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Molecular characterisation of carbapenem resistance of *Acinetobacter* species in an Irish tertiary care hospital.

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January 2010

Declaration

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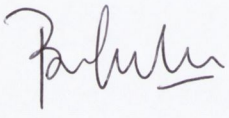

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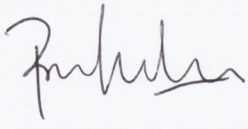

Teck Wee Boo, May 2010.

To Eva and Moira

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Publications

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Boo, T.W., Walsh, F. & Crowley, B. (2009). Molecular characterisation of carbapenem-resistant *Acinetobacter* species in an Irish university hospital: predominance of *Acinetobacter* genomic species 3. *Journal of Medical Microbiology* 58: 209-216.

Boo, T.W. & Crowley, B. (2009). Detection of $bla_{\text{OXA-58}}$ and $bla_{\text{OXA-23-like}}$ genes in carbapenem-susceptible *Acinetobacter* clinical isolates: should we be concerned? *Journal of Medical Microbiology* 58: 839-841.

Summary

This study was undertaken with the primary aim of characterising the molecular epidemiology and mechanisms of resistance to carbapenems in *Acinetobacter* isolates collected at St. James's Hospital, Dublin.

A total of 117 *Acinetobacter* isolates were prospectively collected over a 32-month period from May 2005 to December 2007. Identification of the isolates using VITEK 2 GNI phenotypic method and *rpoB* sequencing method showed the overall error rate of identification at the species level by VITEK 2 method to be 74.4%. However, the VITEK 2 method was highly sensitive and specific (100% and 100%, respectively) in delineating isolates into *Acinetobacter calcoaceticus-baumannii* complex (Acbc) or otherwise. The study highlighted the limitations of phenotypic methods in accurately identifying *Acinetobacter* spp., which could impact on infection control such as the early identification of outbreaks. Using *rpoB* sequencing, *Acinetobacter* genospecies 3 was found to be the predominant *Acinetobacter* species in this hospital. This finding reiterated the clinical importance of this species. Further molecular typing using pulsed-field gel electrophoresis revealed predominantly polyclonal origins of *Acinetobacter baumannii* and *Acinetobacter* genospecies 3 isolates in this study.

This study found the Etest method of susceptibility testing to have very good correlation to the disc diffusion method (97.4% - 100%), while the VITEK 2 method had lower levels of category agreement with the latter method (87.2% - 100%). In particular, an unacceptably high rate of very major errors (11.1%) was found for piperacillin-tazobactam testing by VITEK 2 method. Determination of tigecycline susceptibility by Etest method was also found in the study to be influenced by the

choice of test medium. Only 23.9% of the isolates were susceptible to cefotaxime, while susceptibility rates for ceftazidime, cefepime and ciprofloxacin were 65.0%, 75.2% and 79.5%, respectively. Carbapenem resistance in the *Acinetobacter* isolates was 10.3%, but was 14.1% within the Acbc category. In addition, only 82.9% of all isolates were susceptible to tigecycline.

Carbapenem resistance was found in 12 isolates of different species (*A. baumannii*, *Acinetobacter* genospecies 3 and *A. johnsonii*). The OXA carbapenemase gene $bla_{\text{OXA-23}}$ was found in all 12 isolates, and each had the insertion sequence IS*Aba1* upstream of $bla_{\text{OXA-23}}$. Subsequent qRT-PCR assays of $bla_{\text{OXA-23}}$ expression also demonstrated the constitutive nature of its expression. The findings further supported earlier studies that IS*Aba1* played an important role in the regulation of β-lactamase expression in *Acinetobacter* spp. Plasmid experiments in this study also showed that $bla_{\text{OXA-23}}$ in the carbapenem-resistant isolates was plasmid-borne. Interestingly the transfer of this gene might have occurred between *Acinetobacter* genospecies 3 and *A. johnsonii* as well as between different *Acinetobacter* genospecies 3 strains, since the study found the plasmids carrying $bla_{\text{OXA-23}}$ in the *A. johnsonii* and *Acinetobacter* genospecies 3 strains were all of the same size (40 MDa). This highlighted the different potential modes of transmission of resistance genes among *Acinetobacter* isolates. In addition, $bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-23-like}}$ and $bla_{\text{OXA-58}}$ genes were found in carbapenem-susceptible isolates of *A. baumannii*, *A. radioresistens* as well as *Acinetobacter* genospecies 9 and 10, respectively. This finding showed the potential threat of clinically silent reservoirs of resistance genes. Hence, apart from the therapeutic challenges associated with multidrug-resistant *Acinetobacter* isolates, it is

also particularly challenging to control the dissemination of resistance genes among susceptible and resistant isolates.

Furthermore, through the use of efflux pump inhibitor PAβN and quantitative reverse transcriptase-PCR (qRT-PCR), it was likely that efflux pump mechanisms also played significant roles in meropenem resistance in *Acinetobacter* spp. There were four-fold to eight-fold reductions in meropenem MICs of isolates following exposure to PAβN. The qRT-PCR assay results suggested that resistance-nodulation-division efflux pumps AdeABC and AdeDE had significant roles in meropenem resistance in *A. baumannii* and *Acinetobacter* genospecies 3, respectively. Following exposure to meropenem, *adeB* expression in *A. baumannii* isolates increased by 46% to 73% while *adeE* expression in *Acinetobacter* genospecies 3 isolates increased by 17% to 34%. In addition, CarO outer membrane porin might have a role in carbapenem resistance in *A. baumannii*, as its expression was absent or reduced in isolates with reduced carbapenem susceptibility. Hence, multiple mechanisms were most probably involved in conferring carbapenem resistance in *Acinetobacter* spp.

Acinetobacter spp. represents the paradigm of Gram-negative antimicrobial resistance. In an era of escalating antimicrobial resistance and diminishing options to counter such developments, there is an ever greater urgency to utilise all available measures in infection control, antimicrobial stewardship, diagnostic methodology, therapeutic intervention and scientific research in tackling formidable pathogens such as *Acinetobacter* spp.

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Abbreviations

ABC	ATP-binding cassette
Acbc	*Acinetobacter calcoaceticus-baumannii* complex
AFLP	Amplified fragment length polymorphism
AST	Antimicrobial susceptibility testing
BSAC	British Society of Antimicrobial Chemotherapy
BSI	Bloodstream infection
CCCP	Carbonyl-cyanide *m*-chlorophenylhydrazone
CLSI	Clinical and Laboratory Standards Institute
C_T	Cycle threshold
Da	Daltons
DNA	Deoxyribonucleic acid
EDTA	Ethylene-diamine-tetraacetic acid
EPI	Efflux pump inhibitor
ESBL	Extended-spectrum β-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GNB	Gram-negative bacillus / bacilli
HAI	Hospital-associated infection
ICU	Intensive care unit
IEF	Isoelectric focusing
IS	Insertion sequence
LPS	Lipopolysaccharides
MATE	Multidrug and toxic compound extrusion
MBL	Metallo-β-lactamase
MDR	Multidrug-resistance / Multidrug-resistant
ME	Major error(s)
mE	Minor error(s)
MFS	Major facilitator superfamily
MIC	Minimal inhibitory concentration
MLST	Multi-locus sequence typing
MRSA	Meticillin-resistant *Staphylococcus aureus*
NMP	N-methylpyrrolidone

OMP	Outer membrane protein
PAβN	Phenyl-arginine-β-naphthylamide
PAGE	Polyacrylamide gel electrophoresis
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
RNA	Ribonucleic acid
RND	Resistance-nodulation-division
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS	Sodium dodecyl-sulphate
SMR	Small multidrug resistance
spp.	Species (plural)
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TIFF	Tagged image file format
UK	United Kingdom
US	United States
UTI	Urinary tract infection
VAP	Ventilator-associated pneumonia
VME	Very major error(s)
VRE	Vancomycin-resistant enterococci

Chapter 1

General introduction

1.1 History and microbiology of *Acinetobacter*

1.1.1 History of the genus *Acinetobacter*

In 1911, a Dutch microbiologist Beijerinck described an organism named *Micrococcus calcoaceticus* isolated from soil by enrichment in a calcium-acetate-containing minimal medium (Beijerinck, 1911). Over the next few decades, similar organisms were assigned to various genera and species, including *Diplococcus mucosus*, *Alcaligenes haemolysans*, *Mima polymorpha*, *Moraxella lwoffii* and *Achromobacter anitratus* (Henriksen, 1973).

The current designation of *Acinetobacter* (which is derived from the Greek word 'akinetos' meaning non-motile) was proposed by Brison and Prevot to separate non-motile from motile organisms within the genus *Achromobacter* (Brison & Prevot, 1954). In 1968, Baumann *et al.* published a comprehensive survey unifying various species under a single genus of *Acinetobacter*, and concluded that further subclassification into different species based on phenotypic characteristics was unreliable (Baumann *et al.*, 1968).

