

# Molecular characterization of carbapenem-resistant *Acinetobacter* species in an Irish university hospital: predominance of *Acinetobacter* genomic species 3

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A 30 month prospective study of *Acinetobacter* species encountered in the Central Pathology Laboratory of St James's Hospital, Dublin, Ireland, was conducted to investigate the prevalence and molecular epidemiology of carbapenem resistance in such isolates. *Acinetobacter* genomic species 3 (AG3) was found to be the predominant *Acinetobacter* species (45/114, 39%) in our institution. A total of 11% of all *Acinetobacter* species (12/114) and 22% of AG3 isolates (10/45) were carbapenem resistant. Carbapenem resistance was mediated by Ambler class D  $\beta$ -lactamase OXA-23 in all 12 isolates, with insertion sequence IS*Aba1* found upstream of *bla*<sub>OXA-23</sub>. IS*Aba1* was also found upstream of *bla*<sub>ADC-25</sub>, which encodes the enzyme AmpC, in an *Acinetobacter baumannii* isolate, and upstream of the aminoglycoside-acetyltransferase-encoding gene *aacC2* in three AG3 isolates. Inter-species plasmidic transfer was most likely involved in the emergence and spread of *bla*<sub>OXA-23</sub> among the *Acinetobacter* isolates within our institution. The emergence of carbapenem resistance was associated not only with prior carbapenem use but also with the use of other antimicrobial agents, most notably  $\beta$ -lactam/ $\beta$ -lactamase-inhibitor combinations. The study demonstrated the emerging trend of carbapenem resistance in the wider context of the *Acinetobacter* genus, and reiterated the paramount importance of the prudent use of antimicrobial agents, stringent infection control measures and resistance surveillance of pathogens.

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## INTRODUCTION

*Acinetobacter* spp. have become major pathogens in hospital-associated infections, especially in critical care settings such as intensive care units (ICUs) and units for patients with severe burns (Murray & Hospenthal, 2008; Sunenshine *et al.*, 2007). They can survive in the hospital environment for long periods and have a remarkable propensity to develop resistance to multiple classes of

**Abbreviations:** AG3, *Acinetobacter* genomic species 3; CLSI, Clinical and Laboratory Standards Institute; ICU, intensive care unit; MBL, metallo  $\beta$ -lactamase.

The GenBank/EMBL/DDBJ accession numbers for the *Acinetobacter* sequences reported in this paper are as follows: EU827524 (*Acinetobacter* genomic species 3 IS*Aba1*/*bla*<sub>OXA-23</sub>/ATPase), EU827525 (*Acinetobacter johnsonii* IS*Aba1*/*bla*<sub>OXA-23</sub>/ATPase), EU827526 (*Acinetobacter baumannii* IS*Aba1*/*bla*<sub>OXA-23</sub>/ATPase), EU835512 (*Acinetobacter baumannii* IS*Aba1*/*bla*<sub>ADC-25</sub>), EU839488 (*Acinetobacter* genomic species 3 IS*Aba1*/*aacC2*), EU872057 (*Acinetobacter johnsonii* *aacC2*).

A table of primer sequences is available as supplementary material with the online version of this paper.

antimicrobial agents. As a result, they can cause a variety of infections including ventilator-associated pneumonia and bacteraemia. Carbapenems have been a particularly useful group of antibiotics in the treatment of such infections as they are  $\beta$ -lactams with the broadest spectra of activity. They are clinically effective against Gram-negative organisms producing Ambler classes A and C extended-spectrum  $\beta$ -lactamases. However, carbapenem resistance in *Acinetobacter* has been increasingly reported worldwide, attributable mainly to Ambler class D carbapenemases and class B metallo- $\beta$ -lactamases (MBLs) (Poirel & Nordmann, 2006; Walther-Rasmussen & Høiby, 2006). This emerging trend is of grave concern given the prospect of a further reduction in therapeutic options for infections by these multi-drug-resistant organisms. Following the emergence of carbapenem-resistant *Acinetobacter* species in St James's Hospital in 2005 (Boo *et al.*, 2006), a prospective study of *Acinetobacter* isolates encountered in the Central Pathology Laboratory, St James's Hospital, was conducted to ascertain the clinical epidemiology, molecular mechanisms and genetic regulation of these carbapenem-resistant isolates.

