Molecular Epidemiology of Multi-resistant Acinetobacter baumannii in a Single Institution over a Ten Year Period

MRAB in a Single Institution over a Ten Year Period

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Abstract

Multi-resistant *Acinetobacter baumannii* is a worldwide nosocomial menace. We sought to better understand its behaviour through studying the molecular epidemiology of this organism at the Royal Brisbane and Women’s Hospital, Queensland, Australia over a ten year period. Multi-locus sequence typing (MLST), semi-automated rep-PCR and PFGE was performed on a selection of 31 *A. baumannii* isolates collected over the ten year period to determine their relationships to one another. MLST also allowed us to put this information in a global context. The presence or absence of *bla*$_{OXA-23}$ was also established. The presence of *bla*$_{OXA-23}$ closely correlated with carbapenem resistance in our collection. ST92 was the dominant sequence-type and was present in the hospital for nine years. There was also evidence of spread of ST69, ST73 and ST125 (novel) within the hospital, but this was not sustained over long periods. There were single examples only of the novel sequence types ST126 and ST127. The different typing methods clustered the isolates similarly, however PFGE and rep-PCR were more discriminatory than MLST. Worldwide, ST92 and the associated clonal complex CC92 represent the most sampled and widespread sequence type(s) and is also known as the European Clone 2 or Worldwide Clone 2. Antibiotic susceptibility within ST92 is variable, suggesting a role for mechanisms other than antibiotic resistance in its success.
Introduction

Acinetobacter baumannii is a non-fermentative gram-negative bacillus which is notable for its ability to acquire antibiotic resistance determinants and cause hospital outbreaks of infection (23). A. baumannii is an important pathogen of critically ill patients and can cause a range of infections including ventilator-associated pneumonia, bloodstream infection, wound infection and nosocomial meningitis (13, 15, 23). In many institutions substantial difficulties arise because A. baumannii strains have become resistant to all beta-lactam antibiotics (including carbapenems), all fluoroquinolones, trimethoprim-sulfamethoxazole and most, if not all, aminoglycosides (24). Thus, empiric treatment choices are extremely limited.

In order to better control multi-resistant A. baumannii, an understanding of the molecular epidemiology of the infection is necessary. From a global perspective, it is known that A. baumannii is typically clonal in nature (11). Three clonal complexes have predominated in Europe for more than a decade (7, 28); these clonal complexes have more recently also been documented in North America, Asia, Africa and Australia (11). The precise origin of these clonal lineages will likely never be known. From this broader geographic perspective it is remarkable how successful clones have spread, likely through the international transfer of patients (11, 22).

The molecular epidemiology of A. baumannii has been typically studied in the context of outbreaks of infection. However, an understanding of the epidemiology of the infection over longer time periods may allow new insights into the behaviour of this emerging pathogen. In this paper, we have undertaken a longitudinal evaluation of the molecular
epidemiology of multi-resistant \textit{A. baumannii} in a single institution over a ten year period.

Materials and Methods

Setting

The Royal Brisbane and Women’s Hospital (RBWH) is a 900 bed teaching hospital of the University of Queensland. The hospital is a major referral centre for trauma, burns and hematologic transplantation. Within the hospital is a 19 bed long-stay Intensive Care Unit (ICU) which has a bed occupancy of 550-670 patient-days per month.

Isolates and Time Period

Isolates were selected from a collection of all \textit{A. calcoaceticus-A. baumannii} complex strains with non-wild type susceptibility profiles that had been cultured at the Royal Brisbane Hospital between September 1998 and November 2008. A non-wild-type susceptibility profile was defined as any isolate with resistance by VITEK GNI card to trimethoprim-sulfamethoxazole, gentamicin, tobramycin, amikacin, ceftazadime, cefepime, timentin, tazocin, ciprofloxacin or meropenem.