1.1.2 Taxonomy of *Acinetobacter*

The genus *Acinetobacter* comprises Gram-negative, strictly aerobic, non-fermentative, non-motile, catalase-positive and oxidase-negative bacteria with a DNA

G+C content of between 39% and 47%. The genus *Acinetobacter* is currently classified in the family *Moraxellaceae* within the order of *Gammaproteobacteria*, which includes the genera *Moraxella*, *Acinetobacter* and *Psychrobacter* (Rossau *et al.*, 1991).

Until the 1980s, as a result of insufficient identification criteria, the customary practice has been to regard all *Acinetobacter* isolates as members of a single species, *Acinetobacter calcoaceticus*. A major breakthrough was achieved in 1986 when Bouvet and Grimont delineated the genus into 12 DNA groups (or genospecies) using DNA-DNA hybridisation studies (Bouvet & Grimont, 1986; see Table 1-1). In 1989, Tjernberg and Ursing, using DNA-DNA hybridisation, reported that most of their *Acinetobacter* strains could be identified as members of the DNA groups previously described by Bouvet and Grimont, along with three new DNA groups, numbered 13 through 15 (Tjernberg & Ursing, 1989). However, they could not distinguish between DNA groups 8 and 9, hence DNA group 9 was omitted from their system. Within the same year, Bouvet and Jeanjean also reported five new DNA groups which were numbered 13 through 17 (Bouvet & Jeanjean, 1989). Further confusion arose when it was discovered that DNA group 13 in the Bouvet and Jeanjean study and DNA group 14 in the Tjernberg and Ursing study were synonymous. For clarification, most investigators now include the initials of the investigators of the above studies to the respective DNA groups, for example, DNA group 13TU or DNA group 15BJ. More recently, 10 additional species were described using DNA-DNA hybridisation and 16S rDNA sequencing methods. They include three species of human origin, *A.*

Table 1-1. Taxonomy of *Acinetobacter* species.

	1986[1]	1989[2]	1989[3]	2001[4]	2003[5]	2003[6]
	***Acinetobacter* DNA groups:**					
1)	1 (*A. calcoaceticus*)	1				
2)	2 (*A. baumannii*)	2				
3)	3	3				
4)	4 (*A. haemolyticus*)	4				
5)	5 (*A. junii*)	5				
6)	6	6				
7)	7 (*A. johnsonii*)	7				
8)	8 (*A. lwoffii*)	8/9				
9)	9					
10)	10	10				
11)	11	11				
12)	12 (*A. radioresistens*)	12				
13)		13TU				
14)		14TU	13BJ			
15)		15TU				
16)			14BJ			
17)			15BJ			
18)			16			
19)			17			
20)				*A. ursingii*		
21)				*A. schindleri*		
22)					*A. baylyi*	
23)					*A. bouvetii*	
24)					*A. gerneri*	
25)					*A. grimontii*	
26)					*A. tandoii*	
27)					*A. tjernbergiae*	
28)					*A. towneri*	
29)						*A. parvus*

[1]Bouvet & Grimont, 1986
[2]Tjernberg & Ursing, 1989
[3]Bouvet & Jeanjean, 1989
[4]Nemec *et al.*, 2001
[5]Carr *et al.*, 2003
[6]Nemec *et al.*, 2003

ursingii, *A. schindleri* and *A. parvus*, as well as seven species from activated sludge, namely *A. baylyi*, *A. bouvetii*, *A. grimontii*, *A. tjernbergiae*, *A. towneri*, *A. tandoii and A. gerneri* (Carr *et al.*, 2003, Nemec *et al.*, 2001; Nemec *et al.*, 2003).

The taxomony and nomenclature of *Acinetobacter* is still evolving. Recently, more novel species such as *A. soli*, *A. beijerinckii* and *A. gyllenbergii* have been added to the ever increasing number species within this genus (Kim *et al.*, 2008; Nemec *et al.*, 2009).

1.1.3 Speciation within the genus

Acinetobacter can be identified presumptively to the genus level as Gram-negative, catalase-positive, oxidase-negative, non-motile and non-fermentative coccobacilli. They are short, plump, Gram-negative rods that are difficult to destain and may be misidentified as either Gram-negative or Gram-positive cocci. *Acinetobacter* species generally grow well on solid media that are routinely used in clinical microbiology laboratories, such as sheep blood agar and tryptic soy agar, at an incubation temperature of 37°C. They form smooth or mucoid colonies that are white, pinkish or grey in colour. Colonies of the *A. calcoaceticus-baumannii* complex resemble those of *Enterobacteriaceae* in size (colony diameter of 1.5 mm to 3 mm after overnight culture), while most other species produce smaller and more translucent colonies. In the stationary phase of growth and on non-selective agars, coccobacillary forms

predominate, while early growth in fluid media and growth on plates in the presence of cell-wall-active antibiotics yield mostly rods (Bergogne-Bérézin & Towner, 1996).

Various methods have been used for the speciation of *Acinetobacter* genus. Phenotypic methods based on biochemical reactions are commonly used for the identification of organisms. A phenotypic identification scheme based on 28 tests was also proposed for the speciation of *Acinetobacter*, but the scheme could not distinguish between *A. baumannii* and *Acinetobacter* genospecies 13TU, or between *A. calcoaceticus* and genospecies 3 (Bouvet & Grimont, 1986). Further simplification of phenotypic tests based on growth temperature and carbon source assimilation tests was attempted, but could only identify 78% of isolates (Gerner-Smidt *et al.*, 1991). Manual and automated commercial identification systems such as API 20NE, Biolog, VITEK 2 systems are also available for the identification of *Acinetobacter* spp. They are based largely on biochemical tests such as carbon source assimilation tests (Bergogne-Bérézin & Towner, 1996). However, the substrates in the phenotypic tests commonly used in diagnostic laboratories for bacterial species identification have not been tailored specifically for adequate distinction between *Acinetobacter* spp. (Bernards *et al.*, 1995; Bernards *et al.*, 1996). In addition, the databases of the systems contain limited numbers of species. As a result, problems of sensitivity, specificity and reproducibility have been encountered in the identification of *Acinetobacter* spp. using phenotypic tests, and unambiguous speciation of even the commonest *Acinetobacter* spp. may not be achieved (Bergogne-Bérézin & Towner, 1996;

Bernards *et al.*, 1996; Gerner-Smidt *et al.*, 1991; Joyanes *et al.*, 2001; Peleg *et al.*, 2008).

The gold standard of delineation of *Acinetobacter* isolates into the respective species is currently based on DNA-DNA relatedness using several different DNA hybridisation methods such as nitrocellulose filter method, S1 endonuclease method, hydroxyapatite method and quantitative bacterial dot filter method (Bergogne-Bérézin & Towner, 1996; Dijkshoorn *et al.*, 2007). Moreover, DNA-DNA hybridisation methods remain the mainstay of diagnostic methods in the characterisation of new species within the *Acinetobacter* genus (Carr *et al.*, 2003; Kim *et al.*, 2008; Nemec *et al.*, 2009; Nemec *et al.*, 2001; Nemec *et al.*, 2003). However, DNA-DNA hybridisation is far too laborious and time-consuming for the routine use in diagnostic laboratories. Other molecular methods have thus been developed and validated for identification of *Acinetobacter* spp., such as amplified ribosomal DNA restriction analysis (ARDRA) (Dijkshoorn *et al.*, 1998; Koeleman *et al.*, 1998; Vaneechoutte *et al.*, 1995), amplified fragment length polymorphism (AFLP) (Janssen *et al.*, 1997; Koeleman *et al.*, 1998; Nemec *et al.*, 2001), tRNA spacer fingerprinting (Ehrenstein *et al.*, 1996) and sequencing of the *rpoB* gene and its flanking spacers (La Scola *et al.*, 2006).

ARDRA and AFLP analyses are currently the most widely used methods for identification of most *Acinetobacter* spp. In ARDRA, PCR amplification of the 16S rRNA gene is performed and subjected to endonuclease restriction by at least five

restriction enzymes, of which *Alu*I, *Cfo*I, *Mbo*I, *Rsa*I and *Msp*I are the most commonly utilised (Dijkshoorn *et al.*, 1998; Koeleman *et al.*, 1998; Vaneechoutte *et al.*, 1995). Restriction fragment patterns are then analysed by gel electrophoresis. Gels are photographed and patterns are compared visually, while calculation of molecular sizes of restriction fragments is performed with specific software such as GelCompar software (Applied Maths, Kortrijk, Belgium) (Koeleman *et al.*, 1998; Vaneechoutte *et al.*, 1995). There are several disadvantages of ARDRA. It is laborious and time-consuming, and also requires the use of at least five endonucleases for restriction analysis as well as the need for specialised software for accurate analysis of restriction patterns (Dijkshoorn *et al.*, 1998; Koeleman *et al.*, 1998; Vaneechoutte *et al.*, 1995). Moreover, ARDRA also lacks discriminatory power between certain genospecies, such as species 4 *versus* 7, species 5 *versus* 17 and species 10 *versus* 11 (Dijkshoorn *et al.*, 1998; Vaneechoutte *et al.*, 1995). Variations in banding patterns of the same species using the same restriction enzymes were also observed, making inter-laboratory comparison of results difficult and unreliable (Koeleman *et al.*, 1998; Dijkshoorn *et al.*, 1998).