## METHODS

**Collection of *Acinetobacter* isolates.** Isolates were prospectively screened and collected in the Central Pathology Laboratory of St James's Hospital over a 30 month period from May 2005 to October 2007. In cases where there were two or more specimens yielding the same organism in a patient, the first isolate from each patient episode was chosen.

**Identification of organisms.** *Acinetobacter* isolates were presumptively identified using the Vitek-2 GNI identification system (bioMérieux). Confirmatory identification and speciation was carried out by PCR and sequencing of the *rpoB* gene and flanker regions, as described by La Scola *et al.* (2006). In this study, a 500 bp segment of the *rpoB* gene, as well as a region of variable size flanking the *rpoB* and *rpoC* genes, were selected for PCR and sequencing.

**Antimicrobial susceptibility testing.** Antimicrobial susceptibility testing was performed using Vitek GNI susceptibility test system (bioMérieux) for the following antimicrobial agents: ampicillin, amoxicillin-clavulanate, piperacillin-tazobactam, cefoxitin, cefotaxime, ceftazidime, cefepime, meropenem, ciprofloxacin, trimethoprim-sulphamethoxazole, gentamicin and amikacin. MICs were determined using Etest strips (bioMérieux) for the above antimicrobial agents, as well as for the following agents: tigecycline, colistin and imipenem. Carbapenem-resistant isolates were also screened phenotypically for the presence of MBL using imipenem versus imipenem + EDTA Etest MIC strips (bioMérieux). Resistance was defined using Clinical and Laboratory Standards Institute (CLSI) breakpoints (CLSI, 2006).

**Screening for carbapenem-resistance genes.** PCR was performed using primers for Ambler class B  $\beta$ -lactamase-encoding genes *bla<sub>VIM</sub>*, *bla<sub>IMP</sub>*, *bla<sub>SPM</sub>*, *bla<sub>GIM</sub>* and *bla<sub>SIM</sub>* (Castanheira *et al.*, 2004; Lee *et al.*, 2005; Pitout *et al.*, 2005), as well as Ambler class D carbapenemase-encoding genes *bla<sub>OXA-23-like</sub>*, *bla<sub>OXA-24-like</sub>*, *bla<sub>OXA-51-like</sub>* and *bla<sub>OXA-58</sub>* (Afzal-Shah *et al.*, 2001; Coelho *et al.*, 2006; Héritier *et al.*, 2005) (Supplementary Table S1 available with the online journal). The PCR was carried out in the GeneAmp 9700 PCR system thermal cycler (Applied BioSystems) using a Qiagen PCR core kit (Qiagen) under the conditions specified by the manufacturer.

**Screening of other resistance genes.** PCR was also performed to screen for other resistance genes that might be present in carbapenem-resistant isolates, including those encoding Ambler class A  $\beta$ -lactamase-encoding genes (*bla<sub>TEM</sub>*, *bla<sub>SHV</sub>*, *bla<sub>CTX-M</sub>*, *bla<sub>VEB</sub>* and *bla<sub>PER</sub>*) (Hopkins *et al.*, 2006; Naas *et al.*, 2006) and Ambler class C *Acinetobacter*-derived cephalosporinase-encoding genes (*bla<sub>ADC</sub>*) (Hujer *et al.*, 2005). The presence of integrons was screened using primers specific for class 1 and class 2 integrases (Koeleman *et al.*, 2001). Aminoglycoside resistance was investigated by screening for genes encoding aminoglycoside-modifying enzymes, *aacC1*, *aacC2* and *aphA6* (Vila *et al.*, 1999). The primers are shown in Supplementary Table S1 available with the online journal.

**Screening for insertion sequence IS*Aba1*.** The presence of the IS*Aba1* insertion sequence, upstream of the resistance genes and in the correct orientation to promote resistance-gene expression, was sought using a forward primer homologous to sequences located within IS*Aba1* and reverse primers homologous to the respective resistance genes.