There were 483 isolates from unique patients available. The vast majority of isolates in the collection had been cultured from patients in the ICU or burns ward. The dominant phenotype was susceptibility only to amikacin or to amikacin and tobramycin (colistin and tigecycline susceptibility were not routinely tested). More than half of the isolates collected over the eleven year period were from 2001-2002 ($n = 204$) and 2006 ($n = 84$); these periods corresponded to \textit{A. baumannii} outbreaks in the hospital.
From this collection, a total of 33 isolates were selected for study. Seventeen isolates were from outbreak periods. Of these, seven had a PFGE profile demonstrating a close or possible relationship to the outbreak strain, six were different to the outbreak strain by PFGE and four isolates were not typed by PFGE. Sixteen isolates were from sporadic cases. These isolates were selected because there was a point of difference in the antibiotic susceptibility profile or epidemiology (known international transfer or lack of association with the burns unit or ICU), or to allow representation of all years.

**Species Identification, Antimicrobial Susceptibility and Detection of Carbapenemases**

Phenotypic identification and antibiotic susceptibility testing were performed by VITEK or VITEK 2 (bioMérieux, France) and interpreted according to CLSI criteria (4).

Genotypic identification as *A. baumannii* was confirmed by detection of *bla*<sub>OXA-51-like</sub> by a real-time PCR assay adapted from the gel based method previously described (26). PCR reactions were performed using 20 µl reaction volumes with 10 pmol each of forward and reverse primer, 7 µl of water, 10 µl of Platinum SYBR Green qPCR Supermix-UDG (Invitrogen, Carlsbad, CA) and 1 µl of extracted DNA. Amplification and detection was performed on a Rotor-Gene 6000 real time system (Corbett Research, Sydney) with the following conditions: 94°C for 3 minutes, 35 cycles of 94°C for 45 seconds, 60°C for 45 seconds, 72°C for 1 minute, followed by a final extension of 72°C for 1 minute.

Detection of *bla*<sub>OXA-23</sub> (commonly associated with carbapenem resistance) was performed as previously described (2).
Multilocus Sequence Typing

MLST was performed as previously described (3) with the following exceptions:

Amplification of *gyrB* and *gpi* was performed with an annealing temperature of 50°C for some isolates as product generation was inefficient at 55°C. The amplification product was purified with ExoSAP-IT (Axygen, Union City, CA, USA). Sequencing was performed with Big Dye Terminator pre-mix version 3 and an ABI 3730 DNA Analyzer (Applied Biosystems). For four isolates, sequencing of *gdhB* required use of diluted amplification primers as sequencing was unsuccessful with the internal sequencing primers.

Editing and interpretation of electropherograms was performed visually and with the assistance of the following software: Finch TV (http://www.geospiza.com/Products/finchtv.shtml), Readseq (http://www.ebi.ac.uk/cgi-bin/readseq.cgi) and Clustal X (16). Analysis of allele sequences and ST assignment made use of the Oxford *Acinetobacter baumannii* MLST website (http://pubmlst.org/abaumannii/) (14). The eBURST diagram was constructed by V3 software (http://eburst.mlst.net/) using all available data from previous publications (3, 9, 12, 18, 21, 29) and unpublished data from the online database where the submitter of the data consented to its use.

Typing Using rep-PCR

rep-PCR was performed with the semi-automated Diversilab® system (bioMerieux, Melbourne) according to the manufacturer’s instructions. Diversilab fingerprints were
analysed with the Diversilab software using the Pearson correlation statistical method to
determine clonal relationships.

_Pulsed field gel electrophoresis_
PFGE was performed after digestion of genomic DNA with ApaI (New England Biolabs,
Beverley, MA) as previously described (25) and analysed using Bionumerics software
(Applied Maths, Belgium).