The AFLP method involves the digestion with restriction endonucleases *Hind*III and *Taq*I followed by ligation of restriction half-site-specific adaptors to all restriction fragments (Janssen *et al.*, 1997). Selective PCR amplification of restriction fragments is performed and amplified fragments are analysed by gel electrophoresis. Gels are digitally scanned and processed using specific software such as GelCompar software

(Applied Maths). Images are normalised using *A. calcoaceticus* type strain ATCC 23055 as the reference strain. Comparison of AFLP banding patterns is performed with the software, and the similarity between pairs and clustering of strains are calculated with Pearson correlation coefficient and unweighted-pair group method with arithmetic averages, respectively (Janssen *et al.*, 1997). While AFLP has been found to have higher discriminatory power than ARDRA, it is also laborious and time-consuming (Koeleman *et al.*, 1998). The requirement for specialised equipment and analytical software also makes this method rather costly (Janssen *et al.*, 1997; Koeleman *et al.*, 1998).

DNA-sequence analysis is increasingly used as a method to assess microbial relationships such as the different species within a genus. The main advantages are the relative simplicity in methodology and the generation of universally comparable data through the use of sequence databases such as GenBank® (Dijkshoorn *et al.*, 2007). 16S rDNA sequencing was investigated as a method of speciation of *Acinetobacter* isolates (Ibrahim *et al.*, 1997). However, discrepancies with previous DNA-DNA hybridisation data were found whereby some unrelated species were found to have high 16S rDNA sequence similarities while conversely, closely-related species were found in different branches of the 16S rDNA phylogenetic tree. This would suggest potential inadequacy of 16S rDNA sequence analysis as a method of *Acinetobacter* speciation (Ibrahim *et al.*, 1997).

More recently, the sequencing of parts of *Acinetobacter rpoB* gene and the flanking spacer regions has enabled accurate delineation of most genomic species compared with previous DNA-DNA hybridisation data (La Scola *et al.*, 2006). Following PCR amplification of *rpoB* or the spacer regions, sequencing of the amplicon is performed. The degree of similarity of the sequencing results with existing data in sequence databases such as GenBank® (www.ncbi.nlm.gov/GenBank) can be compared using sequence alignment programmes such as the Basic Local Alignment Search Tool (BLAST) programme of the National Center of Biotechnology Information (www.blast.ncbi.nih.gov). This DNA-sequence-based method is relatively rapid, inexpensive and uncomplicated. The requirement for specific equipment and analytical software is also less than the ARDRA and AFLP methods. Comparison of sequencing results with data from other sources can be performed expediently via the internet. Moreover, it has the advantage of validation of method for the more recently reported species such as *A. ursingii*, *A. parvus* and *A. baylyi* (see Table 1-1) (La Scola *et al.*, 2006). Validation of this partial *rpoB* sequencing method on 86 reference *Acinetobacter* strains of various species produced impressive results with 100% correct identification of strains and with 98.9% - 100% intra-species relatedness (Gundi *et al.*, 2009). Further work on 99 *Acinetobacter* strains also demonstrated the very low level of *rpoB* sequence variance of 0% - 2.6% from the respective type strains (Gundi *et al.*, 2009). Hence partial *rpoB* sequencing has been shown to be an accurate method of speciation of *Acinetobacter* isolates. The data generated by the method are universally comparable, and the method also has additional advantages of being less labour-intensive and less time-consuming. However, all the above

molecular methods are still too specialised or laborious to be applied routinely in most diagnostic microbiology laboratories, and their use is currently confined mainly to reference or research laboratories.

1.2 Clinical Aspects of *Acinetobacter* infections

1.2.1 Natural habitats

Many *Acinetobacter* spp. are considered ubiquitous organisms, having been recovered from various sources including soil, vegetables, surface water as well as human clinical specimens (Giamarellou *et al.*, 2008; Peleg *et al.*, 2008). They are often considered part of the human skin flora, since carriage rates of *Acinetobacter* spp. in non-hospitalised individuals were found to be between 10% and 40% (Berlau *et* al., 1999; Giamarellou *et al.*, 2008; Seifert *et al.*, 1997). The most frequently isolated species were *A. lwoffii*, *A. johnsonii*, *A. junii* and *Acinetobacter* genospecies 15BJ (Berlau *et* al., 1999; Seifert *et al.*, 1997). Carriage rate in hospitalised patients was found to be even higher at 75% (Seifert *et al.*, 1997). The clinically significant isolates belonging to the *A. calcoaceticus-baumannii* complex (ie. *A. baumannii*, *Acinetobacter* genospecies 3 and 13TU) are detected at lower frequencies on human skin (Berlau *et al.*, 1999; Giamarellou *et al.*, 2008; Seifert *et al.*, 1997). While most of the species described above are widely distributed in nature and reported in various

samples including environmental, organic and clinical specimens (Dijkshoorn *et al.*, 2007), other species have only been isolated from activated sludge (eg. *A. baylyi*, *A. bouvetii*), soil (*A. soli*) and human specimens (*A. ursingii* and *A. schindleri*) respectively (Carr *et al.*, 2003; Kim *et al.*, 2008; Nemec *et al.*, 2001).

1.2.2 Global epidemiology and clinical significance of *Acinetobacter* species

Most *Acinetobacter* spp. recovered from human clinical specimens have at least been reported as human pathogens, but the majority of *Acinetobacter* infections are due to three species within the *A. calcoaceticus-A. baumannii* complex; namely, *A. baumannii*, *Acinetobacter* genospecies 3 and *Acinetobacter* genospecies 13TU (Dijkshoorn *et al.*, 2007; Peleg *et al.*, 2008; Traub & Bauer, 2000). Other species such as *A. johnsonii* and *A. ursingii* have occasionally been implicated in infections (Dortet *et al.*, 2006; Seifert *et al.*, 1993b; Seifert *et al.*, 1994). While *A. baumannii* is the most commonly reported species in infections and outbreaks worldwide, *Acinetobacter* genospecies 3 has on some occasions been reported to be the predominant species (Tjernberg & Ursing, 1989; Traub & Bauer, 2000).

Multidrug-resistant (MDR) *Acinetobacter* spp. (mainly *A. baumannii*) have been reported from hospitals from most regions of the globe (Giamarellou *et al.*, 2008; Perez *et al.*, 2007). They have spread to cause outbreaks in cities and countries, and

importation of MDR strains from endemic areas (such as the Mediterranean countries) to areas with historically low rates, such as northern Europe, has also occurred (Lolans *et al.*, 2006; Peleg *et al.*, 2006; Perez *et al.*, 2007; Schulte *et al.*, 2005). MDR *Acinetobacter* spp. have also caused infections and outbreaks in personnel involved in military operations in Iraq and Afghanistan (Davis *et al.*, 2005; Scott *et al.*, 2007).

A striking feature of *Acinetobacter* spp., particularly *A. baumannii*, is the propensity for persistence in the hospital setting. They are highly effective in the colonisation of the skin, respiratory tracts and wounds of debilitated hospitalised patients (Bergogne-Bérézin & Towner, 1996; Dijkshoorn *et al.*, 2007). Pili and hydrophobic sugars within the outer membrane lipopolysaccharides (LPS) promote adherence to host cells, facilitating colonisation of skin of susceptible patients and healthcare workers (particularly the hands), formation of biofilm in medical equipment as well as contamination of inanimate surfaces (Bergogne-Bérézin & Towner, 1996; Haseley *et al.*, 1997; Tomaras *et al.*, 2003). They have been found on various hospital equipment including ventilators, mattresses, bed rails, intravenous nutrition equipment and temperature probes (Fournier & Richet, 2006; Giamarellou *et al.*, 2008). In addition, their ability to survive on such inanimate surfaces for as long as weeks or months is probably due to their metabolic versatility and/or high level of resistance to dessication and disinfectants (Bouvet & Grimont, 1986; Fournier & Richet, 2006; Jawad *et al.*, 1998; Wisplinghoff *et al.*, 2007). A recent study on the genomes of *A. baumannii* strains confirmed the presence of numerous genes encoding antiseptic resistance (Fournier *et al.*, 2006). The persistence of *Acinetobacter* spp. in the hospital

environment can thus facilitate the rapid spread among patients via various modalities (Dijkshoorn *et al.*, 2007). It is evident from the number of outbreaks caused by these pathogens that *Acinetobacter* spp. represent an enormous infection control challenge (Coelho *et al.*, 2006; Lolans *et al.*, 2006; Scott *et al.*, 2007; Vila *et al.*, 1999).

