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Species identification within *Acinetobacter calcoaceticus - baumannii* complex using MALDI – TOF MS (REVISED MANUSCRIPT)

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Running Title: Species identification of Acb complex using MALDI - TOF MS

Key words: *A. baumannii*, Mass spectra, Rapid diagnostics, *Acb* complex

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Abstract

Acinetobacter baumannii, one of the more clinically relevant species in the *Acinetobacter* genus is well known to be multi-drug resistant and associated with bacteremia, urinary tract infection, pneumonia, wound infection and meningitis. However, it cannot be differentiated from closely related species such as *A. calcoaceticus*, *A. pittii* and *A. nosocomialis* by most phenotypic tests and can only be differentiated by specific, time consuming genotypic tests with very limited use in clinical microbiological laboratories. As a result, these species are grouped into the *A. calcoaceticus* – *A. baumannii* (*Acb*) complex. Herein we investigated the mass spectra of 73 *Acinetobacter* spp., representing ten different species, using an AB SCIEX 5800 MALDI – TOF MS to differentiate members of the *Acinetobacter* genus, including the species of the *Acb* complex. *RpoB* gene sequencing, 16S rRNA sequencing, and *gyrB* multiplex PCR were also evaluated as orthogonal methods to identify the organisms used in this study. We found that whilst 16S rRNA and *rpoB* gene sequencing could not differentiate *A. pittii* or *A. calcoaceticus*, they can be differentiated using *gyrB* multiplex PCR and MALDI – TOF MS. All ten *Acinetobacter* species investigated could be differentiated by their MALDI – TOF mass spectra.

1. Introduction

Acinetobacter baumannii is an opportunistic, aerobic, Gram-negative nosocomial pathogen that has become one of the six most important multi-drug resistant microorganisms worldwide (Antunes et al., 2014). *A. baumannii* is commonly known to cause difficult to treat pneumonia, bacteremia, urinary tract infection, wound infection and meningitis (Maragakis and Perl, 2008).

Accurate identification of *A. baumannii* and its closely related species *A. pittii*, *A. nosocomialis* and *A. calcoaceticus* is important as each of these species may display different characteristics in regards to antimicrobial susceptibilities, pathogenicity and clinical outcomes (Chuang et al., 2011; Sedo et al., 2013). The environmental strain *A. calcoaceticus* has not been reported to cause infection in humans whilst the other organisms are all of clinical significance (Peleg et al., 2008; Peleg et al., 2012). A recent paper has described increased severity and mortality in bacteraemia patients infected with *A. baumannii* compared with those infected with *A. pitii* and *A. nosocomialis*, emphasising the need for accurate differentiation (Fitzpatrick et al., 2015).

However, these four species which together make up the *A. calcoaceticus – A. baumannii* (*Acb*) complex are indistinguishable by phenotypic based tests (Peleg et al., 2008; Wang et al., 2013; Lee et al., 2014).

Molecular methods such as 16S rRNA and *rpoB* gene sequencing and have shown to be useful in differentiating members of the *Acinetobacter* genus (La Scola et al., 2006; Zarrilli et al., 2009; Wang et al., 2014). However, neither method is sufficient to differentiate species such as *A. pittii* and *A. calcoaceticus* (Higgins et al., 2010), and other molecular methods such as PCR amplification of intrinsic resistance genes or *gyrB* are used. However, these methods are mainly applied in research settings and have very limited use in clinical laboratories (La Scola et al., 2006; Higgins et al., 2010; Kamolvit et al., 2014).

Matrix assisted laser desorption ionisation – time of flight mass spectrometry (MALDI – TOF MS) has been shown to be a rapid and highly discriminatory method for the identification of bacteria (Kliem, 2010; Welker et al., 2011; Sedo et al., 2013). MALDI – TOF instruments that are commonly used in clinical settings for bacterial identification rely on the use of proprietary identification software and spectral databases (Martiny et al., 2012).