**Results**

Of, 33 isolates selected for analysis, 31 were _bla_OXA-51-like_ positive. These isolates that
were confirmed to be _A. baumannii_ underwent molecular typing and were examined for
presence of _bla_OXA-23

**MLST, Antibiotic Susceptibility and _bla_OXA-23**
MLST findings and associated antibiograms and _bla_OXA-23_ results are summarised in
Tables 1 and 2. It must be noted that many ST designations have changed on the _A.
baumannii_ MLST website since the publication of relevant studies (9, 10, 18, 20, 21) to
avoid conflict with an earlier publication (3). For example, ST92 was previously referred
to as ST22. Changes relevant to the current study are listed in Table 3.

ST92 was the dominant sequence type (22 of 32 isolates) and was found in the hospital
over the period from 2000-2008. ST92 is also the most frequently isolated and founding
genotype of the largest and most widespread clonal complex (CC92) of _A. baumannii_
Within CC92, ST92 (18), ST6 (3), ST98 (10) and ST118 (18) have previously been shown to be representatives of the “European Clone 2” (EU2) or worldwide clonal lineage 2 (WW2) by other typing methods. All of the isolates with this sequence type were carbapenem and ciprofloxacin resistant, but had variable susceptibility to aminoglycosides. All of the isolates were also $\text{bla}_{\text{OXA-23}}$ positive.

Three isolates were ST69. This is a double locus variant (DLV) of multiple STs within CC92 implying a close relationship; however an intervening SLV has not yet been identified so it cannot be considered a member of CC92 by conservative definition (8). ST69 isolates were ciprofloxacin and amikacin resistant but carbapenem susceptible and $\text{bla}_{\text{OXA-23}}$ negative. This phenotype was only identified in a small number of isolates, namely Q1, Q45 (isolated in 1998 and 1999, respectively) and Q54. Q54 was isolated on the day of admission from a patient transferred from Indonesia in 2002. In spite of the apparent repeated introduction of this ST, there was no evidence of persistence.

ST73 isolates were unique among local carbapenem-resistant strains; they possessed a distinct pulsotype and were ciprofloxacin susceptible. Only four isolates with this phenotype and pulsotype were detected and these were all isolated from patients over a 4 month period in 2006. The index ST73 isolate was cultured from a patient that had been transferred from Papua New Guinea. ST73 is a singleton by MLST and has previously been described in Korea (20).

Three novel singleton STs were identified. ST125 was present in the hospital in 1999 only and was resistant to all aminoglycosides, ciprofloxacin and meropenem. The source
of the index case is unknown. The ST126 isolate (Q55) had a similar antibiotic
susceptibility profile to the ST69 isolates (i.e. it was resistant to amikacin and
ciprofloxacin but susceptible to meropenem), however these two STs were unrelated. The
ST127 isolate (Q22) was cultured from a patient one week after admission to the ICU;
Q22 was resistant to meropenem only and possessed a \( \text{bla}_{\text{OXA-23}} \) resistance determinant.

**Rep PCR and PFGE**

A dendrogram constructed from rep-PCR results is presented in Figure 2. The three
typing methods were generally concordant. By rep-PCR ST69 clustered >95% with
ST92. By PFGE also, ST69 clustered amongst ST92 (data not shown). This suggests
ST69 may belong to CC92 even though an SLV to link it to the complex by MLST has
not yet been identified. Some isolates were indistinguishable by both rep-PCR and PFGE
despite being cultured from patients many years apart (e.g. Q6, Q28 seven years and Q28,
Q53 six years, respectively).

**Discussion**

In this study we have examined the epidemiology of multidrug-resistant *A. baumannii* at
a single institution longitudinally over 10 years. We found that in spite of six different
STs being represented by non-wild type *A. baumannii* isolates tested, ST92 dominated
heavily and was isolated from 2000 to 2008. Within ST92 there were diverse, but related,
pulstotypes and rep-PCR patterns. The only previous comparison of a large group of *A.
baumannii* isolates by PFGE and MLST showed unexpected divergence of sequence
types by PFGE on a geographically diverse collection of isolates (10). In this study the
sequence types cluster together. These differences likely reflect the source of strains comprising the two collections. The only discrepant result between the typing methods was that ST69 clustered amongst ST92 by PFGE and rep-PCR. However these ST69 isolates had a distinct epidemiology and phenotype consistent with the differentiation by MLST.