Acinetobacter spp. are opportunistic pathogens that mainly affect critically ill patients such as patients in ICUs, trauma or burn units. Predisposing risk factors for developing infection include severe underlying diseases, major surgery or trauma, prolonged hospitalisation especially in ICU, invasive procedures such as mechanical ventilation and indwelling devices such as intravascular and urinary catheters, as well as prior broad-spectrum antimicrobial therapy (Bergogne-Bérézin & Towner, 1996; Dijkshoorn *et al.*, 2007; Fournier & Richet, 2006; Garcia-Garmendia *et al.*, 2001; Garnacho-Montero *et al.*, 2005). In infected patients, several features potentiate the micro-organism's virulence and pathogenicity, including antimicrobial resistance, lipid A-induced cytotoxicity, biofilm formation and iron-acquisition mechanisms (Bergogne-Bérézin & Towner, 1996; Brade *et al.*, 1983; Dorsey *et al.*, 2003; Tomaras *et al.*, 2003).

The combination of the propensity for colonisation of various surfaces, environmental resilience and development of resistance to multiple antimicrobial agents presents significant therapeutic and infection control challenges, and renders them particularly formidable nosocomial pathogens (Giamarellou *et al.*, 2008; Peleg *et al.*, 2008; Perez *et al.*, 2007).

1.2.3 Types of clinical infections

Acinetobacter spp. can cause a wide range of infections. Over 90% of these are hospital-associated infections (HAI) and less than 5% represent community-acquired disease (Rodríguez-Baño *et al.*, 2004). Respiratory tract infections are the commonest type of infections; other infections include soft tissue infections, urinary tract infections (UTIs) and bloodstream infections (BSIs) (Rodríguez-Baño *et al.*, 2004).

1.2.3.1 Hospital-acquired pneumonia

The most frequent and important clinical manifestation of *Acinetobacter* infection is hospital-acquired pneumonia, especially ventilator-associated pneumonia (VAP) (Bergogne-Bérézin & Towner, 1996; Garnacho-Montero *et al.*, 2005). In large US surveillance studies, *A. baumannii* was responsible for 5% to 10% of ICU-acquired pneumonia (Gaynes & Edwards, 2005). *A. baumannii* VAP is associated with prolonged ICU hospitalisation and may carry a 50% mortality rate (Garnacho-Montero *et al.*, 2005; Seifert *et al.*, 1995). Bacteraemic *A. baumannii* pneumonia has a particularly poor prognosis (Cisneros *et al.*, 1996; Seifert *et al.*, 1995).

1.2.3.2 Community-acquired pneumonia

A. baumannii has been reported to be an uncommon cause of community-acquired pneumonia mainly in tropical regions such as Southeast Asia and Australia (Anstey *et*

al., 2002; Leung *et al.*, 2006). *A. baumannii* community-acquired pneumonia is characterised by a fulminant clinical course, secondary bloodstream infection and mortality rate of 40% to 60% (Anstey *et al.*, 2002; Leung *et al.*, 2006). Most cases were associated with significant underlying conditions such as alcoholism, smoking, chronic obstructive pulmonary disease and diabetes mellitus (Anstey *et al.*, 2002; Leung *et al.*, 2006).

1.2.3.3 Bloodstream infections

A. baumannii was the tenth commonest pathogen in a large study of nosocomial BSIs in the US and crude mortality rates range from 16% to 44% (Wisplinghoff *et al.*, 2004). Sepsis and/or septic shock are reported in nearly 20% of patients with bacteraemia, demonstrating the pathogenicity of *Acinetobacter* strains (Valero *et al.*, 2001). The majority of *Acinetobacter* BSIs are due to *A. baumannii*, with typical sources being vascular catheters, pneumonia, urinary tract infections or wound infections (Bergogne-Bérézin & Towner, 1996; Cisneros *et al.*, 1996; Seifert *et al.*, 1995). Male gender, age over 50 years and ICU hospitalisation are risk factors associated with the acquisition of *Acinetobacter* BSIs (Blot *et al.*, 2003; Wisplinghoff *et al.*, 2000). Immunocompromised adults and neonates are also at risk of acquiring such infections (Bergogne-Bérézin & Towner, 1996). Other species such as *A. ursingii* and *A. johnsonii* are less commonly reported and are typically catheter-related and more prevalent in patients with haematological malignancies (Dortet *et al.*, 2006; Seifert *et al.*, 1994; Valero *et al.*, 2001).

1.2.3.4 Infections associated with natural disasters and combat casualties

Wound infection, osteomyelitis and bloodstream infections by *A. baumannii* have been associated with disasters as such as earthquakes, bomb blasts and recent military operations such as those in Afghanistan, Iraq and Kuwait (Davis *et al.*, 2005; Fournier & Richet, 2006; Johnson *et al.*, 2007; Oncül *et al.*, 2002; Scott *et al.*, 2007). During the Vietnam War, *A. baumannii* was also the commonest GNB recovered from traumatic injuries of the extremities (Fournier & Richet, 2006). It is a well-known pathogen in burn units and isolates are commonly multidrug-resistant. Contamination of the environment of field hospitals and cross-transmission in healthcare facilities are thought to be mainly responsible for the acquisition of the pathogen in these settings (Fournier & Richet, 2006; Scott *et al.*, 2007).

1.2.3.5 Other infections

Acinetobacter spp. are occasional causes of catheter-associated urinary tract infection (Gaynes & Edwards, 2005), prosthetic valve endocarditis (Olut & Erkek, 2005) and post-neurosurgical meningitis (Bergogne-Bérézin & Towner, 1996; Metan *et al.*, 2007).

1.2.4 Clinical impact of *Acinetobacter* infections

It has been challenging to determine the true clinical impact of *Acinetobacter* spp (mainly *A. baumannii*) since colonised or infected patients are often critically ill and usually have significant co-morbidities or secondary infective complications. Significant methodological heterogeneity exists between studies, making comparison of results difficult (Peleg *et al.*, 2008). Most studies utilise a matched cohort or case control study design, but definitions for cases and controls are very diverse. The rigour of matching for severity of illness and comorbidities also varies (Peleg *et al.*, 2008).

Even though results are not always consistent, there is mounting evidence that *A. baumannii* infection is associated with significant attributable mortality rates ranging from 7.8% to 43% with about half of the results achieving statistical significance (Albrecht *et al.*, 2006; Blot *et al.*, 2003; Garcia-Garmendia *et al.*, 1999; Garnacho *et al.*, 2003; Grupper *et al.*, 2007; Lortholary *et al.*, 1995). In most of these studies *Acinetobacter* infection is also associated with excess length of hospital and/or ICU stay. In addition, a systemic review of matched cohort and case-control studies also found a statistically significantly higher mortality for patients with A. *baumannii* acquisition (ie. colonised or infected) compared with controls without such acquisition (Falagas *et al.*, 2006). Studies comparing *Acinetobacter* infections with MDR strains versus susceptible strains suggested a trend of increased attributable mortality and increased hospitalisation, although again, some of the results were not statistically significant (Abbo *et al.*, 2007; Sunenshine *et al.*, 2007). Since carbapenems are

frequently used for the treatment of such MDR-*Acinetobacter* infections, carbapenem resistance can further contribute to mortality as a result of discordant empirical therapy (Kwon *et al.*, 2007). Two studies have demonstrated that carbapenem resistance in *Acinetobacter* infections is also associated with increased attributable mortality and prolonged hospitalisation (Kwon *et al.*, 2007; Playford *et al.*, 2007).

There is also evidence that *Acinetobacter* bacteraemia carry equal or even increased mortality compared to bacteraemia caused by other GNB (Robenshtok *et al.*, 2006; Wisplinghoff *et al.*, 2000). Another study using Kaplan-Meier analysis showed a significant increase in mortality with MDR-*A. baumannii* infection or colonisation compared to that with MDR-*P. aeruginosa* infection or colonisation (Gkrania-Klotsas & Hershow, 2006).

Overall, it is reasonable to conclude that there is mounting evidence to suggest that *Acinetobacter* infection has an adverse effect on mortality and length of hospitalisation and may be as severe as those caused by other GNB. Antimicrobial resistance in *Acinetobacter* may be a further factor contributing to mortality and morbidity.

