Molecular detection of an atypical, highly resistant, clonal *Pseudomonas aeruginosa* isolate in cystic fibrosis patients

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Abstract

Background: The identification of *Pseudomonas aeruginosa* (*P. aeruginosa*) isolates in sputum from cystic fibrosis (CF) patients can be challenging due to the multitude of phenotypic changes isolates undergo during adaptation to the microenvironment of the CF lung.

Methods: We report the occurrence of shared *P. aeruginosa* isolates which failed identification by phenotypic methodologies and required species specific polymerase chain reaction. *P. aeruginosa* isolates were genotyped by macrorestriction analysis.

Results: Analysis of atypical isolates revealed one clonal *P. aeruginosa* isolate and three smaller clusters. In contrast molecular typing of phenotypically characteristic *P. aeruginosa* isolates revealed only small clusters. Despite exhibiting higher levels of antimicrobial resistance, acquisition of atypical strains was not associated with significant changes in clinical decline.

Conclusions: Our experience highlights the importance of accurate identification of bacterial isolates in CF lung disease to detect clonal spread of atypical isolates.

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Keywords: *Pseudomonas aeruginosa*; Molecular identification; Genotyping

1. Introduction

*Pseudomonas aeruginosa* (*P. aeruginosa*) is an opportunistic pathogen and is the most frequently isolated pathogen from sputum of patients with cystic fibrosis (CF) lung disease. Approximately 80% of CF patients are infected with *P. aeruginosa* and lung infections have been associated with increased morbidity and mortality [1]. Accurate identification of *P. aeruginosa* is important for both patient management and infection control strategies.

Biochemical identification of *P. aeruginosa* in CF lung disease is complicated as *P. aeruginosa* adapts to the microenvironment of the CF lung by undergoing a multitude of phenotypic changes including loss of pigment production, alginate production (mucoidy), loss of motility and synthesis of rough lipopolysaccharide [2]. In addition *P. aeruginosa* modifies its metabolic strategy to adapt to the limited oxygen concentrations available when growing in the thick mucus layers of the CF lung. Energy generation by *P. aeruginosa* in the hypoxic microenvironment of the CF lung can occur through microaerophilic respiration, aerobic and anaerobic denitrification and possibly arginine and pyruvate fermentation [3].

These phenotypic and metabolic adaptations, together with the presence of multiple other bacterial species encountered in the CF lung, complicate biochemical identification of organisms by manual methods (API 20 NE) and commercial identification systems [4]. Several studies have highlighted the need for the development and application of molecular techniques to correctly identify *P. aeruginosa* and other non-fermenter bacteria in CF lung disease [5,6].
2. Material and methods

2.1. Clinical setting

The National Adult Centre at St. Vincent’s University Hospital manages approximately 300 CF patients. 72.4% are infected with P. aeruginosa. At the beginning of this study patients admitted to hospital and patients attending outpatient clinics were segregated by infection status when positive for P. aeruginosa, Burkholderia cepacia complex or meticillin-resistant Staphylococcus aureus. Patients were attending paediatric CF centres in Dublin before being referred to the adult services of this hospital. For the majority of patients sputum specimens were collected at 3 monthly intervals. Isolates analysed in this study were collected from 2005 to 2007. One isolate per patient was included in this study.

2.2. Culture and biochemical identification

Sputum specimens were cultured routinely on MacConkey agar, B. cepacia specific agar, mannitol salt agar and non-selective media (Columbia blood agar, Chocolate agar) (all obtained from Fannin Limited, Dublin, Ireland). P. aeruginosa isolates were phenotypically identified by colony morphology (mucoid or nonmucoid), green/blue pigmentation of colonies, positive oxidase reaction and resistance to C390 (chloro-hydrochloride) (Rosco, Denmark). All Gram-negative, oxidase positive isolates which were not phenotypically identified as P. aeruginosa were subjected to API 20 NE analysis (bioMerieux, Marcy l’Etoile, France). These isolates are hereafter referred to as ‘atypical strains’. Biochemical identification was performed according to the manufacturer’s instructions.

Antibiotic susceptibility testing was performed on Isosensitest agar (Fannin Limited, Dublin, Ireland) according to the British Society of Antimicrobial Chemotherapy guidelines.

