Molecular genotyping of *Acinetobacter* spp. isolated in Arizona, USA, using multilocus PCR and mass spectrometry

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*Acinetobacter* spp. are a diverse group of Gram-negative bacteria frequently implicated in nosocomial infections. Genotypic methods have been instrumental in studying *Acinetobacter*, but few offer high resolution, rapid turnaround time, technical ease and high inter-laboratory reproducibility, which has hampered understanding of disease incidence, transmission patterns and diversity within this genus. Here, we further evaluated multilocus PCR electrospray ionization/mass spectrometry (PCR/ESI-MS), a method that is simple and robust, and provides both species characterization and strain-level resolution of *Acinetobacter* spp. on a single platform. We examined 125 *Acinetobacter* isolates from 21 hospitals, laboratories and medical centres spanning four counties in Arizona, USA, using PCR/ESI-MS. We compared PCR/ESI-MS with an in-house amplified fragment length polymorphism (AFLP) genotyping scheme. PCR/ESI-MS demonstrated that *Acinetobacter* spp. from Arizonan hospitals had similar species and strain distributions to other US civilian hospitals. Furthermore, we showed that the PCR/ESI-MS and AFLP genotypes were highly congruent, with the former having the advantages of robust inter-laboratory reproducibility, rapid turnaround time and simple experimental set-up and data analysis. PCR/ESI-MS is an effective and high-throughput platform for strain typing of *Acinetobacter baumannii* and for identification of other *Acinetobacter* spp., including the emerging nosocomial pathogens *Acinetobacter pittii* and *Acinetobacter nosocomialis*.

INTRODUCTION

Members of the genus *Acinetobacter* are a significant cause of opportunistic nosocomial infections (Bergogne-Bérézin & Towner, 1996). Of the 27 currently named *Acinetobacter* spp., only a handful are clinically significant, with *Acinetobacter baumannii*, *Acinetobacter pittii* (formerly genomic species 3) and *Acinetobacter nosocomialis* (formerly genomic species 13TU) (Nemec et al., 2011) being the most common (Gordon & Wareham, 2010; Nemec et al., 2011). Nosocomial outbreaks of multidrug-resistant *Acinetobacter* infections, especially *A. baumannii*, have been documented worldwide (Fournier et al., 2006; Dijkshoorn et al., 2007). The successful transmission of multidrug-resistant *Acinetobacter* in the hospital setting can in part be attributed to its high tolerance to desiccation, which enables this bacterium to survive for long periods on fomites and even on the hands of healthcare workers (Guenthner et al., 1987; Jawad et al., 1998; Roca et al., 2012).

**Abbreviations:** AFLP, amplified fragment length polymorphism; ESI-MS, electrospray ionization/mass spectrometry; MLST, multilocus sequence typing; PFGE, pulse-field gel electrophoresis; SNP, single-nucleotide polymorphism; ST, sequence type.

Two supplementary tables are available with the online version of this paper.
Multilocus sequence typing (MLST) has been developed for several clinically important bacteria including *A. baumannii*, which has two MLST schemes (Bartual *et al.*, 2005; Diancourt *et al.*, 2010). MLST, which involves DNA sequence comparison of housekeeping loci, is a widely used technique in epidemiological, clinical and research studies due to the robustness of DNA sequence data and the ability to compare sequence types (STs) among laboratories on a global scale (Maiden *et al.*, 1998). The *Acinetobacter* MLST schemes not only differentiate *A. baumannii* strains, but can also be used to speciate *A. baumannii*, *A. nosocomialis* and *A. pittii* (Wisplinghoff *et al.*, 2008; Diancourt *et al.*, 2010). However, despite the attractive nature of MLST for *Acinetobacter* typing, this technique is not viable as a real-time surveillance strategy for most clinical laboratories due to its time-consuming nature and the high cost of Sanger sequencing. Alternative typing methods capable of targeting rapidly evolving loci, such as multilocus variable number tandem repeat analysis, are often used in combination with MLST or pulse-field gel electrophoresis (PFGE) to provide increased resolution (Turton *et al.*, 2009; Minandri *et al.*, 2012).