1.3 Antimicrobial agents and resistance mechanisms

1.3.1 Gram-negative cell wall and antimicrobial resistance

The Gram-negative bacterial cell wall presents quite a formidable barrier to the penetration of agents potentially harmful to the bacterium, such as antimicrobial agents. As it has a much more complex structure than that of Gram-positive bacteria, it is thus also more effective in preventing noxious agents such as antibiotics from traversing it. The general structure of the Gram-negative cell wall is shown in Figure 1-1. The outermost layer is the bacterial outer membrane. It provides a strong barrier against action by lysozymes, bile salts and digestive enzymes. It has an asymmetrical structure composed of phospholipids, lipoproteins and lipopolysaccharides (LPS). Phospholipids and lipoproteins are located mainly in the inner layer of the outer membrane while LPS are located in the outer layer of the outer membrane. LPS form 35-45% of the outer membrane and consist of a hydrophobic end of lipid A, an oligosaccharide core and a polar polysaccharide side-chain extending from the bacterial surface (Madigan & Martinko, 2006a). LPS are also known as endotoxins and are potent virulence factors capable of inducing systemic inflammatory response and shock when released in significant amounts in the bloodstream. Transmembrane proteins can aggregate to form water-filled channels known as porins or outer membrane proteins (OMP) which span the entire thickness of the outer membrane. They allow small molecules with molecular weight of less than 600 Da to traverse an otherwise relatively impermeable outer membrane. In most Gram-negative bacilli (GNB), porins are proteins of 35,000 to 40,000 Da. The periplasmic space lies

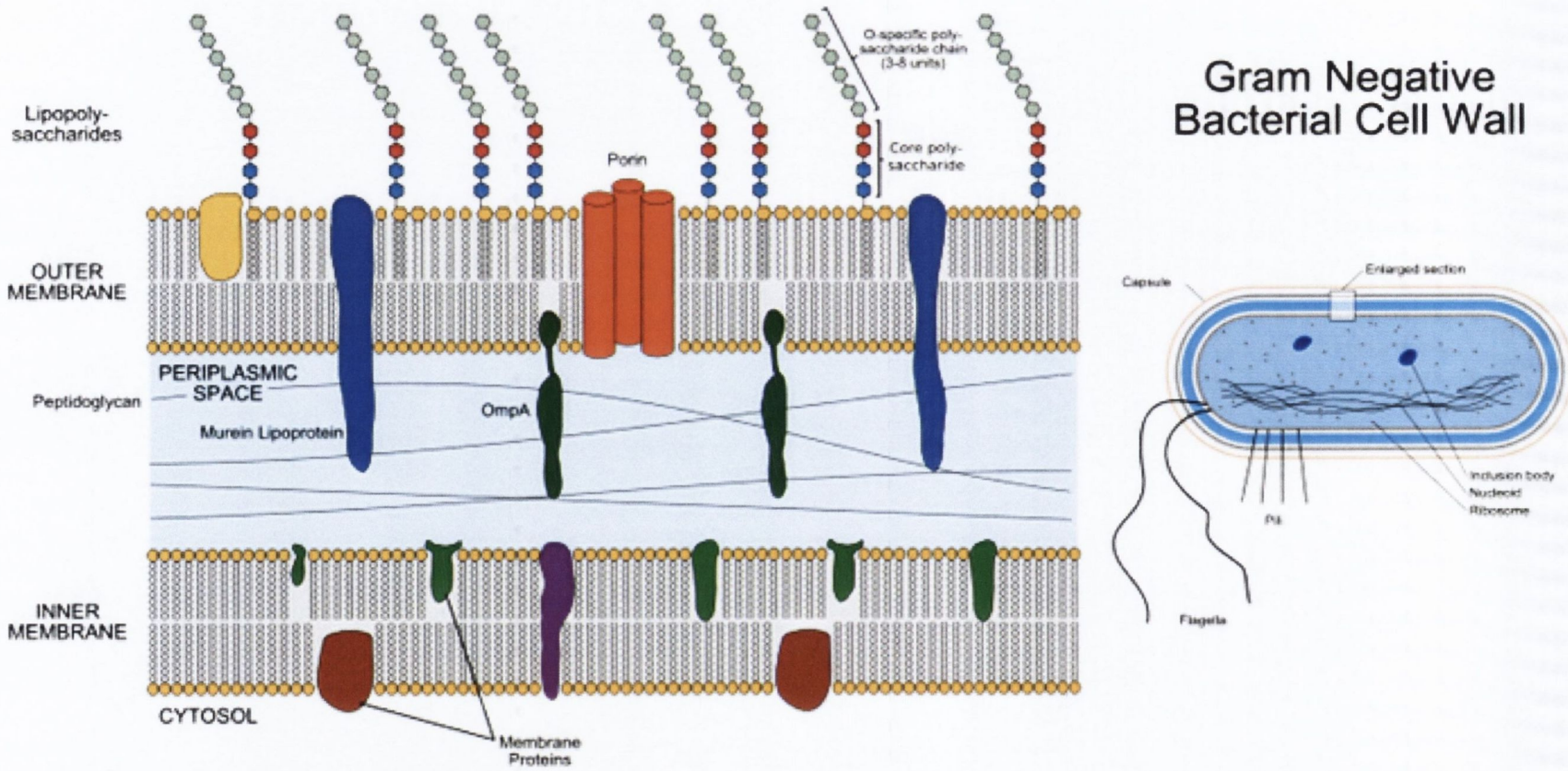


Figure 1-1 . Composition of the Gram-negative bacterial cell wall.
(Figure obtained from wikimedia.org/wikipedia/commons with permission granted by Jeff Dahl under the terms of the GNU Free Documentation License, version 1.3.)

between the (inner) plasma membrane and an outer membrane. The peptidoglycan layer, which is thinner than those of Gram-positive organisms, is located in this compartment and makes up around 5-20% of the cell wall. Hydrolytic enzymes such as autolysins and β-lactamases are also present in this compartment. The innermost structure of the cell wall is the plasma membrane or cytoplasmic membrane. It consists of a lipid bilayer which also contains proteins such as respiratory enzymes, membrane ATPases and penicillin-binding proteins (PBPs) (Madigan & Martinko, 2006a; Sauvage *et al.*, 2008).

Several mechanisms located within the cell wall have been employed to confer resistance to various antimicrobial agents. The lipid outer membrane presents a significant barrier to the more hydrophilic compounds. Although the smaller members of such compounds may be able to utilise porins to traverse the outer membrane, selective loss or reduction of such porins from the membrane would limit the entry of such compounds. A notable example is the loss of OprD porin in *P. aeruginosa* leading to carbapenem impermeability and resistance (Köhler *et al.*, 1999; Quale *et al.*, 2006).

Gram-negative bacteria can also produce enzymes which accumulate in the periplasmic space and degrade the antibiotics that have penetrated the outer membrane. A wide variety of β-lactamases are synthesised and secreted into the periplasmic compartment and as a whole, they constitute a major mechanism of resistance to β-lactams in Gram-negative organisms (see section 1.3.2).

Another mechanism of antimicrobial resistance is the variety of efflux pumps involving in expelling compounds toxic to the bacterial cell. These protein pumps are primarily located in the plasma membrane but some types of efflux pumps may span the entire thickness of the cell wall, such as the resistance-nodulation-division efflux pumps (see below).

Bacterial efflux pumps are mainly classified into five superfamilies (Poole, 2005; Vila *et al.*, 2007). They are: (1) ATP-binding cassette (ABC) family; (2) small multidrug resistance (SMR) family; (3) multidrug and toxic compound extrusion (MATE) family; (4) major facilitator superfamily (MFS); and (5) resistance-nodulation-division (RND) family. Depending on the types of efflux pumps and their respective substrate specificities, the efflux pumps can extrude one or more classes of antimicrobial agents from the cytoplasmic compartment into the periplasmic or extracellular space, or from the periplasmic compartment into the extracellular space (Piddock, 2006). Concentration of the agent in the immediate vicinity of the bacterial cell is thereby reduced significantly.

ABC-type efflux pumps are rarely involved in antimicrobial resistance while MFS and RND efflux pumps are the most abundant of antibiotic efflux pumps. MFS pumps are found in both Gram-positive and Gram-negative bacteria, and are characterised by narrow spectra of substrates; the RND pumps are found exclusively in Gram-negative bacteria and display extremely wide spectra of substrates (ie. poly-selectivity),

including different classes of antibiotics, antiseptic compounds, dyes and detergents (Levy, 2002; Li & Nikaido, 2004; Mahamoud *et al.*, 2007; Poole, 2005). RND family of pumps are three-component efflux pumps that span the entire thickness of the cell wall (Quale *et al.*, 2006; Tikhonova & Zgurskaya, 2004). The multidrug transporter component (located predominantly in the periplasm) interacts with the membrane fusion protein and OMP components, which are located in the plasma and outer membranes, respectively. The RND pumps allow antibiotics to pass across the inner and outer membranes of the bacteria without accumulating in the periplasmic space. Well-characterised RND pumps include the AcrAB-TolC pumps in *Enterobacteriaceae* and the Mex-Opr-type pumps in *P. aeruginosa* (Ghisalberti *et al.*, 2006; Quale *et al.*, 2006; Tikhonova & Zgurskaya, 2004). In *A. baumannii*, efflux pumps of the various families have also been described. Fournier *et al.* found multiple copies of the *qacE1* genes encoding SMR efflux pumps conferring resistance to ammonium antiseptic compounds (Fournier *et al.*, 2006). Tet(A) and CmlA pumps are narrow-spectrum MFS efflux pumps targeting tetracyclines and chloramphenicol, respectively (Vila *et al.*, 2007). Broad-spectrum MATE and RND pumps have also been characterised. AbeM is a recently characterised multidrug efflux pump of the MATE family and recognises several compounds including quinolones, gentamicin, triclosan, doxorubicin, rhodamine and ethidium bromide (Su *et al.*, 2005; Vila *et al.*, 2007). Several RND efflux pumps were also characterised in *A. baumannii* and *Acinetobacter* genospecies 3, namely AdeABC, AdeDE, AdeIJK and AdeXYZ, and substrates of these pumps include β-lactams, aminoglycosides, quinolones,

tetracyclines, chloramphenicol and ethidium bromide (Chau *et al.*, 2004; Chu *et al.*, 2006; Damier-Piolle *et al.*, 2008; Magnet *et al.*, 2001).