2.3. Bacterial DNA isolation

DNA was isolated from cultures of organisms grown overnight on Columbia blood agar at 37 °C. A single isolated colony was suspended in nuclease-free water. Suspensions were boiled for 10 min and subsequently centrifuged at 2500 rpm for 5 min. The supernatant was directly used for PCR analysis.

2.4. PCR amplification

PCR was carried out as described by Spilker et al. using 16S ribosomal DNA as target [5]. Amplification was performed in a 25 μl reaction volume containing 2.5 μl 10× buffer (New England Biolabs, USA), 250 μM of each deoxynucleoside triphosphate (Roche), 0.8 μM of forward (PA-SS-F) and reverse primer (PA-SS-R), 1 U of Taq DNA polymerase (New England Biolabs, USA) and 10 μl of whole-cell bacterial lysate adjusted to a total volume of 25 μl. Amplification was carried out in a Storm thermocycler with an initial denaturation step of 2 min at 95 °C followed by 25 cycles of 20 s at 94 °C, 20 s at 56 °C and 40 s at 72 °C. Amplicons were detected by electrophoresis in a conventional 1.5% agarose gel in 0.5× tris-borate-EDTA buffer. Gels were subsequently stained with ethidium bromide and viewed under UV light.

2.5. Pulsed-field gel electrophoresis (PFGE)

PFGE was performed with contour-clamped homogenous electric field CHEF mapper equipment (Bio-Rad Laboratories) in 1.2% agarose at 14 °C, and a voltage of 200 V and a pulse rate of 5 s to 60 s, using the restriction enzyme SpeI. Gels were imaged as described above and analysed using Fingerprinting software (FPQuest, Version 4.5, Bio-Ra).

2.6. Patient data

All patients infected with atypical P. aeruginosa isolates were identified. Clinical and demographic data were obtained from each patient’s medical chart for the 2-year period before and after infection with atypical P. aeruginosa isolates. Clinical variables analysed included forced expiratory volume (FEV1), forced expiratory vital capacity (FVC) and body mass index (BMI), exacerbation history including total number of inpatient hospital days and total days on IV antibiotics.

2.7. Statistical analysis

Annual rate of change in FEV1 (% predicted) was the primary outcome of interest. Secondary outcomes included rate of change in BMI, frequency of pulmonary exacerbations and total number of days in hospital and total number of days on IV antibiotics. Age, gender, and sputum microbiology were included in the regression analysis as possible confounders/ effect modifiers. Generalised estimating equations were used to analyse longitudinal changes in FEV1 and BMI. Wilcoxon signed-rank test was used to compare exacerbation data pre- and post-exposure to atypical P. aeruginosa isolates. Antibiotic
resistance profiles between atypical and phenotypically characteristic *P. aeruginosa* strains were compared with chi-square test.

3. Results

The occurrence of an increasing number of non-fermenter bacteria from CF sputum specimen, identifying poorly as – ‘Moraxella’ – species on API 20 NE prompted us to initiate molecular identification techniques. Analysis of the first non-fermenter bacteria by species-specific *P. aeruginosa* PCR revealed a high percentage of these strains as *P. aeruginosa* isolates (Table 1). Therefore the conventional identification approach for non-fermenter bacteria from CF sputum specimen was changed, and all non-fermenter isolates with suboptimal (less than ‘excellent’ or ‘very good’) phenotypic identification by API 20 NE were subjected to molecular analysis. Only isolates with – ‘excellent’ – or – ‘very good’ – identification by API 20 NE were reported along with their biochemical identification profile. Sixty-two percent (48 of 78) of the isolates with suboptimal biochemical identification initially tested were reclassified as *P. aeruginosa* by molecular methods (Table 1), whereas no isolate with – ‘excellent’ – or – ‘very good’ – biochemical identification needed to be reclassified after molecular testing (data not shown). The most frequent API 20 NE identification profile recorded for these isolates was ‘Moraxella species’, other biochemical profiles encountered are listed in Table 1. During the study period 11.6% (260 out of 2242) of CF *P. aeruginosa* isolates investigated in the laboratory were classified as ‘Moraxella species’ by API 20 NE. All of the investigated isolates showed the same morphotype: non-mucoid, non-pigmented and oxidase positive.