PCR electrospray ionization/mass spectrometry (PCR/ESI-MS) overcomes many of the disadvantages associated with currently used genotyping methods, such as inter-laboratory reproducibility and throughput, whilst providing comparable resolution to MLST (Ecker *et al.*, 2006). PCR/ESI-MS, which is based on six housekeeping loci that overlap between *Moraixella catarrhalis* and *Acinetobacter* spp., has been used to characterize *Acinetobacter* isolates collected from several settings, including military patients, European outbreaks and reference collections (Ecker *et al.*, 2006). The authors demonstrated that PCR/ESI-MS yielded genotype data congruent with PFGE profiles for this genus, and provided accurate species characterization. Like MLST, PCR/ESI-MS analyses the DNA sequence of conserved regions in housekeeping genes; however, base composition is determined by mass spectrometry. PCR/ESI-MS allows a much more rapid turnaround time and a lower per-sample cost, whilst providing similar resolution to MLST (Ecker *et al.*, 2006). These advantages make PCR/ESI-MS desirable for high-throughput and real-time analyses of *Acinetobacter* spp., and several previous studies have shown the utility of PCR/ESI-MS in characterizing *Acinetobacter* outbreaks (Whitman *et al.*, 2008; Wortmann *et al.*, 2008; Schuetz *et al.*, 2012).

In the current study, we further evaluated PCR/ESI-MS using 125 clinical *Acinetobacter* isolates obtained over an 18-month period from 21 hospitals and medical laboratories in four Arizona counties. We expanded the previously reported comparative analysis of PCR/ESI-MS and PFGE (Ecker *et al.*, 2006) by comparing PCR/ESI-MS with a highly resolving amplified fragment length polymorphism (AFLP) technique (Allender *et al.*, 2008) to determine the robustness of species designations between these methods. AFLP is typically employed for discriminating among closely related strains for a number of different bacteria, including *Acinetobacter* (Vos *et al.*, 1995), but has also been used for species determination (Janssen *et al.*, 1997; van den Broek *et al.*, 2009). We then analysed the distribution of *Acinetobacter* spp. genotypes isolated from Arizona hospitals and compared these genotypes with those observed in hospitals throughout the USA and globally.

**METHODS**

**Sample collection.** The 125 *Acinetobacter* samples used in our study were collected between February 2005 and August 2006 with 99% of isolates collected between December 2005 and August 2006. All isolates were collected from four counties across southern Arizona (Apache, Maricopa, Pima and Yuma). The majority of isolates were from Maricopa county, which encompasses the state capital, Phoenix (87/125 isolates; 70%). Twenty isolates were from Pima county, eight from Yuma county and one from Apache county. The remaining nine isolates were from unidentified counties in Arizona (Table S1, available in JMM Online).

**AFLP typing.** AFLP was conducted as described previously (Allender *et al.*, 2008) with the following modifications: (i) during restriction digestion, samples were incubated for 2 h at 37 °C to ensure complete digestion prior to ligation; (ii) ligations were incubated for 2 h at 16 °C instead of at room temperature; (iii) selective PCR used the following four primer combinations: 6FAM-EcoRI/MseI, 6FAM-EcoRI/MseI, HEX-EcoRI/MseI and HEX-EcoRI/MseG (Allender *et al.*, 2008); (iv) dNTPs (Invitrogen) were increased from 0.1 to 0.2 mM; and (v) Platinum Taq (Invitrogen) was used instead of Taq polymerase to increase amplification efficiency. Capillary gel electrophoresis was performed on a 3730xL DNA analyser (Applied Biosystems) and electropherograms were analysed using the ‘Microsatellite’ module of GeneMapper version 4.0 (Applied Biosystems).

**PCR/ESI-MS.** PCR/ESI-MS was performed as described previously (Ecker *et al.*, 2006). The MLST loci targeted by the *Acinetobacter* PCR/ESI-MS scheme were *adk*, *efp*, *fumC* (twice), *mutY* (twice), *ppa* and *trpE*. ST assignment was made according to the PCR/ESI-MS database, and thus does not correspond with STs from the conventional *Acinetobacter* MLST schemes.

**Phylogenetic analysis.** A maximum-parsimony tree was constructed from AFLP data using PAUP version 4.0. An unrooted radial phylogeny was constructed with TreeView version 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treepview.html) and edited for clarity in Adobe Illustrator version 14.0.0.

**Sequencing.** Amplification of *rpoB* and surrounding regions was performed according to previously published protocols (La Scola *et al.*, 2006). The 16S rRNA gene was amplified using previously described primers (Nadkarni *et al.*, 2002). Sequencing was performed using BigDye Terminator version 3.1 (Applied Biosystems) on an ABI 3130xl instrument according to the manufacturer’s instructions.