1.3.2 Genetic exchange of antimicrobial resistance

Antimicrobial resistance genes may be present in various genetic elements in bacteria. Some of these are located within the bacterial chromosomal DNA. Genes encoding Ambler class C β-lactamases are often found within the chromosomes of Gram-negative organisms including most species of *Enterobacteriaceae*, *P. aeruginosa* and *Acinetobacter* spp.

However, many resistance genes are carried on mobile genetic elements that can be transferred from cell to cell and often between species as well as genera. Plasmids are genetic elements that exist within prokaryotes as free, typically circular, double-stranded DNA molecules. They replicated independently of the host chromosome and vary in copy numbers within the host cells. The main mechanism of plasmid transfer between cells is through the process of conjugation. Plasmids carrying antimicrobial resistance genes are commonly known as resistance plasmids (R plasmids). Host range, which is the extent of a plasmid's ability to transfer to a variety of organisms, varies among plasmids. R plasmids may confer multidrug resistance to a wide variety of bacterial cell populations if they have a broad host range and carry numerous resistance genes as well. Genes conferring resistance to β-lactams, aminoglycosides,

sulphonamides and chloramphenicol are usually plasmid-mediated (Madigan & Martinko, 2006b).

Resistance genes may also be associated with transposable elements, which mediate the process of transposition. This is a process whereby a gene moves from one location to another within the genome. Insertion sequences (IS) and transposons are examples of transposable elements. Both carry genes encoding transposases (enzymes necessary for the transposition process) and have short inverted terminal repeats at the ends of their DNA (Madigan & Martinko, 2006b).

IS are the simplest type of transposable elements and carry no genes other than those required for their movement to new locations. IS are found in both chromosomal and plasmid DNA, and different bacterial species and strains vary in the number and frequency of these elements. The significance of IS in antimicrobial resistance is the presence of promoter sequences in some of them, which can regulate expression of resistance genes if inserted next to them. This is exemplified by the presence of IS elements (such as IS*Aba1*, IS*Aba4*, IS*1999* and IS*18*) upstream of resistance genes in *Acinetobacter* spp. and *K. pneumoniae* (Corvec *et al.*, 2007; Naas *et al.*, 2007; Poirel & Nordmann, 2006a; Segal *et al.*, 2005; Turton *et al.*, 2006; Walther-Rasmussen & Høiby, 2006). These IS elements have been shown to upregulate the expression of resistance genes such as class C and D β-lactamases (Corvec *et al.*, 2007; Naas *et al.*, 2007; Poirel & Nordmann, 2006a; Segal *et al.*, 2005).

Transposons are larger than IS and carry other genes often conferring important properties on the host organism, such as antimicrobial resistance. In addition, some transposons (known as conjugative transposons) carry *tra* genes which allow the transposons to move between bacterial species by the process of conjugation. Bacterial conjugation involves the transfer of genetic material by cell-to-cell contact via surface structures known as sex pili (Madigan & Martinko, 2006b).

A relatively novel mechanism of resistance gene dissemination among bacteria is the integron (Stokes & Hall, 1989). Integrons are transposons that capture and express genes or groups of genes (known as cassettes) from other sources, but unlike other transposons, they are highly selective in their insertion site. A distinguishing feature of an integron is the *int* gene which encodes an integrase which mediates site-specific recombination of genes. Integrons also possess the *attI* gene encoding the cassette integration site and one or more promoters responsible for expression of gene cassettes (Stokes & Hall, 1989). Integrons can be incorporated into plasmids, other transposons and even chromosomes. Based on the sequence of the integrase genes, three classes (class 1 to 3) of integrons containing antimicrobial resistance gene cassettes have been described.

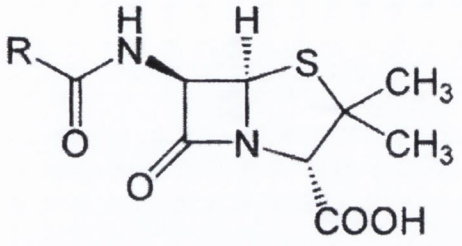
The majority of integrons belong to class 1, and are found mainly in Gram-negative organisms including *Acinetobacter* species (Koeleman *et al.*, 2001; Levesque *et al*, 1995; Sallen *et al.*, 1995). Antimicrobial resistance genes found within integron cassettes include genes encoding aminoglycoside-modifying enzymes, *sul* and *dfr*

genes which mediate sulphonamide and trimethoprim resistance respectively, and *qacEΔ1* gene mediating resistance to quarternary compounds and ethidium bromide (Koeleman *et al.*, 2001; Levesque *et al*, 1995; Turton *et al.*, 2005). Important β-lactamase genes were also found in class 1 integrons, such as those encoding VEB-, PER-, IMP-, VIM- and OXA-type β-lactamases (see section 1.3.3.1 below) (Naas *et al.*, 1998; Naas *et al.*, 2006a; Naas *et al.*, 2006b; Perez *et al.*, 2007; Yum *et al.*, 2002). The efficient accumulation and horizontal transfer of resistance genes through integrons most probably provide selective survival advantage for bacterial strains, especially in antibiotic- and disinfectant-rich environments such as hospitals. This is exemplified by studies demonstrating a close association between the presence of integrons and epidemic strains of multidrug-resistant *A. baumannii* (Koeleman *et al.*, 2001; Turton *et al*, 2005).

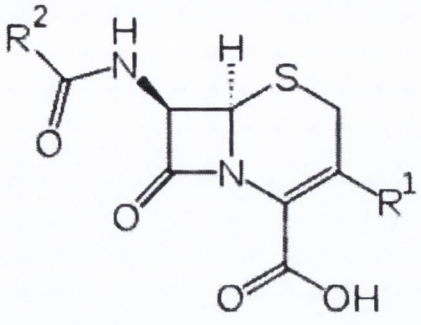
1.3.3 β-lactams

The β-lactam antimicrobial agents consist of a large family of naturally-derived or semi-synthetic molecules whereby the central feature is the β-lactam ring or penam (see Figure 1-2). This is a cyclic amide ring structure with three carbon atoms and one nitrogen atom.

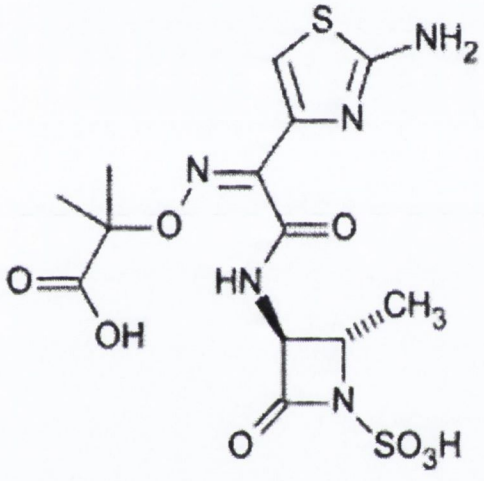
β-lactams act by binding and inhibiting bacterial PBPs which are integral in the synthesis of the peptidoglycan layer (see section 1.3.1 and Figure 1-1) (Wang *et al.*,



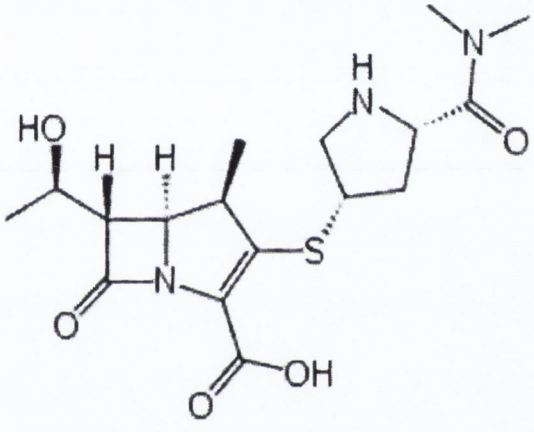
Penicillins



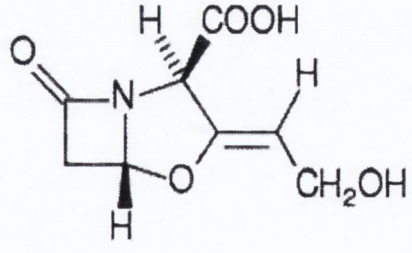
Cephalosporins



Aztreonam (monobactam)



Meropenem (carbapenem)



Clavulanic acid (β-lactamase-inhibitor)

Figure 1-2. Biochemical structures of β-lactam compounds.

