High-throughput single-nucleotide polymorphism-based typing of shared *Pseudomonas aeruginosa* strains in cystic fibrosis patients using the Sequenom iPLEX platform

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Shared strains of *Pseudomonas aeruginosa* are now well recognized in people with cystic fibrosis (CF), and suitable *P. aeruginosa* laboratory typing tools are pivotal to understanding their clinical significance and guiding infection control policies in CF clinics. We therefore compared a single-nucleotide polymorphism (SNP)-based typing method using Sequenom iPLEX matrix-assisted laser desorption ionization with time-of-flight mass spectrometry (MALDI-TOF MS) with typing methods used routinely by our laboratory. We analysed 617 *P. aeruginosa* isolates that included 561 isolates from CF patients collected between 2001 and 2009 in two Brisbane CF clinics and typed previously by enterobacterial repetitive intergenic consensus (ERIC)-PCR, as well as 56 isolates from non-CF patients analysed previously by multilocus sequence typing (MLST). The isolates were tested using a *P. aeruginosa* Sequenom iPLEX MALDI-TOF (PA iPLEX) method comprising two multiplex reactions, a 13-plex and an 8-plex, to characterize 20 SNPs from the *P. aeruginosa* housekeeping genes *acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA* and *trpE*. These 20 SNPs were employed previously in a real-time format involving 20 separate assays in our laboratory. The SNP analysis revealed 121 different SNP profiles for the 561 CF isolates. Overall, there was at least 96% agreement between the ERIC-PCR and SNP analyses for all predominant shared strains among patients attending our CF clinics: AUST-01, AUST-02 and AUST-06. For the less frequently encountered shared strain AUST-07, 6/25 (24%) ERIC-PCR profiles were misidentified initially as AUST-02 or as unique, illustrating the difficulty of gel-based analyses. SNP results for the 56 non-CF isolates were consistent with previous MLST data. Thus, the PA iPLEX format provides an attractive high-throughput alternative to ERIC-PCR for large-scale investigations of shared *P. aeruginosa* strains.

INTRODUCTION

Over the past decade, several studies have indicated that shared strains of *Pseudomonas aeruginosa* are common in patients with cystic fibrosis (CF) and some are associated with increased morbidity (Aaron *et al.*, 2010; Armstrong *et al.*, 2002; Fothergill *et al.*, 2012; Kidd *et al.*, 2012a; O’Carroll *et al.*, 2004; Scott & Pitt, 2004). For the clinical
laboratory, this highlights the need for appropriate tools to conduct ongoing molecular epidemiological surveillance and to help inform local infection control policies for CF clinics. Molecular epidemiological typing tools should ideally have high discriminatory power, high reproducibility and be simple, objective, inexpensive and capable of handling high sample loads (Blanc, 2004; Foxman et al., 2005; Gómez-Díaz, 2009; Struelens, 1998; van Belkum et al., 2001). Common genotyping tools for \textit{P. aeruginosa} include pulse-field gel electrophoresis (PFGE; Scott & Pitt, 2004), multilocus sequence typing (MLST; Kidd et al. 2012b), multiple-locus variable-number tandem-repeat analysis (Turton et al., 2010; Vu-Thien et al., 2007), repetitive element-based PCR typing such as enterobacterial repetitive intergenic consensus (ERIC)-PCR (Syrmis et al., 2004) and ArrayTube-chip (Wielhmann et al., 2007), and, more recently, we described single-nucleotide polymorphism (SNP)-based typing by real-time PCR (Anuj et al., 2011). However, to date, no single \textit{P. aeruginosa} typing method possesses all of the ‘ideal’ attributes. Moreover, with increasing recognition of the prevalence and potential clinical significance of \textit{P. aeruginosa} strains being shared between CF patients, larger-scale studies and ongoing surveillance are needed. This requires a suitable and economical typing tool capable of processing rapidly hundreds and even thousands of isolates.