**Nucleotide sequencing.** Sequencing of amplicons in both directions was performed at the Institute of Molecular Medicine, Trinity College, University of Dublin, with the ABI Prism 3130xl analyser (Applied BioSystems) using the BigDye terminator V3.1 cycle sequencing kit (Applied BioSystems) according to the manufacturer's

specifications. Sequences were aligned using CLUSTAL W software ([www.ebi.ac.uk/Tools/clustalw](http://www.ebi.ac.uk/Tools/clustalw)) and compared with existing GenBank sequences using the BLAST programme ([www.ncbi.nih.gov/blast](http://www.ncbi.nih.gov/blast)). The sequences of our isolates were submitted to GenBank, National Center for Biotechnology Information ([www.ncbi.nih.gov/Genbank](http://www.ncbi.nih.gov/Genbank)).

**Epidemiological typing of isolates.** Epidemiological typing of carbapenem-resistant isolates was carried out using PFGE following digestion of genomic DNA by restriction endonuclease *ApaI* as described by Seifert *et al.* (2005). The PFGE patterns were analysed using BioNumerics software (BioSystematica) according to the criteria described by Tenover *et al.* (1995).

**Plasmid studies.** Plasmid extraction of bacterial suspensions was carried out using a plasmid midi kit (Qiagen) according to the manufacturer's specifications. PCR using the plasmid preparations as templates was performed to ascertain whether the carbapenem-resistance genes were plasmid borne. PCR of the *rpoB* gene was also performed using the same plasmid preparations to look for evidence of chromosomal contamination during the extraction procedure. Following gel electrophoresis, DNA from individual plasmid bands was eluted using a gel extraction kit (Qiagen) and used as template for PCR to ascertain the sizes of the plasmids carrying the resistance genes.

**Clinical and epidemiological data for the patients.** Clinical, epidemiological and demographic data for the patients were obtained from clinical case notes and from the Central Pathology Laboratory surveillance database. Infection was defined as the isolation of the organism from a normally sterile site; or clinical evidence of sepsis originating from a normally non-sterile site where significant colony counts of the organism had been cultured.

## RESULTS AND DISCUSSION

### Species identification of *Acinetobacter* isolates

A total of 114 *Acinetobacter* isolates were collected in the Central Pathology Laboratory over the stipulated period. The Vitek-2 GNI identification system identified the isolates (and their numbers) as follow: *Acinetobacter baumannii* (77), *Acinetobacter lwoffii* (31), *Acinetobacter junii* (2), *Acinetobacter haemolyticus* (4). However, *rpoB* gene sequencing revealed the distribution of the various species as shown in Table 1. All amplicons had sequence concordance of 98 to 100% when compared with the respective reference GenBank sequences (La Scola *et al.*, 2006). A total of 39% (45/114) of all isolates were *Acinetobacter* genomic species 3 (AG3) while *A. baumannii* constituted 22% (25/114) of the total. The predominance of AG3 over *A. baumannii* was an unusual finding but such an epidemiology had occasionally been reported in hospitals (Traub & Bauer, 2000). This study also demonstrated the poor accuracy of the speciation of *Acinetobacter* species by the Vitek-2 GNI identification system, with 75% of isolates erroneously speciated. It highlights the need to regard such results as preliminary data. Accurate speciation using molecular methods not only is important in the investigation of outbreaks caused by *Acinetobacter* species, but also is relevant in epidemiological studies such as this report.

**Table 1.** Speciation and meropenem MICs of *Acinetobacter* isolates collected in the Central Pathology Laboratory, St James's Hospital (May 2005 to October 2007)

<i>Acinetobacter</i> species	No. of isolates	Meropenem and imipenem MICs of $\geq 32$ mg l <sup>-1</sup> * (percentage within species)	Meropenem and imipenem MICs of $\leq 4$ mg l <sup>-1</sup> * (percentage within species)
All species	114	12 (11%)	102 (89%)
<i>Acinetobacter</i> genomic species 3	45	10 (22%)	35 (78%)
<i>Acinetobacter baumannii</i>	25	1 (4%)	24 (96%)
<i>Acinetobacter calcoaceticus</i>	5	0	5 (100%)
<i>Acinetobacter</i> genomic species 13	2	0	2 (100%)
<i>Acinetobacter johnsonii</i>	12	1 (8%)	11 (92%)
<i>Acinetobacter ursingii</i>	8	0	8 (100%)
<i>Acinetobacter</i> genomic species 9	5	0	5 (100%)
<i>Acinetobacter lwoffii</i>	2	0	2 (100%)
<i>Acinetobacter</i> genomic species 10	1	0	1 (100%)
<i>Acinetobacter</i> genomic species 11	4	0	4 (100%)
<i>Acinetobacter haemolyticus</i>	1	0	1 (100%)
<i>Acinetobacter schindleri</i>	1	0	1 (100%)
<i>Acinetobacter tjernbergiae</i>	1	0	1 (100%)
<i>Acinetobacter radioresistens</i>	2	0	2 (100%)

\*Based on the Etest method; no isolate had an MIC of 8 or 16 mg l<sup>-1</sup>.