1999; Zapun *et al.*, 2008). PBPs are anchored within the plasma membrane and emerge on the outer surface of the membrane adjoining the periplasmic space. Numerous types of PBPs exist (for example, PBPs -1a, -1b, -2, -3, -4, -5, -6) and they have molecular weights between 30,000 and 120,000 Daltons. They can be divided into high and low molecular weight PBPs for functional reasons (Sauvage *et al.*, 2008). High molecular weight PBPs such as PBP-1a, -1b, -2 and -3 have transpeptidase activity while low molecular weight PBPs like PBP-4, -5 and -6 are carboxypeptidases. PBP-1a and -1b are bifunctional enzymes catalysing the elongation of glycan chains via transglycosylation in addition to the cross-linking of glycan chains via transpeptidation. β-lactams act by covalently binding to PBPs and inhibition of the latter reaction. PBP inhibition by β-lactam is complex. Different bacterial species have different numbers of PBPs and different proportions of each subtype. Moreover, each β-lactam has different affinities for the different PBPs (Sauvage *et al.*, 2008).

The overall action of β-lactams is the disruption of the integrity of the bacterial peptidoglycan layer. The peptidoglycan layer is in constant turnover with a dynamic balance between synthesis/cross-linking of glycan chains (mediated by PBPs) and the hydrolysis of chains mediated by autolysins such as glycosidases and amidases produced by the bacterial cell. Through the inhibition of PBPs, β-lactams disrupt the equilibrium favouring hydrolysis over synthesis of the peptidoglycan, with consequent bacterial cell lysis (Sauvage *et al.*, 2008; Zapun *et al.*, 2008).

The efficacy of β-lactam activity in Gram-negative bacteria depends on several factors which are summarised in Figure 1-3. The two major factors are the affinity of the β-lactam to the PBPs (which in turn depends on bacterial characteristics as well as the type of β-lactam involved) as well as the concentration of the β-lactam achieved at the target site (ie. periplasmic space).

The first factor, affinity of β-lactam to PBPs, has been discussed in the previous paragraphs. The second factor is in turn dependent on various features such as the penetration through the outer membrane, activity of periplasmic β-lactamases and extrusion of β-lactams into the extracellular space.

Penetration of the outer membrane by a β-lactam depends on its physicochemical properties such as hydrophobicity, size and charge. Although most β-lactams are largely hydrophilic compounds, they do vary in the degree of hydrophobicity. Penetration of the more hydrophobic compounds can be facilitated by outer membranes rich in phospholipids. However, the main passage of most β-lactams into the periplasmic space is via porins. The speed of penetration of β-lactams through the porins in turn depends on the physicochemical properties of the individual β-lactam as well as the numbers and types of porins present in the outer membrane (see section 1.3.1). Within the periplasmic space, the concentration of the β-lactam is dependent on the type and level of efflux activity (see section 1.3.1) as well as the dynamic relationship between β-lactams and periplasmic β-lactamases.

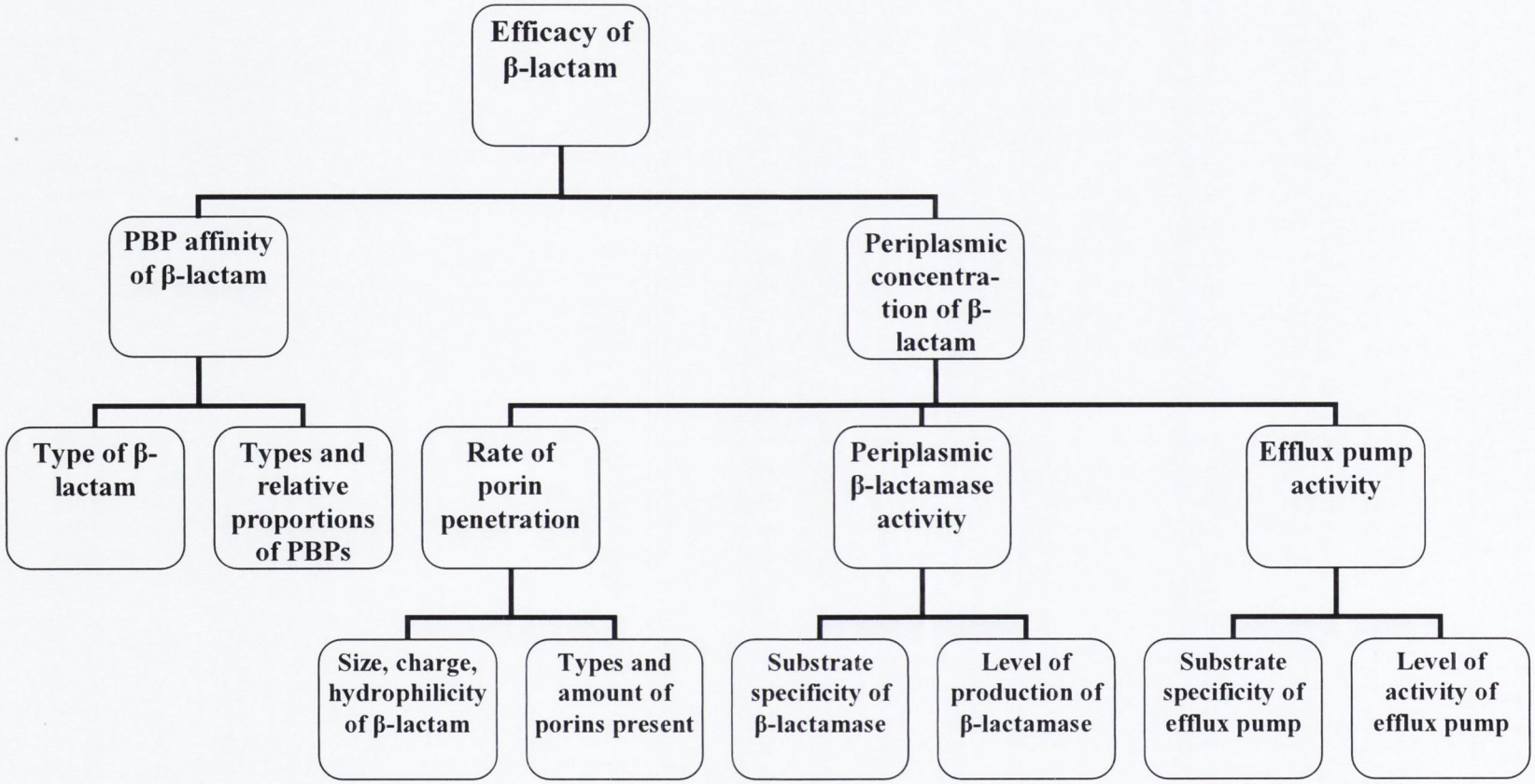


Figure 1-3. Local factors affecting the activity of a β-lactam agent.

β-lactams are usually classified according to the following characteristics:

- presence or absence of fusion of a second ring to the β-lactam ring;
- structure of the second ring;
- nature of side-chain(s) attached to the β-lactam ring system.

Based on the above characteristics, β-lactams can be broadly classified into the following classes (see Table 1-2):

- penicillins;
- cephems such as cephalosporins and cephamycins;
- penems such as carbapenems;
- monolactams such as monobactams;
- β-lactamase-inhibitors.

Penicillin was found to be derived from a mould *Penicillium notatum* in 1928 by Alexander Fleming. Subsequently, other natural penicillins were derived from other *Penicillium* spp. and these closely related compounds differed only in the nature of the acyl side-chain. Numerous semi-synthetic members of this group of β-lactams have since been developed. Penicillins are characterised by the fusion of a 5-membered thiazolidine ring to the β-lactam ring (Bush, 2003). Variations in the side-chains have resulted in wide variations in the spectrum of antimicrobial activity. Introduction of an amino group to the side-chain of benzylpenicillin confers improved activity against GNB and this subgroup of penicillins is known as aminopenicillins. The addition of carboxyl- and ureido- groups to the acyl side-chain of benzylpenicillin

Table 1-2. Classification of β-lactam agents.