To investigate a potential clonal relationship among atypical *P. aeruginosa* isolates, these were analysed by pulsed field gel electrophoresis (PFGE). Among 48 atypical isolates tested, PFGE revealed 16 non-related strains with unique banding patterns. The remaining 32 isolates grouped into one clonal strain (denoted as PFGE pulsotype 1) recovered from 23 patients (only 18 profiles shown in Fig. 1) and 3 smaller clusters (Fig. 1). Isolates with PFGE pulsotype-2 were cultured from 4 patients, pulsotype-3 from 3 patients and pulsotype-4 from 2 patients (Fig. 1). Pulsotype 2, 3 and 4 clusters were unrelated to the major clonal strain and were unrelated to each other.

In contrast PFGE analysis of 100 CF *P. aeruginosa* isolates collected during the same time period with characteristic phenotype (as defined in Material and methods) only revealed miniclusters with up to 2 patients but no larger clonal groupings (Fig. 2). The majority of patients infected with atypical isolates were frequently co-infected with phenotypically characteristic *P. aeruginosa* isolates, only a few patients had not grown identifiable *P. aeruginosa* previously. The frequency of co-infection with characteristic *P. aeruginosa* isolates was 78% in pulsotype-1 group, 75% in pulsotype-2 group, 100% in pulsotype-3 and pulsotype-4 group and 88% among patients infected with atypical unique isolates. Over the subsequent years sputum specimens remained persistently positive for clonal pulsotype-1 strain in the majority of patients.

Atypical *P. aeruginosa* isolates revealed higher levels of antibiotic resistance than phenotypically characteristic *P. aeruginosa* isolates (Table 2). Analysis of clinical data showed that acquisition of atypical *P. aeruginosa* was not associated with a change in rate of decline in FEV\(_1\) (% predicted) or BMI (% baseline) (Table 3). Post-exposure rate of decline in FEV\(_1\) (% predicted) was similar to that reported with colonization with phenotypically characteristic *P. aeruginosa* [12]. There was no significant change in exacerbation frequency, total days in hospital or total days on IV antibiotics after exposure to atypical *P. aeruginosa* isolates (Table 3).

4. Discussion

Over the last decade an increasing number of studies have reported the infection of unrelated patients by distinct strains of *P. aeruginosa* implying patient-to-patient spread or acquisition from a common environmental source [10]. The majority of clonal strains were detected through characteristic antibiograms or through routine genotyping efforts applied to these *P. aeruginosa* isolates [13,8,10].

Ireland has the highest incidence of CF worldwide with an estimated incidence of 1/1353 live births [14]. Despite the high incidence of CF disease clonal *P. aeruginosa* strains have so far only been reported once from a paediatric hospital in the Republic of Ireland. We document here the occurrence of a clonal *P. aeruginosa* strain among an adult CF population, which could only be detected by initiating molecular identification techniques.

The shortcomings of biochemical identification techniques applied to unusual Gram-negative, oxidase-positive rods were published previously [6]. Only 17% of unusual isolates were correctly identified by API 20 NE and an agreement between biochemical and molecular identification was only achieved for isolates with ‘excellent identification’ by API 20 NE [6]. Data recently published by the EuroCareCF quality assessment group reveals problems and delays encountered by many European CF
laboratories in identifying emerging unusual pathogens [15]. The finding of a clonal \textit{P. aeruginosa} strain, which was only correctly identified by species-specific PCR further highlights the need for molecular or mass spectrometry based identification techniques in the routine CF microbiology laboratory to support more routine approaches.

Prompt identification of \textit{P. aeruginosa} isolates is important to be able to initiate effective therapy immediately and to facilitate segregating of CF patients according to their colonization status. Most of the CF patients infected with the clonal \textit{P. aeruginosa} strain were co-infected with typical \textit{P. aeruginosa} strains. All of the patients harbouring the clonal strain had been attending paediatric CF centres in Dublin before being transferred to the service of this hospital. Acquisition of the clonal \textit{P. aeruginosa} isolate could therefore have happened in different locations. The identification of genetically related \textit{P. aeruginosa} strains among CF patients led to the implementation of a strict segregation policy in the hospital to minimize any possible contact between CF patients (October 2007). Typing data obtained by the University of Edinburgh suggested that this isolate had not been detected in other centres in Ireland nor the UK (data not shown).