### Antimicrobial susceptibility of carbapenem-resistant isolates

Vitek-2 MICs corresponded closely with those obtained with the Etest method. Of the 114 isolates, 12 (11% of the total) were resistant to both meropenem and imipenem (MICs of  $\geq 32$  mg l<sup>-1</sup>) (Table 1). Ten isolates were AG3, while the remaining two were *A. baumannii* and *Acinetobacter johnsonii*. The remaining 102 isolates were susceptible to both meropenem and imipenem (MICs of  $\leq 4$  mg l<sup>-1</sup>). A significant proportion (22%) of the AG3 isolates was thus found to be carbapenem resistant, and to the authors' knowledge, this study is the first report of a carbapenem-resistant *A. johnsonii* clinical isolate. Worldwide, the overwhelming majority of carbapenem-resistant isolates are *A. baumannii* isolates. AG3 has rarely been reported to be carbapenem resistant (Boo *et al.*, 2006; Marti *et al.*, 2008b). The unusual predominance of AG3 among the carbapenem-resistant isolates in our institution probably reflects the relative distribution of the various *Acinetobacter* species locally. This study adds to the emerging reports of carbapenem resistance in clinical isolates of *Acinetobacter* species other than *A. baumannii*, such as *Acinetobacter* genomic species 13TU and genomic species 16 (Marti *et al.*, 2008a; Martinovich *et al.*, 2008). Since species such as AG3 and 13TU were also associated with clinically important infections (Traub & Bauer, 2000), the development of carbapenem resistance in these species is yet another worrisome development in multidrug resistance within the *Acinetobacter* genus.

The antimicrobial susceptibility results of the 12 isolates can be divided into 4 antibiograms (Table 2). All 12 isolates demonstrated full resistance to ampicillin, amoxicillin–

clavulanate, piperacillin–tazobactam, ceftazidime, meropenem, imipenem and ciprofloxacin, while all isolates had colistin MICs of  $\leq 0.5$  mg l<sup>-1</sup>. Ratios of  $\geq 8$  with the MBL Etest (imipenem MIC versus imipenem + EDTA MIC) were obtained for the 12 isolates, giving presumptive positive results for MBL. Seven AG3 isolates (antibiogram 1) were susceptible to ceftazidime, aminoglycosides and trimethoprim–sulphamethoxazole, and had intermediate or full resistance to cefotaxime and cefepime. Antibiogram 2 (*A. johnsonii*) had a similar profile as antibiogram 3 (three remaining AG3 isolates) but with lower cephalosporin MICs and a higher gentamicin MIC. Of note, all AG3 and *A. johnsonii* isolates had cefepime MICs that were two to eight times higher than those of ceftazidime. The *A. baumannii* isolate was highly resistant to cephalosporins, trimethoprim–sulphamethoxazole and aminoglycosides (antibiogram 4). All AG3 and *A. johnsonii* isolates had tigecycline MICs of  $\leq 0.5$  mg l<sup>-1</sup>, while the *A. baumannii* isolate had a tigecycline MIC of 2 mg l<sup>-1</sup>.

### Carbapenem-resistance genes and their genetic surroundings

The resistance genes of the various isolates are summarized in Table 3. PCR was negative for *bla*<sub>VIM</sub>, *bla*<sub>IMP</sub>, *bla*<sub>SPM</sub>, *bla*<sub>GIM</sub>, *bla*<sub>SIM</sub>, *bla*<sub>OXA-24-like</sub> and *bla*<sub>OXA-58</sub>. False-positive results were thus obtained with the MBL Etest, which serves as a reminder that PCR confirmation of such results is still required as OXA carbapenemases can produce false-positive MBL results (Segal & Elisha, 2005). To date, there is still no reliable phenotypic method distinguishing MBLs from OXA carbapenemases.