To assist with surveillance of the shared \textit{P. aeruginosa} strains in our local CF patient population, our laboratory examined the utility of several typing schemes. Briefly, whilst PFGE remains the gold standard for genotyping \textit{P. aeruginosa}, it suffers from high workload, expense and subjectivity. Overall, it is better suited for short-term outbreak situations involving epidemiologically related isolates than strain surveillance in geographically dispersed CF centres over prolonged time periods (Blanc, 2004; Kidd et al., 2011a, 2012a; Tenover et al. 1995). ERIC-PCR is also suitable for rapid, small-scale epidemiological analyses of \textit{P. aeruginosa} isolates from patients with CF (Kidd et al., 2011a; Syrmis et al., 2004) but, similar to PFGE, is also gel based and therefore subjective and prone to error in larger-scale studies, even if digital gel analysis software is used (Kidd et al., 2011b). MLST is extremely useful as it is objective, provides a categorized analysis and the data are transportable between laboratories (Foxman et al., 2005; Kidd et al., 2011a; Li et al., 2009). However, it is limited by its complexity and cost, making it particularly unsuitable for larger-scale analyses (Li et al., 2009). SNP typing by real-time PCR, whilst effectively a cheaper ‘mini-MLST’ approach and able to discriminate between the predominant shared strains AUST-01 and AUST-02 among patients attending Australian CF clinics (Anuj et al., 2011), is still restricted in its utility for larger-scale analyses. This is because it requires at least 10 and, depending on the purpose, up to 20 separate real-time PCR assays per isolate to be performed.

The MassARRAY iPLEX format by Sequenom (Oeth et al., 2009) combines single-base extension PCR with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The benefit over real-time PCR is that this system enables cost-effective multiplexing of significantly more SNPs per reaction and thus is better suited for larger-scale typing analyses. We have been successful previously in converting a similar SYBR Green-based 16-SNP real-time PCR method for meticillin-resistant \textit{Staphylococcus aureus} (MRSA) to a MALDI-TOF format (Syrmis et al., 2011). To determine whether this could also be achieved for \textit{P. aeruginosa} to facilitate molecular epidemiological surveillance within CF clinics, we adapted our previously described 20-SNP \textit{P. aeruginosa} real-time PCR assay to a MassARRAY iPLEX method and applied it to 625 local clinical isolates.

**METHODS**

**Control isolates.** Twenty-four \textit{P. aeruginosa} control isolates were used to validate the MassARRAY iPLEX method. These isolates were tested previously by SYBR Green-based real-time PCR assays and collectively were representative of each of the 20 SNPs (Anuj et al., 2011). The control isolates included examples of the predominant strains shared by Australian CF patients (i.e. two AUST-01, five AUST-02 and two AUST-06 isolates, known previously as the AES-1, AES-2 and pulsotype 42, respectively; Kidd et al. 2012a). In addition, one isolate each from strains shared less frequently by CF patients in Brisbane (AUST-07, AUST-11 and AUST-13; previously known as pulsotypes 5, 58 and 3, respectively; Kidd et al. 2012a), the Manchester (MAN) strain C3425 and the Liverpool (LES) strain H190 were used, as well as ten unique strain types isolated only from single patients (Syrmis et al., 2004). The reproducibility of the iPLEX method was investigated by testing the control isolates in two separate test runs.

**Test isolates.** These included 561 \textit{P. aeruginosa} isolates from 235 patients attending paediatric (131 isolates) or adult (430 isolates) CF clinics in Brisbane, Australia, between 2001 and 2009. Each had been typed previously by ERIC-PCR (Anuj et al., 2011; Kidd et al., 2011a; Syrmis et al., 2004). A further 56 clinical isolates from patients without CF and typed by MLST as part of another study were also included (Kidd et al. 2012b). These comprised 18 blood culture isolates and a further 38 respiratory isolates, 14 of which were from 13 patients with bronchiectasis and one with chronic obstructive pulmonary disease.