In this study, we evaluated the use of a research-use-only (RUO) 5800 MALDI – TOF MS (AB SCIEX, Concord, ON, Canada) to differentiate and identify various species in the *Acb* complex, as well as other *Acinetobacter* spp. using only the standard instrument software and freely available open-source software for the acquisition, processing and interpretation of spectra.

2. Materials and Methods

2. 1. Bacterial isolates and reference strains

The strains investigated in our study included thirty two multilocus sequence typed (MLST typed) *A. baumannii* isolates, six *Acinetobacter* reference strains and thirty five additional *Acinetobacter* isolates as described below:

The MLST typed *A. baumanni* isolates included sixteen isolates of sequence type (ST)110, four isolates of ST92 and three isolates of ST109 (Huber et al., 2014). In addition to this, four isolates of from the Arabian peninsula were added to the study, including one isolate from Saudi Arabia of the ST195 and one of ST436, respectively, plus one isolate of ST208 from Kuwait and one of ST229 from Qatar (Zowawi et al., 2015).

Two additional isolates of ST208 were of Japanese origin, and one isolate of ST208 was from Thailand, and two Singaporian strains were of ST491 (Kamolvit et al., unpublished data). The isolates from Japan, Thailand and Singapore were collected between 2008 and 2010, and the

MLST typing was performed in silico. The Kenyan strains and the strains from the Arabian peninsula were collected and MLST typed as previously described (Huber et al., 2014; Zowawi et al., 2015). MLST typing has been performed according to the Oxford scheme for all isolates (http://pubmlst.org/a*baumannii*/).

The following reference strains and previously published isolates and were added to the study; *A. baumannii* ATCC 19606, *A. calcoaceticus* ATCC 14987, *A. lwoffi* ATCC 15309 and ATCC 17986, *A. johnsonii* ATCC 17909, *A. junii* ATCC 17908, *A. baylyi* (*n*=1), *A. calcoaceticus* (*n*=1), *A. pitii* (*n*=1) (Peleg et al., 2012) and *A. nosocomialis* (*n*=2) (Peleg et al., 2012; Carruthers et al., 2013).

Various additional *Acinetobacter* spp. from Kenya (*n*=4, provided by the AGA KHAN University hospital in Nairobi, Kenya and collected between 2010-2011), Japan (*n*=12, provided by the Toho University in Tokyo, Japan and collected in 2010), Australia (*n*=2, collected at the Royal Brisbane and Women's hospital in Brisbane, Australia in 2004 and 2006 respectively), Thailand (*n*=7, provided by the Siriaj Hospital in Bangkok), Singapore (*n*=5, collected in 2008 and provided by the National University of Singapore). All isolates were grown on Mueller Hinton agar and incubated for 24 hours in a 37^{o}C incubator, and identification and confirmation of species was performed as described in chapter 2. 2.

2. 2. 16S rRNA identification

All *Acinetobacter* spp. isolates were initially identified by the sequencing of the 16S rRNA gene as previously described (Misbah et al., 2005). Sequencing was performed by Macrogen Inc,

Seoul, Korea, and sequences were blasted on NCBI using the megablast function against the 16S ribosomal RNA sequences database with maximum target sequences being set at 100. If 16S rRNA sequencing was unable to identify an isolate using the highest percentage identity, score and an E-value of 0 resulting in a sequence that matches two species with identical lengths, *rpoB* gene sequencing of zone 1 and 2 was performed as previously described (La Scola et al., 2006). A previously described *gyrB* multiplex PCR (Higgins et al., 2010) was used to differentiate *A. calcoaceticus* and *A. pittii*.

2. 3. MALDI - TOF MS

MALDI – TOF MS analyses were conducted on a 5800 TOF/TOF set in linear positive mode running the TOF/TOF Series Explorer acquisition software (AB SCIEX, Framingham, Massachusetts) at a laser frequency of 100 Hz with a set mass range of 3,000 to 20,000 Da. A continuous stage motion set in a random pattern at 600 µm/sec was used for sampling.