**RESULTS AND DISCUSSION**

**PCR/ESI-MS analysis of isolates**

According to PCR/ESI-MS, 97 (78%) isolates were *A. baumannii* (Table 1). Of the 28 (22%) non-*A. baumannii* isolates, 18 (14%) were *A. pittii*, two typed as *Acinetobacter berezniae*, one isolate each was identified as *Acinetobacter*...
*Lwoffii* and *A. nosocomialis*, two were of unknown species and four isolates genotyped as mixtures (Table 1). The base composition of amplicons generated by PCR/ESI-MS is summarized in Table S2. As expected, *A. baumannii* was the most frequently implicated species in clinical *Acinetobacter* infections, followed by *A. pittii*, *A. nosocomialis* and *A. bereziniae*. The predominance of *A. baumannii* has also been observed in other studies of *Acinetobacter* spp. from both North American (Higgins et al., 2012; Wisplinghoff et al., 2012) and European hospitals (Gundi et al., 2009).

The *A. baumannii* isolates could be further divided into 17 STs, with ST10 being predominant (60 %), followed by ST12 (9 %), ST66 (9 %) and ST68 (5 %). All other STs were present at a frequency of <2 % (Table 1). ST10 was the only clone observed in all hospitals and counties sampled (data not shown). Its dominance and widespread nature indicate that ST10 is a successful clone in Arizonan hospitals. *A. baumannii* ST10 and ST12 belong to clone II, which has generally been associated with outbreaks in Europe. Their presence in our isolate set indicates the successful global dispersion of these strains, an observation supported by other studies (Dijkshoorn et al., 1996; van Dessel et al., 2004; Higgins et al., 2010). ST10 and ST12 are single-locus variants and only differ by one single-nucleotide polymorphism (SNP) within the MLST loci interrogated by the PCR/ESI-MS method (Ecker et al., 2006). Interestingly, other STs from clone II (i.e. ST1, -11 and -47), which also differ from ST10 by single SNPs, were not present in our collection; however, ST1 has been observed previously in Ohio (Perez et al., 2010). In addition, no sequence types associated with European clone I (ST15, -16, -45 and -46) or III (ST8 and -14) were characterized in our study. This distribution is consistent with previous studies, which have reported the presence of these STs in military hospitals but not in civilian hospitals (Hujer et al., 2006; Wortmann et al., 2008).

Unlike *A. baumannii*, *A. pittii* did not show dominance or clustering of specific STs. Interestingly, the degree of diversity within *A. pittii* was remarkably high, with 14 different STs identified across 18 isolates (Table 1).

### AFLP analysis and comparison with PCR/ESI-MS

AFLP was performed on 121 isolates with isolates previously determined as mixtures removed from analysis. The phylogeny generated with AFLP mostly agreed with ST designations assigned by PCR/ESI-MS (Fig. 1). *A. baumannii* was distinct from all other species with the exception of one isolate (unknown A), which was termed ‘unknown’ by PCR/ESI-MS due to its novel sequence composition, with seven out of eight loci showing previously unidentified signatures (Table S2); it clustered with *A. baumannii* according to AFLP. However, the AFLP branch length for this isolate was long, indicating possible long-branch-length attraction due to the absence of more closely related taxa in the AFLP analysis. Alternatively, this isolate may represent an incorrect species designation using AFLP.

### Table 1. *Acinetobacter* spp. and STs from Arizona hospitals according to PCR/ESI-MS

<table>
<thead>
<tr>
<th>Species</th>
<th>PCR/ESI-MS ST</th>
<th>No. of samples for each ST</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. baumannii</em></td>
<td>10*</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>12*, 66†</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>68†</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>26*, 54†, 69‡</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>5*, 50†, 67‡, 70‡, 71‡, 72‡, 73‡, 74‡, 75‡, 76‡</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Total: 97</td>
<td></td>
</tr>
<tr>
<td><em>A. pittii</em></td>
<td>B7*</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>B2*, B3*</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>B13‡, B14‡, B15‡, B16‡, B17‡, B18‡, B19‡, B20‡, B21‡, B22‡, B23‡</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Total: 18</td>
<td></td>
</tr>
<tr>
<td><em>A. bereziniae</em></td>
<td>X3‡, X4‡</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Total: 2</td>
<td></td>
</tr>
<tr>
<td><em>A. nosocomialis</em></td>
<td>A6</td>
<td>1</td>
</tr>
<tr>
<td><em>A. lwoffii</em></td>
<td>NA</td>
<td>1</td>
</tr>
<tr>
<td>Unknown</td>
<td>NA</td>
<td>2</td>
</tr>
<tr>
<td>Mixture</td>
<td>NA</td>
<td>4</td>
</tr>
</tbody>
</table>