**Preparation.** Isolates were grown from –80 °C storage on blood agar plates incubated at 37 °C for 24 h and subcultured onto blood agar for another 24 h at 37 °C. Heat-denatured suspensions of each strain were made as described previously (Anuj et al., 2011). Briefly, a 1 ml 1.00 McFarland standard of each strain in sterile water was heated at 100 °C for 10 min, vortexed and centrifuged at 3000 r.p.m. in a microfuge for 5 min, and the supernatant was used for PCR.

**\textit{P. aeruginosa} iPLEX (PA iPlex) MassARRAY MALDI-TOF MS**

**iPLEX design.** The assays were developed to target the 20 SNPs described previously by Anuj et al. (2011). Primers and extension primers for each target were designed using the MassARRAY Designer 4.0 software (Sequenom). Two multiplex assays (an 8-plex and a 13-plex) were subsequently developed. The 34 amplification primers and 21 extension primers are given in Tables 1 and 2. Two extension primers were used for SNP 8 to accommodate proximal SNP variation (Table 2). All amplification primers commenced with the 5’
Table 1. Primers used for primary PCR

<table>
<thead>
<tr>
<th>SNP*</th>
<th>Forward (5'→3')</th>
<th>Reverse (5'→3')</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10mer-TGACGCCAGAACAGCGAGGAACGAG</td>
<td>10mer-AAGTCTAATCGGCTCAGAGG</td>
<td>117</td>
</tr>
<tr>
<td>2</td>
<td>10mer-AGTGCGATCTAGGCGACGACGAGC</td>
<td>10mer-ACCGCCGCTAGGCGACGACGAGC</td>
<td>118</td>
</tr>
<tr>
<td>3, 4</td>
<td>10mer-AGTGCGATCTAGGCGACGACGAGC</td>
<td>10mer-ACCGCCGCTAGGCGACGACGACG</td>
<td>117</td>
</tr>
<tr>
<td>5</td>
<td>10mer-ATGACGCCAGAACAGCGAGGAACGAG</td>
<td>10mer-ACCGCCGCTAGGCGACGACGACG</td>
<td>119</td>
</tr>
<tr>
<td>6</td>
<td>10mer-ATGACGCCAGAACAGCGAGGAACGAG</td>
<td>10mer-ACCGCCGCTAGGCGACGACGACG</td>
<td>117</td>
</tr>
<tr>
<td>7</td>
<td>10mer-ATGACGCCAGAACAGCGAGGAACGAG</td>
<td>10mer-ACCGCCGCTAGGCGACGACGACG</td>
<td>119</td>
</tr>
<tr>
<td>8</td>
<td>10mer-ATGACGCCAGAACAGCGAGGAACGAG</td>
<td>10mer-ACCGCCGCTAGGCGACGACGACG</td>
<td>117</td>
</tr>
<tr>
<td>9</td>
<td>10mer-ATGACGCCAGAACAGCGAGGAACGAG</td>
<td>10mer-ACCGCCGCTAGGCGACGACGACG</td>
<td>117</td>
</tr>
<tr>
<td>10</td>
<td>10mer-ATGACGCCAGAACAGCGAGGAACGAG</td>
<td>10mer-ACCGCCGCTAGGCGACGACGACG</td>
<td>117</td>
</tr>
<tr>
<td>11, 12</td>
<td>10mer-AGTACGCCAGAACAGCGAGGAACGAG</td>
<td>10mer-ACCGCCGCTAGGCGACGACGACG</td>
<td>117</td>
</tr>
<tr>
<td>13</td>
<td>10mer-ACACCCCTCGCCTAGGCGACGACG</td>
<td>10mer-ACCGCCGCTAGGCGACGACGACG</td>
<td>117</td>
</tr>
<tr>
<td>14</td>
<td>10mer-ACACCCCTCGCCTAGGCGACGACG</td>
<td>10mer-ACCGCCGCTAGGCGACGACGACG</td>
<td>117</td>
</tr>
<tr>
<td>15, 16</td>
<td>10mer-ACACCCCTCGCCTAGGCGACGACG</td>
<td>10mer-ACCGCCGCTAGGCGACGACGACG</td>
<td>117</td>
</tr>
<tr>
<td>17</td>
<td>10mer-ACACCCCTCGCCTAGGCGACGACG</td>
<td>10mer-ACCGCCGCTAGGCGACGACGACG</td>
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</tr>
<tr>
<td>18</td>
<td>10mer-ACACCCCTCGCCTAGGCGACGACG</td>
<td>10mer-ACCGCCGCTAGGCGACGACGACG</td>
<td>117</td>
</tr>
<tr>
<td>19</td>
<td>10mer-ACACCCCTCGCCTAGGCGACGACG</td>
<td>10mer-ACCGCCGCTAGGCGACGACGACG</td>
<td>117</td>
</tr>
<tr>
<td>20</td>
<td>10mer-ACACCCCTCGCCTAGGCGACGACG</td>
<td>10mer-ACCGCCGCTAGGCGACGACGACG</td>
<td>117</td>
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</table>