An in-house sinapinic acid matrix consisting of 10mg of sinapinic acid (>99.0% for MALDI-MS, Fluka 85429) in 500 µL acetonitrile, 475 µL distilled water and 25 µL 80% trifluoroacetic acid (TFA, LC-MS grade, Fluka 40967) was adapted from a previously published protocol (Freiwald and Sauer, 2009). Calibration was performed using calibration mixture 2 (AB SCIEX, Framingham, Massachusetts) which contained Angiotensin I, ACTH (1-17 clip), ACTH (18-39 clip), ACTH (7-38 clip) and insulin (bovine) to ensure mass accuracy within 5 ppm.

2. 4. Acquisition of mass spectra

A small amount of bacteria (approximately 10^6 – 10^8 cfu) was transferred from a 24 hour culture by spreading a thin layer onto a sample spot on an Opti-TOF 384 MALDI plate insert (AB

SCIEX, Framingham, Massachusetts) and overlaid with 1 µL of sinapinic acid matrix. Each isolate was spotted in quadruplicate and each replicate scanned once. Laser intensity was set at 4322 units and at a pulse rate of 100 Hz with a total of 1000 spectra accumulated for each sample. A mass range of *m/z* 3,000 to *m/z* 20,000 and a continuous stage motion set in a random pattern at 600 µm/sec was used for sampling. The TOF/TOF Series Explorer acquisition software (AB SCIEX, Framingham, Massachusetts) was used to acquire mass spectra.

2. 5. Processing of spectra

Mass spectra files were non-manipulatively converted from t2d files to mzXML files using a t2d converter (http://www.pepchem.org) and processed using mMass version 5.50 (Strohalm et al., 2008) (http://www.mmass.org/). Processing of raw spectra was conducted in mMass 5.5 (Martin Strolham) with a peak picking algorithm that used baseline correction, Savitzky-Golay smoothing and a signal to noise ratio of 3. Replicates of the same isolates were averaged to form a consensus spectrum.

3. Results

3. 1. Bacterial identification

Using 16S rRNA identification, we confirmed the following *Acinetobacter* spp. in our collection; *A. baumannii* (*n*=37), *A. nosocomialis* (*n*=15), *Acinetobacter junii* (*n*=3), *Acinetobacter lwoffii* (*n*=2), *Acinetobacter johnsonii* (*n*=1), *Acinetobacter baylyi* (*n*=1), *Acinetobacter soli* (*n*=1) and *Acinetobacter bereziniae* (*n*=1).

Using *rpoB* gene sequencing and *gyrB* multiplex PCR we determined the remaining fourteen strains to be *A. pittii* (*n*=12) and *A. calcoaceticus* (*n*=2). The results are as summarized in Table 1.

3. 2. MALDI – TOF MS based characterisation

All ten *Acinetobacter* species investigated in our study had sufficient differences in their mass spectra to be characterised and differentiated using MALDI – TOF MS. In all of the *A. baumannii* strains investigated (*n* = 35) we observed the presence of a characteristic, high intensity mass of *m/z* 5743.05, as well as two other specific masses of *m/z* 8583.00 and *m/z* 8715.00 that could be used to distinguish *A. baumannii* from other *Acinetobacter* spp. (Figure 1). Additionally, all the species that were not part of the *Acb* complex could also be characterised and differentiated (Summarised in Table 1).

Nine of the twelve *A. pittii* isolates studied had a representative high intensity mass of *m/z* 5780.03 and two other specific masses of *m/z* 8620.00 and *m/z* 8822.00. In addition, three of the twelve isolates were also characterized by a mass at *m/z* 5743.05, with similar intensity to that of the one in *A. baumannii*. However, the mass at *m/z* 5780.03 was absent (Figure 1).

In *A. nosocomialis* we observed a representative mass at *m/z* 8134.00 in all isolates, and another mass at *m/z* 8542.00 in fourteen of fifteen isolates (Figure 2). In *A. calcoaceticus*, we observed a representative prominent peak at *m/z* 5829.00 that occurred in both isolates of this species (Figure 2).