**Table 2.** Antimicrobial susceptibility patterns of the carbapenem-resistant isolates

Susceptibility was determined according to CLSI interpretation criteria (CLSI, 2006). I, intermediate; R, Resistant; S, susceptible.

Antimicrobial agent	Antibiogram 1 (MIC value)	Antibiogram 2 (MIC value)	Antibiogram 3 (MIC value)	Antibiogram 4 (MIC value)
Cefotaxime	I/R (32–64 mg l <sup>-1</sup> )	I (16 mg l <sup>-1</sup> )	R (128–≥256 mg l <sup>-1</sup> )	R (≥256 mg l <sup>-1</sup> )
Ceftazidime	S (4–8 mg l <sup>-1</sup> )	S (8 mg l <sup>-1</sup> )	I/R (16–32 mg l <sup>-1</sup> )	R (≥256 mg l <sup>-1</sup> )
Cefepime	I/R (16–64 mg l <sup>-1</sup> )	R (32 mg l <sup>-1</sup> )	R (64–128 mg l <sup>-1</sup> )	R (≥256 mg l <sup>-1</sup> )
Trimethoprim–sulfamethoxazole	S (≤1/19 mg l <sup>-1</sup> )	R (≥32/608 mg l <sup>-1</sup> )	R (≥32/608 mg l <sup>-1</sup> )	R (≥32/608 mg l <sup>-1</sup> )
Gentamicin	S (0.25–1 mg l <sup>-1</sup> )	R (128 mg l <sup>-1</sup> )	R (32 mg l <sup>-1</sup> )	R (≥256 mg l <sup>-1</sup> )
Amikacin	S (0.5–2 mg l <sup>-1</sup> )	S (1 mg l <sup>-1</sup> )	S (0.5–2 mg l <sup>-1</sup> )	R (≥256 mg l <sup>-1</sup> )
Types of isolates (no.)	AG3 (7)	<i>A. johnsonii</i> (1)	AG3 (3)	<i>A. baumannii</i> (1)
Isolate identifier	S659, A629, S448, S748, S165, A594, A616	A868	A540, A277, A357	U437

*A. baumannii* was positive for both *bla*<sub>OXA-23-like</sub> and *bla*<sub>OXA-51-like</sub> genes, while the other 11 isolates were positive for the *bla*<sub>OXA-23-like</sub> gene only. Nucleotide sequencing of the *bla*<sub>OXA-23-like</sub> amplicons revealed that all 12 isolates carried the classical *bla*<sub>OXA-23</sub> gene, which has also been reported in various continents including Europe, Asia and America (Coelho *et al.*, 2006; Corvec *et al.*, 2007; Meric *et al.*, 2008; Valenzuela *et al.*, 2007; Villegas *et al.*, 2007; Zong *et al.*, 2008). In addition, the insertion sequence *ISAbal* was found immediately upstream of *bla*<sub>OXA-23</sub> in all 12 isolates (separated by 17 nucleotides); while the ATPase-encoding gene was found downstream of it. The regions between *ISAbal* and *bla*<sub>OXA-23</sub> of all 12 isolates were identical in sequence, as were the regions between *bla*<sub>OXA-23</sub> and ATPase-encoding gene (104 nucleotides in length). *ISAbal* was not present upstream of the *bla*<sub>OXA-51-like</sub> gene in the *A. baumannii* isolate. Hence the  $\beta$ -lactamase-encoding gene contributing to carbapenem resistance for

all 12 isolates from St James's Hospital was *bla*<sub>OXA-23</sub>, since *ISAbal* was shown to possess promoter sequences that upregulated expression of the  $\beta$ -lactamase-encoding genes downstream (Corvec *et al.*, 2007; Héritier *et al.*, 2006). Additional mechanisms of resistance that have not been investigated in this study, such as loss of membrane porins and multidrug efflux pump, may also play important roles in the development of carbapenem resistance (Dupont *et al.*, 2005; Magnet *et al.*, 2001; Siroy *et al.*, 2005).