*The 13-plex primary PCR comprised SNPs 1, 2, 6, 8, 9, 10, 11, 12, 13, 14, 15, 16 and 17, and the 8-plex primary PCR comprised SNPs 3, 4, 5, 7, 8, 18, 19 and 20.

Table 2. Primers used for extension PCR

Mass (Da) is provided for the unextended extension primer (UEP), as well as associated extension products (EP) 1, 2 and, where relevant, 3.

<table>
<thead>
<tr>
<th>SNP*</th>
<th>UEP†</th>
<th>Mass</th>
<th>EP1 (mass)</th>
<th>EP2 (mass)</th>
<th>EP3 (mass)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TACATCGTCTATGGGCCC</td>
<td>5450.6</td>
<td>C (5697.7)</td>
<td>T (5777.6)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>CTGCCTAGTCTCTGCTTC</td>
<td>4818.1</td>
<td>C (5065.3)</td>
<td>T (5145.2)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>GCCGATGACAGCTGGGCT</td>
<td>5236.4</td>
<td>C (5483.6)</td>
<td>T (5563.5)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>CTCACGTCGCTGCTTCAG</td>
<td>5515.6</td>
<td>T (5786.8)</td>
<td>C (5802.8)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>CCGTGCCGCAAGGAAGGA</td>
<td>5837.8</td>
<td>C (6085.0)</td>
<td>A (6109.0)</td>
<td>G (6125)</td>
</tr>
<tr>
<td>6</td>
<td>aggtTGGCCCGCTCGCTCAA</td>
<td>6094.0</td>
<td>C (6341.1)</td>
<td>A (6421.1)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>gaaGCCGATGCGCTGCTGC</td>
<td>6158.0</td>
<td>T (6429.2)</td>
<td>C (6445.2)</td>
<td></td>
</tr>
<tr>
<td>8proxA‡</td>
<td>ggaaGCACCTCCTAGCATGGA</td>
<td>6455.2</td>
<td>C (6702.4)</td>
<td>G (6742.4)</td>
<td>T (6782.3)</td>
</tr>
<tr>
<td>8proxG‡</td>
<td>ctcaGCcaCTTCATCGAGT</td>
<td>6052.9</td>
<td>C (6300.1)</td>
<td>G (6340.2)</td>
<td>T (6380.0)</td>
</tr>
<tr>
<td>9</td>
<td>gaTGCAGTCGAGGCTGTCGA</td>
<td>6374.1</td>
<td>T (6645.3)</td>
<td>C (6661.3)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>ccgcACCTGCTCTGCTGCGC</td>
<td>6020.9</td>
<td>T (6292.1)</td>
<td>C (6308.1)</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>AGCAAGACCTGAGCGAGCA</td>
<td>5911.9</td>
<td>A (6183.1)</td>
<td>G (6199.1)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>TGCCCTCGGCTGTGGAG</td>
<td>5009.2</td>
<td>T (5280.5)</td>
<td>C (5296.5)</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>cggGGTGAGGAAGGTCGCG</td>
<td>5605.6</td>
<td>C (5852.8)</td>
<td>A (5876.8)</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>CTGCCCTAGGCTGAG</td>
<td>4858.2</td>
<td>C (5105.3)</td>
<td>T (5185.3)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>GACCGCGGAGCCTGAGAACG</td>
<td>5221.4</td>
<td>A (5492.6)</td>
<td>G (5508.6)</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>GATCGTCCGCTGGGACCT</td>
<td>5459.6</td>
<td>A (5730.8)</td>
<td>G (5746.8)</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>ACCGATGCTGCTGGAAG</td>
<td>5813.8</td>
<td>C (6061.0)</td>
<td>T (6140.9)</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>GtCGAGCTCCTGCGCTAC</td>
<td>5420.5</td>
<td>G (5667.7)</td>
<td>A (5747.6)</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>GCAGATGTCGCTCGAG</td>
<td>4842.2</td>
<td>G (5089.3)</td>
<td>C (5129.4)</td>
<td>A (5169.3)</td>
</tr>
<tr>
<td>20</td>
<td>ggGTGGTGTCTACCGCGG</td>
<td>5627.6</td>
<td>C (5874.8)</td>
<td>T (5954.7)</td>
<td></td>
</tr>
</tbody>
</table>