4. Discussion

Retrospective studies found that *A. baumannii* was associated with higher mortality rates, greater antimicrobial resistance and different clinical characteristics than the ones of *A. nosocomialis* and *A. pittii* (Chuang et al., 2011; Lee et al., 2011; Yang et al., 2013). This raises the importance of being able to differentiate the organisms within the *Acb* complex to allow for treatment to be optimised for specific organisms.

A. calcoaceticus – A. baumannii complex organisms are hard to differentiate because of high phenotypic similarities (Gerner-Smidt et al., 1991). Molecular methods such as 16S rDNA and *rpoB* sequencing as well as *gyrB* PCR amplification are possible methods to discriminate between these strains. We observed that 16S rRNA sequencing or *rpoB* gene sequencing were not sufficient to differentiate *A. pittii* and *A. calcoaceticus*, and that the identification of these two organisms required *gyrB* multiplex PCR. However, in our investigation these species showed MALDI-TOF mass spectra that are clearly distinct from each other.

MALDI is a soft desorption ionization method which may result in the formation of ions without significantly breaking chemical bonds, which is particularly useful in protein analysis. MALDI instruments commonly used for bacterial identification use licenced software packages that are incompatible with instruments of other brands. Such instruments are usually single TOF mass analysers that do not allow for efficient structure and sequence investigation of analytes. The ABSciex 5800 instrument in our laboratory is a TOF/TOF instrument which can overcome the limitations of a single TOF mass analyser by linking two TOF mass analysers in series, making it a much more powerful tool in protein research (Ng et al., 2014). However, due to the lack of a database, bacterial identification is not readily performed with this instrument.

The use of freeware to augment the abilities of RUO instruments such as the 5800 TOF/ TOF greatly increase the functionality and availability of these instruments for bacterial identification. The ability to differentiate species without using automated systems may be a step back from the automated systems that the VITEK – MS and the Biotyper offer, but it validates the ability of MALDI – TOF instruments to visualise spectral differences for greater discrimination between closely related species.

As we have seen in our study, MALDI – TOF was able to differentiate closely related species such as *A. pittii* and *A. calcoaceticus*. It appears that MALDI – TOF may also differentiate various *Acinetobacter* spp. from each other, as well as organisms that fall within the *Acb* complex. Automated processes would be required if this method is to be used as part of a diagnostic procedure.

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Figure 1: Spectra acquired from *A. baumannii* and *A. pittii*.