### Other resistance genes

No Ambler class A  $\beta$ -lactamase-encoding genes (*bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M</sub>, *bla*<sub>VEB</sub> and *bla*<sub>PER</sub>) were detected by PCR. The Ambler class C ADC-encoding genes of the AG3 and *A. baumannii* isolates were sequenced. All 10 AG3 isolates were positive for *bla*<sub>ADC-18</sub> (>99% concordance with GenBank accession number AM283523), while the *A.*

**Table 3.** Resistance genes of carbapenem-resistant isolates

Isolate identifier	Species	Resistance gene/cassette encoding:			
		OXA	ADC	Aminoglycoside-modifying enzymes	Class 1 integron
A540	AG3	<i>ISAbal</i> + OXA-23	ADC-18	<i>ISAbal</i> + <i>aacC2</i>	–
S659	AG3	<i>ISAbal</i> + OXA-23	ADC-18	–	–
A629	AG3	<i>ISAbal</i> + OXA-23	ADC-18	–	–
S448	AG3	<i>ISAbal</i> + OXA-23	ADC-18	–	–
S748	AG3	<i>ISAbal</i> + OXA-23	ADC-18	–	–
A594	AG3	<i>ISAbal</i> + OXA-23	ADC-18	–	–
A616	AG3	<i>ISAbal</i> + OXA-23	ADC-18	–	–
A277	AG3	<i>ISAbal</i> + OXA-23	ADC-18	<i>ISAbal</i> + <i>aacC2</i>	–
A357	AG3	<i>ISAbal</i> + OXA-23	ADC-18	<i>ISAbal</i> + <i>aacC2</i>	–
S165	AG3	<i>ISAbal</i> + OXA-23	ADC-18	–	–
U437	<i>A. baumannii</i>	OXA-51 group; <i>ISAbal</i> + OXA-23	<i>ISAbal</i> + ADC-25	<i>aphA6</i>	+
A868	<i>A. johnsonii</i>	<i>ISAbal</i> + OXA-23	??	<i>aacC2</i>	–

+, Present; –, absent

\*PCR product not obtained with study primers.

*baumannii* isolate was positive for *bla*<sub>ADC-25</sub> (>99% concordance with GenBank accession number EF016355). We were unable to obtain an amplicon for the *A. johnsonii* isolate with the *bla*<sub>ADC</sub> primers used in this study. Since OXA-23 has little activity on third and fourth generation cephalosporins, the ADC enzymes are responsible for hydrolysing these  $\beta$ -lactams. The cephalosporin susceptibility profiles (where cefepime MICs were higher than ceftazidime MICs) of the AG3 isolates were different from those of most Ambler class C enzymes of other Gram-negative organisms as well as some ADC enzymes such as ADC-7 (Hujer *et al.*, 2005). This suggests that different ADC subtypes may have different affinities for the various cephalosporins. *ISAbal* was found upstream of the *A. baumannii bla*<sub>ADC-25</sub>, but not of the *bla*<sub>ADC-18</sub> of the AG3 isolates.

The *A. baumannii* isolate was the only isolate to possess a class 1 integron and also carried the aminoglycoside phosphotransferase-encoding gene *aphA6* (99% concordance with GenBank accession number X07753), which accounted for the resistance to amikacin as well as gentamicin. The three gentamicin-resistant AG3 isolates were positive for the aminoglycoside-acetyltransferase-encoding gene *aacC2* (100% concordance with GenBank accession number AY138987). *ISAbal* was found upstream of the *aacC2* gene in all three isolates, with a truncated 3' end of insertion sequence IS1133 found between *ISAbal* and *aacC2* (GenBank accession number EU839488). The *aacC2* gene was also found in the *A. johnsonii* isolate, but without *ISAbal* upstream of it. This study demonstrates the role of *ISAbal* in the regulation of various  $\beta$ -lactamase-encoding genes in *A. baumannii* and also in other *Acinetobacter* species. Not only was it found upstream of the *bla*<sub>OXA-23</sub> of various species, but was also found upstream of the *bla*<sub>ADC-25</sub> gene in *A. baumannii*. Upregulation of *bla*<sub>ADC-25</sub> was the most likely explanation for the higher cephalosporin MICs of *A. baumannii* in contrast to those of the AG3 isolates, which did not possess *ISAbal* upstream of *bla*<sub>ADC-18</sub>. The role of *ISAbal* in the regulation of genes encoding aminoglycoside-modifying enzymes was less clear. Gentamicin MICs of AG3 isolates possessing *ISAbal* upstream of *aacC2* were lower than MICs observed in *A. baumannii* and *A. johnsonii* where *ISAbal* was absent upstream of *aphA6* and *aacC2*, respectively. This would suggest that *ISAbal* did not upregulate the expression of such genes. Other mechanisms of resistance may also have contributed to aminoglycoside resistance in our isolates. In particular, overexpression of multidrug efflux pumps such as AdeABC confers resistance to aminoglycosides and several other classes of antimicrobial agents in *A. baumannii* (Magnet *et al.*, 2001).