*The 13-plex extension PCR comprised SNPs 1, 2, 6, 8proxA, 9, 10, 11, 12, 13, 14, 15, 16 and 17, and the 8-plex reaction comprised SNPs 3, 4, 5, 7, 8proxA, 18, 19 and 20.

†Lowercase bases in UEP oligonucleotides indicate non-template bases and were added to modify UEP mass.

‡Note that SNP 8 had two extension primers (8proxA and 8proxG).
10mer tag (5′-ACGTTGGATG-3′) to ensure they fell outside the observed mass window.

**PCR amplification and shrimp alkaline phosphatase (SAP) treatment.** The PA iPLEX initial PCR assays were performed in 384-well microplates (Thermo Fisher Scientific) using the Sequenom PCR Reagent Set (Sequenom) on a Gene AMP PCR system 9700 (Applied Biosystems). Briefly, each reaction contained 1× PCR buffer containing 2 mM MgCl₂, each primer at 0.1 µM (Table 1), 2 mM additional MgCl₂, 500 µM dNTP mix, 0.5 U PCR enzyme and 1 µl bacterial suspension and was adjusted to 5 µl using HPLC-grade water. The PCR step commenced with an initial denaturation at 95 °C for 2 min, followed by 45 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 60 s, with a final extension at 72 °C for 5 min. Residual dNTPs were dephosphorylated by treatment with 0.5 U SAP (Sequenom) and incubated at 37 °C for 40 min followed by an enzyme deactivation step at 85 °C for 5 min.

**Single-base extension and desalting.** Before the single-base extension reaction, all extension primers were adjusted linearly by concentration using Sequenom’s MALDI-TOF MS and Typer 4.0 software to ensure that all were equilibrated according to signal-to-noise ratios as per the manufacturer’s instructions (Sequenom). The single-base extension step was performed on a GeneAMP PCR system 9700 (Applied Biosystems) using an iPLEX Gold Reaction kit (Sequenom) following the manufacturer’s instructions. Each PCR was supplemented with a 2 µl iPLEX extension primer cocktail, containing 0.2 µl iPLEX buffer, 0.25 µl iPLEX terminator mix, 0.05 µl iPLEX enzyme and 0.94 µl of the adjusted iPLEX extension primer mix (Table 2). The PCR conditions were 94 °C for 30 s, followed by 40 cycles of one step at 94 °C for 5 s with five subcycles of 52 °C for 5 s and 80 °C for 5 s and a final extension step at 72 °C for 3 min. Each extension reaction was desalted using 6 µg CLEAN Resin (Sequenom) in 16 µl HPLC-grade water. The cleaned primer extension products were then spotted onto a 384-format SpectroCHIP (Sequenom) using a MassARRAY Nanodispenser RS 1000 (Sequenom).

