four of the patients were observed to harbour additional P. aeruginosa strains in throat swab specimens and one patient harboured a distinct P. aeruginosa strain cultured from sputa which was not detected in a throat swab specimen from the patient.

**Phenotype and antibiotic resistance**

The clonal strain was exclusively non-mucoid in all 13 patients (40 isolates) from which it was identified. This is in contrast to the P. aeruginosa isolates of distinct genotype (i.e present in only one patient) where 50 of the 150 isolates displayed the mucoid phenotype. The results of antibiotic sensitivity testing are presented in Table 2. Isolates of the clonal strain from sibling pairs did not display identical antibiograms. While more resistant to a number of antibiotics than non-clonal isolates, the exclusively non-mucoid clonal strain could not be identified by a distinctive antibiogram, although 100.0% (40/40) of clonal isolates were observed to be susceptible to piperacillin/
tazobactam and colistin (Table 2). Two isolates of the clonal strain (from two children) remained susceptible to all antibiotics tested.

**DISCUSSION**

This study provides what is believed to be the first published report exploring the genetic relatedness of *P. aeruginosa* isolates among a CF patient cohort in Ireland. The routine identification of clonal, predominant or transmissible strains is not possible based on phenotypic identification and instead requires epidemiological surveillance and molecular genotyping techniques. Consistent with previous reports that individual patients harbouring isolates with different colonial morphology mostly belonged to the same genotype (Van Daele et al., 2005; da Silva Filho et al., 2001), this study has confirmed the lack of relationship between *P. aeruginosa* phenotype and genotype. Seventy-six per cent (47/62) of patients from whom more than one *P. aeruginosa* isolate was examined by PFGE were observed to harbour just one *P. aeruginosa* genotype.

The longitudinal design of this study has corroborated the results of previous research demonstrating that most CF patients are colonized for long periods with a single clone (Speert et al., 2002), as patients were observed to harbour indistinguishable *P. aeruginosa* strains over the 32-month period of the research study (up to 14 isolates examined per patient).

Thirteen patients (including three sets of sibling pairs) were identified as harbouring an indistinguishable clonal strain of *P. aeruginosa* (40 isolates, all non-mucoid) corresponding to 19.1% of the patient cohort. This study is believed to

<table>
<thead>
<tr>
<th>Patient age</th>
<th>Number of patients</th>
<th>Number of patients with clonal strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤3 years</td>
<td>2</td>
<td>1*</td>
</tr>
<tr>
<td>&gt;3–6 years</td>
<td>7</td>
<td>1*</td>
</tr>
<tr>
<td>&gt;6–9 years</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>&gt;9–12 years</td>
<td>4</td>
<td>2†</td>
</tr>
<tr>
<td>&gt;12–15 years</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>&gt;15–18 years</td>
<td>19</td>
<td>2</td>
</tr>
<tr>
<td>&gt;18–21 years</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>68</td>
<td>13</td>
</tr>
</tbody>
</table>

*Patient with older sibling harbouring the clonal strain.
†One of the two patients had an older sibling harbouring the clonal strain.

Table 2. Antimicrobial resistance (%) of *P. aeruginosa* isolates

<table>
<thead>
<tr>
<th>Antibiotic*</th>
<th>Clonal strain (n=40)†</th>
<th>Unrelated (distinct) strains‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mucoid (n=50)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>75.0</td>
<td>24.0 (P&lt;0.0001)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>87.5</td>
<td>26.0 (P&lt;0.0001)</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>32.5</td>
<td>4.0 (P=0.0004)</td>
</tr>
<tr>
<td>Colistin</td>
<td>0</td>
<td>2.0 (P=1)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>40.0</td>
<td>10.0 (P=0.002)</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>92.5</td>
<td>36.0 (P&lt;0.0001)</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>55.0</td>
<td>12.0 (P&lt;0.0001)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>70.0</td>
<td>0.0 (P&lt;0.0001)</td>
</tr>
<tr>
<td>Meropenem</td>
<td>47.5</td>
<td>0.0 (P&lt;0.0001)</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>55.0</td>
<td>12.0 (P&lt;0.0001)</td>
</tr>
<tr>
<td>Penicillin/tazobactam</td>
<td>0</td>
<td>2.0 (P=1)</td>
</tr>
</tbody>
</table>