### Epidemiological typing and plasmid studies

The ten AG3 isolates were divided into two genotype groups according to their PFGE banding patterns. Group 1 contained seven isolates S659, A629, A616, A594, S448,

S748 and S165 that had  $\geq 85\%$  similarity and therefore were closely related. Within this group isolates S448 and S748 were clonally related with identical PFGE patterns. In group 2, isolates A540, A277 and A357 had indistinguishable PFGE patterns and hence were clonal. The two groups had <70% similarity to each other and therefore were not related. PFGE groups 1 and 2 corresponded closely with antibiogram patterns 1 and 3, respectively (Table 2).

Four isolates [*A. baumannii* (U437), *A. johnsonii* (A868), and representative AG3 isolates of the two PFGE clusters ([A357 and S448])] were used for the plasmid studies. PCRs of plasmid preparations for all four isolates were positive for *bla*<sub>OXA-23</sub>, while the PCR for the *rpoB* gene was negative for all four isolates. This indicated that the plasmid preparations were free of chromosomal contamination and that *bla*<sub>OXA-23</sub> was plasmid borne for the four isolates. Following gel electrophoresis of the plasmid preparations, *A. johnsonii* and AG3 (isolate A357) demonstrated two plasmid bands of about 40 MDa and about 95 MDa. No bands could be visualized for *A. baumannii* and the other AG3 isolate (S448), which might be the result of lower copy numbers within the preparations. The plasmid bands from the former two isolates were eluted and PCR was positive for *bla*<sub>OXA-23</sub> for the 40 MDa bands in both isolates, implying that *bla*<sub>OXA-23</sub> was carried on the 40 MDa plasmids in these isolates. Thus, while PFGE demonstrated the two different clones and closely related isolates of carbapenem-resistant AG3, plasmid studies also revealed that plasmidic transfer of *bla*<sub>OXA-23</sub> between different *Acinetobacter* species (at least between AG3 and *A. johnsonii*) most probably occurred in our institution as well. This is further supported by the similarities of the genetic surroundings of *bla*<sub>OXA-23</sub> among the different species. This presents a worrying prospect that multiple mechanisms, such as inter-species plasmidic transfer and to a lesser degree, clonal spread, are likely to be involved in promoting the spread of carbapenem resistance within the *Acinetobacter* population in our hospital.

### Clinical and epidemiological data

The microbiological data of the isolates, and the clinical and demographic data of the respective patients are summarized in Table 4. Carbapenem-resistant *Acinetobacter* were isolated from a variety of specimens including sputum, urine, swabs and third-space fluids. There was a male-to-female patient ratio of 2:1 and most were hospital in-patients for longer than 2 weeks when culture-positive. The majority of the patients were infected with the organisms and most had significant co-morbidities and/or major surgery. Epidemiologically, the patients were located in a wide variety of settings, not just the critical-care settings. Most notably, the *A. baumannii* isolate was isolated from a patient from the community, although it was unclear whether there was a history of prior