**MALDI-TOF MS analysis and data quality control.** Data acquisition and analysis were performed on a MassArray Compact Analyser (Sequenom) using SpectroAcquire and MassARRAY typer software version 4.0.3. (Sequenom), respectively. Nucleotide calls (i.e. A, T, C or G) were considered final if called automatically by the software as A, conservative; B, moderate; C, aggressive; or N, no allele. User calls E and F were made manually. The 20 SNP markers were coded (1−20) as described by Anuj et al. (2011).

**Discordant result analyses.** Any isolates typed as AUST-01, AUST-02, AUST-06, AUST-07, AUST-11 and AUST-13 by either method were investigated further if there was a discrepancy between the results of the iPLEX and ERIC-PCR typing. Briefly, the original gel image of the ERIC-PCR assay was re-examined or, if unavailable, the ERIC-PCR assay was repeated (Syrmis et al., 2004).

## RESULTS

**Validation of PA iPLEX using control isolates**

The PA iPLEX results for the 24 control isolates produced identical results in two separate test runs, with a call rate of 478/480 (99.6%) SNP calls. All 478 SNPs called by the iPLEX method were consistent with the known results for these isolates based on previous real-time PCR testing (Anuj et al., 2011). The two SNPs that failed to be called in both test runs were SNP 5 from both AUST-06 isolates. Sequence variation in the AUST-06 SNP 5 extension primer target was responsible for the call failure (data not shown). However, the AUST-06 isolates could still be distinguished from the other isolates using the remaining 19 SNP profiles, as described previously (Anuj et al., 2011).

**CF isolates**

For the 561 CF isolates, the MassARRAY call rate was 97.8% (10 976/11 220 total possible calls; see Table S1, available in JMM Online). Overall, there were 121 different SNP profiles observed. The 244 failed calls from 75 CF isolates included 160 ‘no allele calls’ (i.e. no extension product) and 84 mixed calls (i.e. more than one call for a given SNP). There were 49 CF isolates for which only SNP 5 was not called and the remaining 19 SNPs were consistent with the AUST-06 profile. The other 26 CF isolates comprised 16 unique isolates, nine AUST-02 isolates and one AUST-13 isolate.

The initial comparison of the PA iPLEX and ERIC-PCR typing results showed concordance for 485/561 (86.5%) CF isolates, including 53 AUST-01, 160 AUST-02, 52 AUST-06, 18 AUST-07, seven AUST-11 and five AUST-13 isolates. Twenty-six isolates could not be typed due to incomplete SNP profiles. A further 50 isolates provided discordant results. The ERIC-PCR results for these 50 isolates were reinvestigated by either reviewing the original ERIC-PCR gel image (n=17) or by repeating the assay (n=33). Following this reinvestigation, 34 of these 50 isolates became concordant with both PA iPLEX and ERIC-PCR assays (Table 3). An overall summary of the PA iPLEX and ERIC-PCR assay results for all 561 CF isolates following reanalysis of the ERIC-PCR data is shown in Table 4 and detailed in Table S1.

**Non-CF isolates**

For the 56 non-CF clinical isolates, the call rate was 99.2% (1111/1120 total possible calls). Overall, 53 isolates provided complete 20 SNP profiles, of which 46 different SNP profiles were observed (data not shown). Interestingly, 19 of these SNP profiles (from 25 non-CF isolates) were shared with those observed for the CF isolates (see Table S1). One non-CF isolate was consistent with an AUST-02 SNP profile, but, based on previous MLST typing (ST-914), it was instead a closely related single-locus variant of AUST-02 (Kidd et al., 2012b). Another isolate was deemed to be AUST-11 by SNP profile, which was consistent with its known MLST type (ST-803; Kidd et al., 2012b).