Atypical \textit{P. aeruginosa} strains appeared to be highly adapted to the CF lung and had an increased antibiotic resistance profile compared to phenotypically characteristic \textit{P. aeruginosa} strains. More than 50\% of isolates were resistant to tobramycin, meropenem, ceftazidime and aztreonam. The highest level of resistance among $\beta$-lactam antibiotics was observed for meropenem (79\% of isolates resistant), which is higher than reported for other transmissible strains [9]. 31\% of atypical isolates were resistant to colistin. Similar colistin resistance rates had been reported previously for CF patients treated with nebulized colistin [16].

Acquisition of such a highly adapted strain of \textit{P. aeruginosa} is likely to have adverse consequences compared to the acquisition of a unique environmental strain, which may be successfully eradicated if detected early with oral and nebulised antibiotics. Previous studies reported clonal isolates to be more virulent and to be associated with a more rapid decline in lung function [13,17]. Patients infected with the clonal strain in this study did not show significant changes in clinical outcomes for CF patients. There was no significant change in FEV1 (% predicted) or body mass index (% baseline), exacerbation frequency, total days in

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**Fig. 1.** Cluster analysis of SpeI macrorestriction profiles of 27 atypical \textit{P. aeruginosa} isolates available for analysis. Four distinct pulsotypes were identified by PFGE. Pulsotype 1 was isolated from 23 patients [only 18 patient profiles shown], pulsotype 2 from 4 patients, pulsotype 3 from 3 patients and pulsotype 4 from 2 patients.
hospital or total days on IV antibiotics after acquisition of the clonal isolate.

**Acknowledgment**

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**Table 2**

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Atypical P. aeruginosa</th>
<th>Typical P. aeruginosa</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meropenem</td>
<td>79</td>
<td>48</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>71</td>
<td>37</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Piperacillin/Tazobactam</td>
<td>35</td>
<td>21</td>
<td>p = 0.0009</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>62</td>
<td>32</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>35</td>
<td>37</td>
<td>p = 0.7147</td>
</tr>
<tr>
<td>Colistin</td>
<td>31</td>
<td>5</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>77</td>
<td>28</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>90</td>
<td>52</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Amikacin</td>
<td>86</td>
<td>45</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Cotrimoxazol</td>
<td>88</td>
<td>100</td>
<td>p &lt; 0.0001</td>
</tr>
</tbody>
</table>

Antibiotic susceptibilities of 260 atypical P. aeruginosa strains (identifying as ‘Moraxella’ – species on API 20 NE) isolated during the study period were compared with 260 phenotypically characteristic P. aeruginosa strains. Data are presented as % of isolates resistant to the given antibiotic. Chi-square test was used to compare resistance data.

**Table 3**

Effect of atypical P. aeruginosa acquisition on clinical outcomes of CF patients.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Pre-exposure</th>
<th>Post-exposure</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔFEV₁ [% predicted per year]</td>
<td>1.0 ± 1.1</td>
<td>−1.47 ± 0.76</td>
<td>0.07</td>
</tr>
<tr>
<td>ΔBMI [% of baseline per year]</td>
<td>0.96 ± 1.05</td>
<td>−1.87 ± 1.32</td>
<td>0.11</td>
</tr>
<tr>
<td>Number of exacerbations</td>
<td>5 [IQR 2–7]</td>
<td>5 [IQR 1–6]</td>
<td>0.32</td>
</tr>
<tr>
<td>Total days on IV antibiotics</td>
<td>79 [IQR 29–115]</td>
<td>64 [IQR 19–133]</td>
<td>0.41</td>
</tr>
<tr>
<td>Total no. of days in hospital</td>
<td>34 [IQR 13–71]</td>
<td>53 [IQR 9–133]</td>
<td>0.39</td>
</tr>
</tbody>
</table>

Fig. 2. Cluster analysis of SpeI macrorestriction profiles of phenotypically characteristic P. aeruginosa isolates.
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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jcf.2012.07.007.

References