*Breakpoints for antibiotic resistance in accordance with CLSI criteria (CLSI, 2009).
†All *P. aeruginosa* isolates of the clonal strain identified in this study were non-mucoid.
‡Chi-square with Yates’ correction or Fisher’s two-tailed exact tests were employed to compare resistance rates of the 40 clonal strain isolates to the resistance rates of the mucoid and non-mucoid unrelated (distinct) isolates.
be the first report detailing the identification of a clonal P. aeruginosa isolate among CF patients in Ireland. The clonal strain did not exhibit a distinctive antibiogram. The antibiotic susceptibility pattern of isolates varied within an individual patient from the same specimen and also over time, which is expected and clearly described within the CF literature (Cheng et al., 1996; Lewis et al., 2005; Fothergill et al., 2005). Two patients, the youngest and an 11 year old, consistently harboured a fully sensitive clonal strain as their single Pseudomonas isolate.

The clonal strain identified in this study had a tendency to exhibit greater resistance to aminoglycosides, quinolones, cephalosporins and carbapenems as compared with other strains, both mucoid and non-mucoid, present in this CF patient cohort. Increased resistance is a typical characteristic of epidemic strains (Cheng et al., 1996; Fothergill et al., 2005; Fothergill et al., 2010). The lack of resistance to colistin and piperacillin/tazobactam is surprising as both are first-line agents used to treat clinical exacerbations within this CF unit. None of the clonal strain isolates exhibited a mucoid phenotype, which is unusual, and it remains to be seen whether a mucoid phenotype will emerge over time. While the emergence of a predominantly non-mucoid clonal strain is considered more favourable for CF patient well-being than a mucoid variant, Van Daele et al. (2005) have underlined the significance that such non-mucoid clones form a ‘microbial reservoir’ from which the mucoid phenotype, biofilms and ultimately chronic colonization of the CF lung are founded.

The relevance of the newly identified clonal strain and the long-term morbidity linked to its carriage remain to be determined. At this point, it is not clear how long this isolate has been present within our CF unit; however, 3 of the 13 patients harbouring the clonal isolate also harboured additional P. aeruginosa strains with a mucoid phenotype. Future investigations must focus on continued surveillance to monitor the prevalence of this dominant strain within both this CF patient cohort and the environment, in an effort to differentiate possible person-to-person transmission versus acquisition from a common nosocomial or environmental source, and ultimately to examine P. aeruginosa isolate diversity among other CF centres in Ireland. O’Carroll et al. (2004) commented that whilst it is not certain that all clonal strains are detrimental, the potential for transmission, regardless of pathogenicity, mandates the separation of patient groups harbouring clonal strains from those without P. aeruginosa infection.

Reports of epidemic strains of P. aeruginosa in a host of countries in recent years have highlighted the potential for patient-to-patient transmission of the organism. The LES has in recent years been identified in Northern Ireland (Kakinuma et al., 2010), and in Canada (Aaron et al., 2010), where the strain was found to be associated with a greater risk of death or lung transplantation. The clonal strain identified in this study has to date not been compared genotypically with the well-characterized epidemic strains identified in the UK such as the LES and MES. Previous findings by the authors of this current study have identified, using real-time PCR, that the P. aeruginosa isolates that are the subject of this study are distinct from the LES and the MES (Logan et al., 2010), with no evidence of the LES or MES found amongst the paediatric patient cohort examined.

In conclusion, this study has demonstrated the presence of a clonal strain of P. aeruginosa among a paediatric CF patient cohort in Ireland. The results underline the importance of ongoing longitudinal molecular typing of P. aeruginosa strains as a central element to infection control strategies within CF patient cohorts. Molecular surveillance has the potential to monitor the emergence of clonal strains within the population and ultimately aid efforts aimed at minimizing the potential for the spread of P. aeruginosa strains.

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