**Table 4.** Clinical data for patients with carbapenem-resistant *Acinetobacter* isolates

Isolate	Specimen data			Patient data						
	Organism	Date of isolation (month/year)	Specimen type	Age (years)	Sex	Location	Duration of stay in hospital*	Infection status of organism	Recent anti-Gram-negative antibiotics	Underlying condition
S659	AG3	05/05	Sputum	49	M	ICU	19 days	Colonized	Mer, Cip	Crohn's disease; pancolectomy
A540	AG3	08/05	Biliary fluid	62	F	Surgical ward A	37 days	Infected	Ptz, Cip	Pancreatic carcinoma; gastrojejunostomy
A629	AG3	03/06	Biliary fluid	71	F	Surgical ward B	15 days	Infected	Aug, Cip	Pancreatic carcinoma; biliary tract surgery
A868	<i>A. johnsonii</i>	05/06	Chest drain fluid	44	M	Medical ward A	61 days	Infected	Cip	Liver failure, recurrent empyema
A616	AG3	06/06	Wound swab	44	M	ICU	29 days	Infected	Ptz, Cip	Severe trauma, rhabdomyolysis, viral hepatitis
A594	AG3	07/06	Leg wound	49	M	Surgical ward C	22 days	Colonized	Pen, Clox	Lower limb cellulitis
S448	AG3	08/06	Sputum	56	F	Oncology ward	58 days	Infected	Mer, Cip	Oesophageal carcinoma, diabetes mellitus
S748	AG3	09/06	Sputum	73	M	Oncology ward	5 days†	Colonized	Aug, Cip	Lung carcinoma
A357	AG3	10/06	Leg swab	62	M	ICU	24 days	Colonized	Ptz, Cip	Abdominal surgery, cardiovascular disease
A277	AG3	12/06	Leg swab	57	M	Surgical ward D	33 days	Colonized	Cip	Major trauma and surgery
S165	AG3	05/07	Sputum	64	M	Medical ward B	27 days	Infected	Ptz, Cip	Wegener's granulomatosis, diabetes mellitus
U437	<i>A. baumannii</i>	08/07	Urine	71	F	Community	NA	Infected	Data unavailable	Data unavailable

Aug, Amoxicillin–clavulanate; Cip, ciprofloxacin; Mer, meropenem; NA, not applicable; Pen, penicillin; Ptz, piperacillin–tazobactam.

\*Duration from time of admission to the time of organism isolation in the current patient episode.

†Transferred from another hospital.

hospitalization in the patient. However, there was also some degree of clonal persistence within the institution. AG3 isolates S448 and S748 were isolated in the oncology ward a month apart; while A357 and A277 were isolated 2 months apart in the ICU and surgical ward, respectively, although the patient with isolate A277 had been in the ICU prior to transfer to the surgical ward (ward transfer data are not included in Table 4). Nonetheless, the sporadic nature and different settings for most of the isolates that emerged, together with the findings that different species and clones were involved, would suggest that there was a reservoir of carbapenem-resistant *Acinetobacter* isolates within the patient population served by the hospital. These organisms might then have manifested following antimicrobial therapy.

Of the 11 patients for whom there were data on antimicrobial therapy prior to the isolation of the organisms, only 2 had prior meropenem treatment. Six had prior therapy with  $\beta$ -lactam/ $\beta$ -lactamase-inhibitor combinations. This raises the possibility of co-selection of carbapenem-resistant isolates by these antimicrobial agents through the acquisition of OXA carbapenemases, since these enzymes possess greater activity against these agents (as well as the carbapenems) than the Ambler class C *Acinetobacter*-derived cephalosporinases. As ten patients had prior therapy with ciprofloxacin, it may also have a role in selecting out quinolone-resistant isolates concomitantly carrying the OXA-carbapenemase-encoding genes or isolates overexpressing multidrug efflux pumps with resistance to multiple classes of antimicrobial agents. These observations emphasize the importance of the prudent use of all antimicrobial agents, as non-carbapenem agents may have a role in promoting the emergence of carbapenem-resistant *Acinetobacter* isolates.

There are now mounting data to suggest that the presence of *Acinetobacter* can contribute adversely to the prognosis of patients, especially in the presence of significant comorbidities (Grupper *et al.*, 2007; Murray & Hospenthal, 2008), and multidrug resistance in such isolates is associated with increased mortality and prolonged hospitalization (Lee *et al.*, 2007; Sunenshine *et al.*, 2007). Furthermore, since carbapenems are frequently used for the treatment of such multidrug-resistant *Acinetobacter* infections, carbapenem resistance can further contribute to mortality as a result of discordant empirical therapy (Kwon *et al.*, 2007). In view of such findings, there is now an even greater urgency for resistance surveillance of such organisms as well as the prudent use of antimicrobial agents.

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