**DISCUSSION**

We sought to develop a high-throughput method for typing *P. aeruginosa* isolates from patients attending CF clinics. Our laboratory has previously investigated other approaches for this purpose (Anuj et al., 2011; Kidd et al.,...
2011a; Syrmis et al., 2004) and have, to date, relied mainly upon ERIC-PCR for screening of shared *P. aeruginosa* strains in our local patient population (Syrmis et al., 2004). Although simple and rapid, we identified several limitations with this method when applied to large-scale studies involving several hundred isolates. The main problem was comparing multiple gel images, even with computer-based software assistance, which proved cumbersome and ultimately rate-limiting. Such problems have been encountered elsewhere (Meacham et al., 2003). We have also used MLST and found that, whilst it provided a high level of discriminatory power and objectivity, it was too expensive for routine use (Kidd et al., 2011a). More recently, we have investigated SNP-based profiling using real-time PCR for typing *P. aeruginosa* isolates from CF patients (Anuj et al., 2011). Although fast, simple and objective, and an improvement on ERIC-PCR, the sheer number of real-time PCRs (at least ten) required still made it unsuitable for larger-scale investigations. Here, we adapted our previously described 20-SNP profiling system to the Sequenom platform. We found this method was well suited for high-throughput typing of *P. aeruginosa* clinical isolates. In particular, we found it could be used on heat-denatured isolates (removing the need for a commercial DNA extraction kit) and could genotype up to 384 isolates within one working day and at a lower cost than that of real-time PCR [~AU$14.00 (US$14.50/€11.50) per isolate for PA iPLEX compared with AU$20.00 (US$21.00/€16.50) per isolate for real-time PCR].

Applying the PA iPLEX to 561 CF *P. aeruginosa* isolates typed previously by ERIC-PCR showed that the predominant epidemiological strain types were identified easily by the 20 SNPs. Importantly, we were able to distinguish the predominant shared strains AUST-01, AUST-02 and AUST-06 and the less frequently encountered strains AUST-07, AUST-11 and AUST-13. The PA iPLEX results and ERIC-PCR profiles were concordant for all AUST-01 and AUST-06 isolates and for most AUST-02 isolates, which are our predominant local shared strains. However, the PA iPLEX also highlighted some additional limitations

<table>
<thead>
<tr>
<th>Original ERIC type</th>
<th>iPLEX type</th>
<th>ERIC type following reinvestigation</th>
<th>No. isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUST-02</td>
<td>AUST-07</td>
<td>AUST-07</td>
<td>6</td>
</tr>
<tr>
<td>AUST-02</td>
<td>AUST-11</td>
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<tr>
<td>AUST-02</td>
<td>U</td>
<td>U</td>
<td>12</td>
</tr>
<tr>
<td>AUST-06</td>
<td>AUST-02</td>
<td>AUST-02</td>
<td>1</td>
</tr>
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**Table 4. Summary of the iPLEX and ERIC-PCR assay results following discordant results analysis for the 561 *P. aeruginosa* isolates from CF patients**

Incomplete, incomplete SNP profile due to one or more SNPs failing to be characterized.
of the ERIC-PCR. For example, several isolates that were classified initially by ERIC-PCR as AUST-02 were identified by PA iPLEX as AUST-07. Reanalysis of the ERIC-PCR results for these isolates showed subsequently that these were indeed AUST-07 rather than AUST-02 strains. Overall, the results highlight the subjective nature of the gel-based ERIC-PCR assays in contrast with the objective, categorized results provided by SNP profiling.