*STs first identified by Ecker et al. (2006).†ST previously identified by Wortmann et al. (2008). ‡STs first identified in this study.
Such incorrect genotypes may arise as a consequence of the inherent ambiguity in interpreting electrophoretic banding patterns or, alternatively, may reflect homoplastic character states due to the highly resolving nature of AFLP (Bergsten, 2005). Unsurprisingly, AFLP provided greater resolution than PCR/ESI-MS, providing unique genotypes belonging to the same ST. AFLP clustered *A. baumannii* into a single species and clearly distinguished this clade from other species. *A. pittii* (formerly genomic species 3) loosely clustered isolates into three different groups and yielded the most diverse intra-species genotypes. *A. nosocomialis* (formerly genomic species 13TU), *A. lwoffii*, *A. bereziniae* and one of the unknown species grouped into separate clades with both the PCR/ESI-MS and AFLP. (Note that isolates identified as mixtures by PCR/ESI-MS were removed from analysis and isolates with an asterisk show possible instances of long-branch-length attraction and incorrect grouping based on the AFLP data.)

On the intra-species level, two notable discrepancies were observed between the methods. Specifically, using AFLP, a single *A. baumannii* ST68 was shown to group with ST10, and an ST66 isolate grouped with ST12 isolates. Although not confirmed in this study, we suspect that these discrepancies represent homoplastic character states detected by our AFLP method, as ST10 and -68, and ST66 and -12, differ by four out of six and five out of six loci, respectively, according to PCR/ESI-MS. Our results indicated that reliance on a single method such as AFLP...
can occasionally lead to misleading or erroneous genotypes, although this effect can be mitigated when coupled with more slowly evolving loci such as MLST housekeeping genes, which are less prone to homoplasy (Keim et al., 2004). Further investigation using different molecular techniques, which are beyond the scope of the current study, would be helpful in determining the precise cause for these discrepancies.

Interestingly, AFLP failed to reveal significant differences or epidemiological patterns within the ST10 clade (data not shown). ST10 was found in all sampled hospitals and at different times throughout the study, and was evident across multiple counties in Arizona. Although there is only one SNP difference between ST10 and ST12, AFLP indicated greater overall genomic diversity within the ST12 group (Fig. 1), suggesting that ST12 has been present in Arizona hospitals for a longer period than ST10. Indeed, PCR/ESI-MS analysis of isolates from Ohio hospitals showed that ST12 has persisted since 2000, whereas ST10 has emerged only recently (Decker et al., 2012). Antibiotic resistance characterization of ST10 and ST12 isolates from Arizona hospitals would further aid in determining the epidemiological relationships with European clones and isolates from other hospitals in the USA.

16S rRNA gene and rpoB sequencing identify potentially novel species

In an effort to characterize further the two unknown isolates in our study, we sequenced regions of the 16S rRNA gene and the conserved RNA polymerase, rpoB. Both genes have been used previously to speculate Acinetobacter spp. (La Scola et al., 2006). 16S rRNA gene sequencing confirmed that both unknown samples belonged to the genus Acinetobacter. However, BLAST analysis (nr/nt database) of the rpoB region (performed 10 August 2012) against all Acinetobacter spp. showed incomplete or inconsistent matches across the four rpoB regions, indicating that these isolates are either poorly characterized in DNA databases or they are novel Acinetobacter spp. Although beyond the scope of our study, further analysis would be required to accurately speculate these clinically relevant samples.

In conclusion, PCR/ESI-MS provides a rapid, less-laborious approach to MLST with comparable resolution (Ecker et al., 2005). PCR/ESI-MS is gaining momentum in the Acinetobacter research community due to its simplicity, rapidity and capacity for robust inter-laboratory comparison of genotypes. We recognize that a disadvantage to the PCR-ESI/MS approach is that the ESI/MS platform is expensive; however, per-sample costs are considerably more cost-effective than conventional sequence-based methods such as MLST, and turnaround time is substantially shorter due to less hands-on time and a greater degree of automation. In future, the initial expense of this platform should decrease, allowing more clinical and research laboratories to access this method. Implementation of informative, cost-effective, rapid and accurate typing schemes will ultimately lead to better tracking and understanding of this important pathogen, facilitating improved infection control both within and between hospitals in the USA and globally.

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REFERENCES