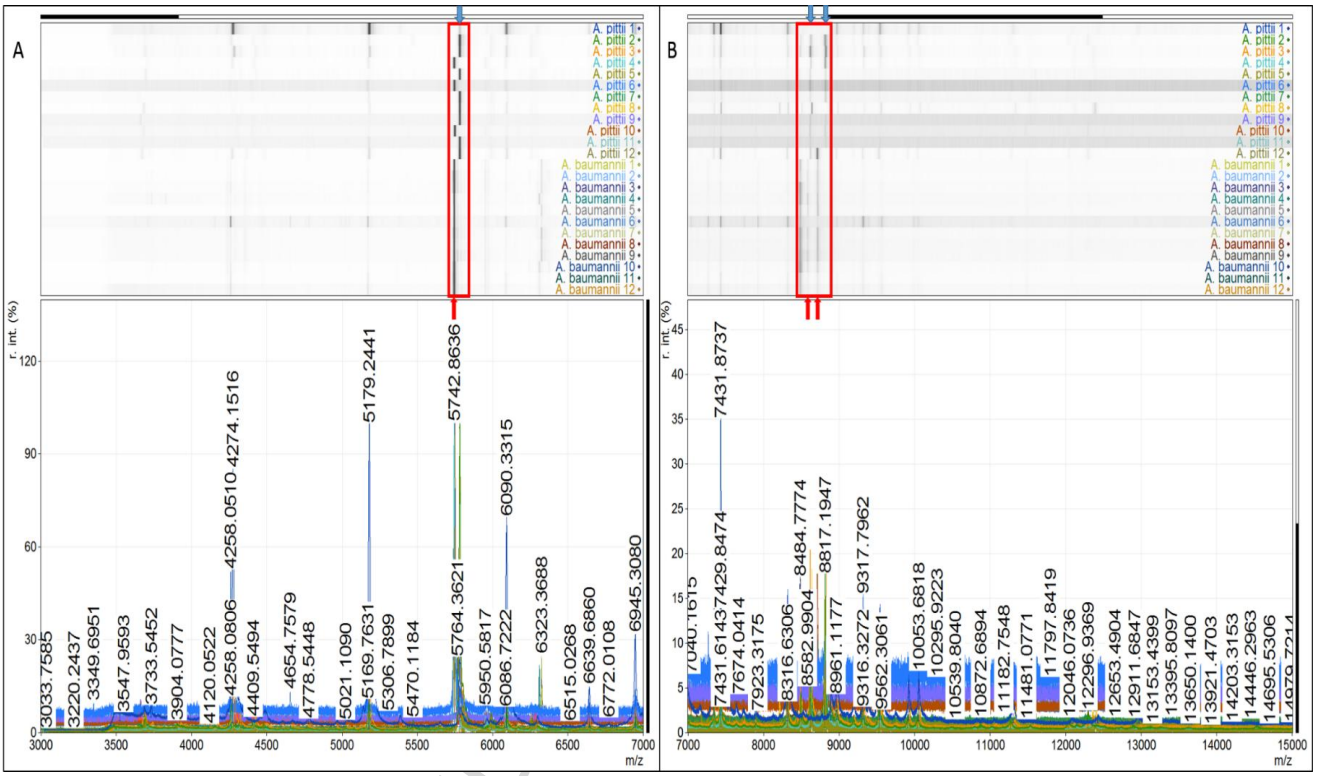

Figure 1: (A) Mass range of *m/z* 3000 – 7000 of *A. baumannii* and *A. pittii*. The red arrow indicates the mass of *m/z* 5743.05 and the blue arrow indicates the mass of *m/z* 5780.03. Three *A. pittii* isolates have the mass of *m/z* 5743.05, normally indicative of *A. baumannii*. However, none of the *A. pittii* isolates have both masses. (B) Mass Range of *m/z* 7000 – 15000 of *A. baumannii* and *A. pittii*. The red arrows indicate the masses of *m/z* 8583.00 and *m/z* 8715.00 that are representative of *A. baumannii*. The blue arrows indicate the masses of *m/z* 8620.00 and *m/z* 8822.00 which are representative of *A. pittii.*

Figure 2: Spectra acquired from *A. nosocomialis* and *A. calcoaceticus*.