Our analysis identified isolates with indistinguishable pulsotypes or rep-PCR patterns separated by 6 and 7 years, respectively. This suggests clonal spread of a successful A. baumannii strain. In contrast, although there was introduction and transmission of ST69 (on two occasions), ST73 and ST125, we did not find evidence of persistence of any of these STs for more than 1 year. Possible explanations for the repeated isolation of ST92 for nearly 10 years include re-admission of previously colonised local patients (the potential duration of carriage is currently uncertain), re-introduction from inter-hospital transfers and long term persistence in the hospital environment in spite of intensive infection control efforts. Our results and the known ability of this organism to resist desiccation (23) support the later. Previous studies using an alternative MLST scheme have also shown repeated isolation of ST2, which are also representatives of EU2 including isolates that directly correspond to ST92 (18), in a single location (6) or geographic region (5) over long periods of time, but without the continuity at one site presented here.

On a global scale, ST92 is the predicted founder of CC92 – the largest and most geographically diverse clonal complex by MLST. The results presented here confirm our facility in Australia is included in this global epidemic. CC92 corresponds to EU2 /
WW2 based on previous typing by other methods of members of the clonal complex including ST92 and ST118 (18). Further, a EU2 reference strain RUH 134 has been reported both as ST6 (3) and with an allelic profile corresponding to ST98 (10) by this MLST scheme in previous publications. It is unclear why the reference strain has given two STs, but ST6 and ST98 are SLVs that differ by only one nucleotide in the \textit{gyrB} locus. Our rep-PCR results also show similarities between ST92 and WW2 (11), though this is an inferior method for comparison between laboratories.

Although ST92 is heavily represented in the current MLST dataset, we cannot rule out the possibility that it may not be truly representative of the diversity and relative abundance of \textit{A. baumannii} STs. There is presumably a bias towards investigation and typing of strains resistant to multiple antimicrobials. Indeed, this is the case for our collection of isolates, as the antibiotic-resistant strains were those that had been stored for potential future study, and half the strains we tested were isolated during outbreaks. We attempted to overcome this by including a number of diverse sporadic strains in our analyses; over half of these were in fact ST92. We note also that we only tested a representative subset of the total number of \textit{A. baumannii} isolates collected from 1998-2008 and thus it is possible that some important clones were missed. Globally, there is also likely to be temporal and geographic bias related to the availability of sequencing technology. For example, eBURST analysis of European strains in the database identifies ST98 as the founder of the dominant carbapenem-resistant clonal complex, whereas ST92 is identified as the founder if Asia/Oceania strains are analysed. The diversity within clonal complexes and the designation of putative founders may change with the collection of more sequence typing data.
In the *A. baumannii* isolates examined here, similar antibiotic resistance profiles were seen within STs. The presence of *bla*<sub>OXA-23</sub> correlated closely with carbapenem resistance, as has previously been the case where Australian strains have been tested (17, 27) including a single ST92 isolate (16). However, antibiotic resistance profiles are known to be an inaccurate predictor of clonality for *A. baumannii* (1, 7, 11, 13, 19) and there is also diversity of antibiotic resistance determinants within strains that are related by MLST. For example, carbapenem susceptible ST92 are widespread in China (9) and carbapenem resistant ST69 have been identified in Korea (21), in contrast to the antibiotic susceptibility patterns of our isolates. The large number of carbapenem susceptible ST92 in China supports the notion that antibiotic resistance may not be the primary determinant of hospital adaptiveness for this clone.