A limitation of the PA iPLEX method was that two isolates exhibited the expected SNP profile for AUST-02, but ERIC-PCR identified them clearly as unique strains (see result profile 94; Table S1). A possible reason for these observations is that the SNPs were designed to reflect changes within the highly conserved core genome, whereas ERIC-PCR captures a genome-wide perspective of diversity, including the highly variable accessory genome. The latter is particularly relevant when applying typing methods to P. aeruginosa from patients with CF, given that many such patients suffer chronic P. aeruginosa infections over years or even decades, during which time strains may undergo multiple mutations, particularly involving the accessory genome. Fothergill et al. (2010) demonstrated recently how P. aeruginosa genomic instability can negatively impact on genetic fingerprinting methods. They found that PFGE, random amplified polymorphic DNA-PCR and BOX-PCR were unable to fully resolve the identity of some LES isolates, the predominant shared strain in the UK. Variations in the possession of both bacteriophages and genomic islands were the likely explanation for the failure to type these strains accurately. Whilst the PA iPLEX targets highly conserved housekeeping genes that are therefore likely to remain more stable over time, it is possible that the two isolates yielding discordant typing results were indeed not AUST-02 strains and that the 20 SNPs selected for this assay lacked sufficient discrimination to differentiate between them (Kidd et al., 2011a). None the less, the remaining 165 CF isolates called AUST-02 by the PA iPLEX were also categorized as AUST-02 by ERIC-PCR, and so these two discordant results are not believed to be a major limitation.

The PA iPLEX also performed well when applied to the 56 non-CF P. aeruginosa clinical isolates, highlighting its potential utility outside CF surveillance. However, one non-CF isolate was also misidentified as an AUST-02 strain by the PA iPLEX where MLST had shown it previously to be a single-locus variant of AUST-02 (Kidd et al., 2012b). This illustrates that SNP typing by the PA iPLEX is not necessarily definitive and, depending on local population data and assay performance characteristics, further confirmatory methods such as MLST may be needed for genotypes of potential clinical importance.

A technical limitation of the PA iPLEX method was that some SNPs could not be called. This has been observed previously for iPLEX assays in other pathogens and is caused typically by sequence variation (Syrmis et al., 2011). The overall error rate of 2.4% observed for the PA iPLEX was similar to the MRSA-iPLEX system we designed previously (Syrmis et al., 2011). For the PA iPLEX the errors were once again contained within specific SNP profiles (particularly AUST-06), further suggesting that the errors arose mainly from sequence variation in these particular genotypes. On re-examination of the aro gene sequence data for AUST-06 (MLST type ST801, with aro sequence type 126), two proximal SNPs were observed adjacent to the targeted SNP and, more importantly, were within the extension primer sequence target. Based on previous experience, these changes would be sufficient to lead to the observed extension failure. Overall, we do not believe that these failed calls detract from the utility of the system. Rather, in our opinion, this limitation is outweighed by its high throughput, and the few SNPs that failed to be called can be retested easily by real-time PCR methods.

It should be noted that other SNP-based P. aeruginosa typing strategies have been described recently and include the ArrayTube, a multimarker microarray targeting 13 SNPs on the core genome and 38 variable genetic markers on the accessory P. aeruginosa genome (Ballarini et al., 2012; Fothergill et al., 2010; Wiehlmann et al., 2007). This technique is also reported to be rapid, relatively inexpensive and robust (Fothergill et al., 2010); however, its suitability for discriminating our local shared strains is yet to be evaluated.

In conclusion, the PA iPLEX offers an overall high-throughput and unambiguous method for discerning both predominant and less frequently encountered shared P. aeruginosa strains in our local patient population. Notwithstanding these results, validation of the tool in other clinical settings and geographical regions may be warranted. Nevertheless, the increasing availability of the Sequenom platform in clinical and reference laboratory settings means this method could be considered as a frontline tool for P. aeruginosa genotype surveillance.

ACKNOWLEDGEMENTS

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with cystic fibrosis by the use of real-time PCR and high-resolution melting curve analysis. Clin Microbiol Infect 17, 1403–1408.