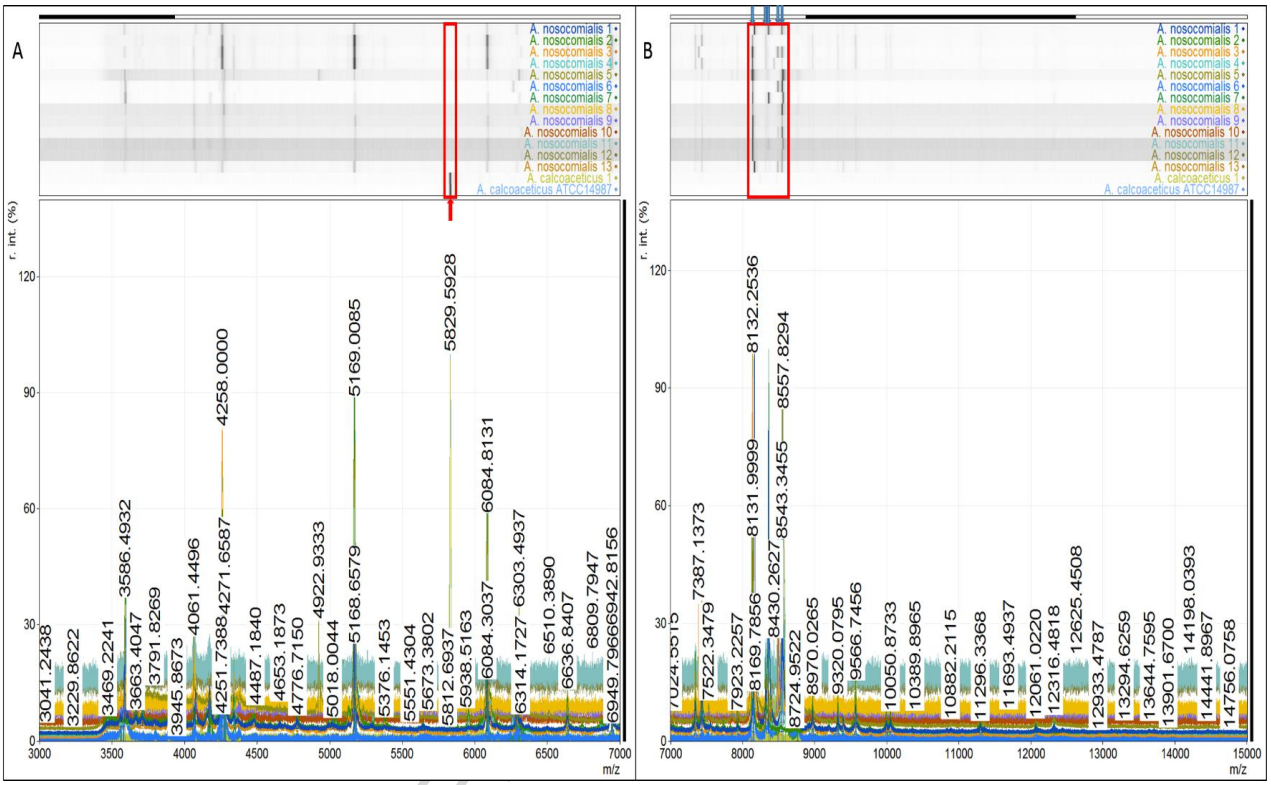

Figure 2: (A) Mass range of *m/z* 3000 – 7000 of *A. nosocomialis* and *A. calcoaceticus*. The red arrow indicates the mass of *m/z* 5829.00 which is indicative of *A. nosocomialis*. (B) Mass Range of *m/z* 7000 – 15000 of *A. nosocomialis* and *A. calcoaceticus*. The blue arrows indicate the masses *m/z* 8134.00, *m/z* 8542.00, *m/z* 8315.00 or *m/z* 8356.00 or *m/z* 8481.00 that are indicative of *A. nosocomialis.*

Table 1:

Bacterial Species	**Methods of species confirmation**	**Indicative MALDI peak masses (*m/z*)**
A. baumannii (*n* = 35)	16S rRNA gene sequencing	5743.05, 8583.00 and 8715.00
A. nosocomialis (*n* = 15)	16S rRNA gene sequencing	8134.00, 8542.00 and 8315.00 or 8356.00 or 8481.00
A. pittii (*n* = 12)	16S rRNA gene sequencing, *rpoB* gene sequencing and *gyrB* multiplex PCR	5743.05 or 5780.03, 8620.00, 8822.00
A. junii (*n* = 3)	16S rRNA gene sequencing	10,849.00
A. calcoaceticus (*n* = 2)	16S rRNA gene sequencing, *rpoB* gene sequencing and *gyrB* multiplex PCR	5829.00
A. lwoffii (*n* = 2)	16S rRNA gene sequencing	7613.00, 7662.00, 9101.00 and 9219.00
A. johnsonii (*n* = 1)	16S rRNA gene sequencing	4309.00 and 6720.00
A. baylyi (*n* = 1)	16S rRNA gene sequencing	5669.00
A. soli (*n* = 1)	16S rRNA gene sequencing	5519.00
A. bereziniae (*n* = 1)	16S rRNA gene sequencing	8018.00 and 9247.00

Table 1: *Acinetobacter* spp. investigated in this study with the methods that were used to identify each species and their indicative MALDI peak masses

Highlights

- *A. baumannii* is hard to differentiate from closely related *Acinetobacter* species.
- MALDI – TOF based identification usually relies on proprietary software.
- Here we differentiated *A. baumannii* from closely related *Acinetobacter* species.
- Differentiation was done using MALDI-TOF and freely available open-source software.