For *A. baumannii* of CC92 / WW2 / Euro 2 the ability to persist for over 9 years in a single hospital, and the variable antibiotic susceptibility of CC92 (and within ST92), suggests adaptation to the hospital environment, as well as antibiotic resistance, may be important for the success of *A. baumannii* as a nosocomial pathogen. This raises the question of why this clonal complex has been so successful. With the progressive accumulation of global data, MLST is proving to be a powerful tool for the study of *A. baumannii* epidemiology and the addition of Australian data adds new evidence for the intercontinental spread of the most successful clonal complex. Colistin-resistant isolates of ST92 have recently been described in Korea (21), a further step towards a truly pan-resistant epidemic *A. baumannii* clone. In order to fully understand and combat multi-
resistant nosocomial *A. baumannii* the mechanisms of hospital adaptiveness beyond antibiotic resistance demand more attention.

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Technical assistance provided by the following people is also gratefully acknowledged: Narelle George, Jacqueline Schooneveldt, Haakon Bergh, Fatimah Haslina, Tarrant Hansen and Angela Duffy.
References


Figure 1. eBURST population snapshot of *A. baumannii* constructed from previous publications where official sequence types have been assigned, plus unpublished data from the *A. baumannii* MLST website where permission for use was granted by the submitter of the data. CC92 is the largest clonal complex currently. STs 19, 69, 9, 123, 28, 29, 30 have double locus variant relationships with members of CC92 and are therefore closely related, although not considered members of the CC by conservative definition.

Figure 2. Dendrogram of *A. baumannii* rep-PCR patterns by the Pearson correlation method. Isolate number, year of isolation and ST are also shown.
Table 1. Sequence types (ST) and allele numbers of *Acinetobacter baumannii* isolates by year isolated.

<table>
<thead>
<tr>
<th>Isolate Number</th>
<th>Year</th>
<th>ST</th>
<th>gltA</th>
<th>gyrB</th>
<th>gdhB</th>
<th>recA</th>
<th>cpn60</th>
<th>gpi</th>
<th>rpoD</th>
</tr>
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<tbody>
<tr>
<td>Q48, Q47, Q57</td>
<td>2000</td>
<td>92</td>
<td>1</td>
<td>3</td>
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<td>3</td>
</tr>
<tr>
<td>Q5, Q50, Q6, Q7</td>
<td>2001</td>
<td>92</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Q51, Q52, Q53</td>
<td>2002</td>
<td>92</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>2</td>
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<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Q10, Q11</td>
<td>2004</td>
<td>92</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
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<td>1</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Q13, Q15, Q16, Q17, Q19, Q21</td>
<td>2006</td>
<td>92</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>7</td>
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<td>3</td>
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<td>73</td>
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<td>4</td>
<td>64</td>
<td>5</td>
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<td>33</td>
<td>57*</td>
<td>11</td>
<td>26</td>
<td>11</td>
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* novel
Table 2. Antibiogram and \( bla_{OXA-23} \) result by sequence type.

<table>
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<th>ST</th>
<th>n</th>
<th>Antibiotic</th>
<th>( bla_{OXA-23} )</th>
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<tr>
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<td>22</td>
<td>21/22 R</td>
<td>12/22 R</td>
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<tr>
<td>69</td>
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<td>2/3 R</td>
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<tr>
<td>73</td>
<td>2</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>125</td>
<td>2</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>126</td>
<td>1</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>127</td>
<td>1</td>
<td>S</td>
<td>S</td>
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</table>

R non-susceptible (including resistant and intermediate); S susceptible
Table 3. New designations for previously published sequence types discussed within the text. During December 2009 / January 2010 changes were made to the MLST website (http://pubmlst.org/abaumannii/) to correct doubly allocated numbers to avoid conflict with an earlier publication (3). A complete list of changes is available at the website.

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<th>Previous Designation</th>
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<td>1-3-3-2-2-7-3</td>
<td>Park 2009 (20), Mugnier 2010 (18), Fu 2010 (9), Ho 2009 (12), Hamouda 2010 (10)</td>
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<td>ST 69</td>
<td>1-46-3-2-2-58-3</td>
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<td>ST 73</td>
<td>1-47-53-1-1-59-32</td>
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