Group	Sub-group	Examples
Penicillins	benzylpenicillins	benzylpenicillin, penicillin V
	aminopenicillins	ampicillin, amoxicillin, mecillinam
	ureidopenicillins	piperacillin, azlocillin
	carboxypenicillins	ticarcillin, carbenicillin
	methoxypenicillins	temocillin
	anti-staphylococcal penicillins	oxacillin, cloxacillin, flucloxacillin, meticillin, nafcillin
Cephems	cephalosporins	1st generation: cefaclor, cephradine
		2nd generation: cefuroxime
		3rd generation: cefotaxime, ceftazidime, cefixime
		4th generation: cefepime, cefpirome
	cephamycins	cefoxitin, cefotetan
	other cephems	oxacephems, carbacephems
Penems	carbapenems	meropenem, imipenem, ertapenem, doripenem
	other penems	oxapenems, sulphopenems
Monolactams	monobactam	aztreonam
	other monolactams	nocardicins, monocarbams
β-lactamase inhibitors		clavulanic acid, tazobactam, sulbactam

result in carboxypenicillin (such as ticarcillin) and ureidopenicillin (such as piperacillin) subgroups respectively. These subgroups are characterised by enhanced activity against *P. aeruginosa*. Other derivatives with phenyl rings in the side chains are relatively resistant to hydrolysis by staphylococcal β-lactamase, and hence are known as anti-staphylococcal penicillins (Bush, 2003).

Cephalosporins were originally derived from a sewage-cultivated mould *Cephalosporium acremonium*. Together with cephamycins and other structurally related compounds, they form the cephem group of β-lactams. They are characterised by the fusion of a 6-membered dihydrothiazine ring to the β-lactam ring. Alterations at different positions of the ring structure result in changes in the spectrum of antimicrobial activity and/or resistance to β-lactamase (Greenwood, 2003). Cephalosporins are commonly classified into four generations based upon increasing activity (generally against GNB) and enhanced β-lactamase stability. Some third-generation cephalosporins such as cefotaxime have potent activity against *Enterobacteriaceae* while other members within this subgroup such as ceftazidime are also highly active against *P. aeruginosa*. Fourth generation cephalosporins such as cefepime have potent activity against *Enterobacteriaceae*, enhanced stability against Ambler class C β-lactamases (see section 1.3.3.1) as well as enhanced anti-staphylococcal activity. However, third- and fourth-generation cephalosporins are still susceptible to hydrolysis by some β-lactamases with extended spectra of activity (Grrenwood, 2003). Cephamycins are compounds related to cephalosporins with activity against GNB and have enhanced stability against Ambler class A β-lactamases (see below).

Monolactams are β-lactams that do not have a second ring fused to the central β-lactam ring. The most commonly encountered member is the monobactam, aztreonam. It is active against many aerobic GNB including *Enterobacteriaceae* and *P. aeruginosa* (Brogden & Heel, 1986). It can be hydrolysed by class A and class C ESBLs but has *in vitro* stability against Ambler class B β-lactamases (also known as metallo-β-lactamases) (Nordmann & Poirel, 2002). However, it has no significant activity against Gram-positive organisms or strict anaerobes (Brogden & Heel, 1986; Hellinger & Brewer, 1999).

Carbapenems are β-lactams characterised by the replacement of the sulphone in the four-position of the thiazolidone moiety of β-lactams with a carbon atom (Bonfiglio *et al.*, 2002). The very first carbapenems discovered were olivanic acids derived from *Streptomyces olivaceus*, followed in 1976 by the discovery of thienamycin produced by a previously unknown *Streptomyces* species called *Streptomyces cattleya* (Kattan *et al.*, 2008). Clinically useful carbapenems such as imipenem and meropenem are semi-synthetic derivatives of thienamycin. They are rapidly bactericidal agents that are highly potent against a wide range of Gram-negative, Gram-positive and anaerobic organisms (Hellinger & Brewer, 1999; Shah & Isaacs, 2003). The enhanced activity of carbapenems is due to several factors, including a greater ability to penetrate Gram-negative outer membrane because of their smaller sizes compared to cephalosporins and because they exist as zwitterions (ie. possessing both negative and positive charges in solution) (Hellinger & Brewer, 1999). They also have high affinity for essential PBPs such as PBP-1b, -2, -3 and -4 from a broad range of bacteria as

well as being highly resistant to hydrolysis by a broad range of Gram-negative and Gram-positive β-lactamases including Ambler classes A and C β-lactamases (see below) (Bonfiglio *et al.*, 2002; Hellinger & Brewer, 1999; Kattan *et al.*, 2008). A recently proposed classification of carbapenems divided them into two groups: group 1 carbapenems such as ertapenem are broad-spectrum β-lactams with limited activity against non-fermentative GNB, while group 2 carbapenems such as imipenem, meropenem and doripenem (S-4661) are defined as broad-spectrum β-lactams that are significantly active against non-fermentative GNB (Shah & Isaacs, 2003). Doripenem (S-4661) is a parenteral carbapenem that has completed phase 3 trials for nosocomial pneumonia (including VAP), complicated intra-abdominal infections and complicated UTI and is licensed for adults for the treatment of complicated intra-abdominal infections and UTIs (Kattan *et al.*, 2008). It has a wide spectrum of activity, combining the *in vitro* activity of imipenem against Gram-positive pathogens and of meropenem against Gram-negative organisms (Jones *et al.*, 2004b; Jones *et al.*, 2005; Mushtaq *et al.*, 2004; Pillar *et al.*, 2008). It is also highly active against organisms carrying different β-lactamases of Ambler classes A and C (Jones *et al.*, 2005; Mushtaq *et al.*, 2004; Pillar *et al.*, 2008). Doripenem has lower MICs for *P. aeruginosa* than several anti-pseudomonal agents and is associated with the lowest rate of spontaneous resistance *in vitro* (Kattan *et al.*, 2008). The emerging trend worldwide of carbapenem resistance in GNB is a particularly worrying development since carbapenems are a very versatile and potent class of β-lactams (see sections 1.3.3.1 and 1.4).

β-lactamase inhibitors are β-lactams that bind irreversibly to β-lactamases, thereby inactivating these enzymes. Examples in clinical use are clavulanic acid, tazobactam and sulbactam (Bonnet, 2004; Livermore, 1995). When combined with another β-lactam (for example, ticarcillin or piperacillin), they can maintain activity of the β-lactam component which would otherwise be hydrolysed by the β-lactamase (Lee *et al.*, 2003). Sulbactam is different from clavulanic acid or tazobactam in that it also has significant affinity to penicillin-binding proteins, whereas the latter two do not. This suggests that in addition to β-lactamase-inhibitory activity, sulbactam also has intrinsic antimicrobial activity. *In vitro*, β-lactamase inhibitors are useful for the detection of Ambler class A β-lactamases of micro-organisms (Bonnet, 2004; Bradford, 2001). Therapeutically, they are active against a range of organisms but their clinical efficacy for infections caused by organisms producing Ambler class A β-lactamases is less reliable than other agents such as the carbapenems (Lee *et al.*, 2003).

1.3.3.1 β-lactamases

β-lactamases and PBPs belong to a large family of peptidases that bind to β-lactams. The rate of hydrolysis of the bound complex distinguishes β-lactamases from the PBPs (Livermore, 1995). PBPs are bound to β-lactams covalently and hence, the complexes do not dissociate readily. The β-lactamase/β-lactam complexes undergo hydrolysis more readily resulting in the disruption of the β-lactam ring and regeneration of the β-lactamase (Livermore, 1995). Synthesis of β-lactamase is mediated either chromosomally or via transferable elements such as plasmids and

transposons (see section 1.3.2). Transferable elements can facilitate more rapid and widespread dissemination of β-lactamase genes among different strains, species or even genera.

There are numerous β-lactamases and their biochemical characteristics are diverse. Logical categorisation and classification of these enzymes are very challenging.

Richmond and Sykes classified β-lactamases according to an expanded substrate profile and proposed five major groups (I-V) (Richmond & Sykes, 1973). Bush *et al.* proposed a modified version of the classification of four groups (1-4) based on several characteristics such as spectrum of activity, biochemical properties, molecular structure and nucleotide sequence (Bush *et al.*, 1995).

However, classifications based on phenotypic characteristics face the challenge that point mutations may greatly alter the substrate and inhibitor profiles of the enzyme. A simpler and more user-friendly method of classification was proposed by Ambler in 1980, whereby β-lactamases were classified according to the nucleotide sequence into four molecular classes (A, B, C and D) (Ambler, 1980). In addition to simplicity, such sequence-based classification methods are more stable as the relationships are much less likely to be distorted by point mutations. The Ambler classification system of β-lactamases will henceforth be adopted in subsequent sections of this thesis.

Enzymes of Ambler classes A, C and D have a serine residue in their active sites and are called serine peptidases, while Ambler class B enzymes have a divalent cation (usually Zn^{2+}) in their catalytic sites and are also called metallo-β-lactamases.

1.3.3.1.1 Ambler class A β-lactamases

These β-lactamases have the conserved motif of Ser-Xxx-Xxx-Lys (where Xxx is an unconserved amino acid) in their active sites as do serine-β-lactamases of Ambler classes C and D (Bonnet, 2004; Bradford, 2001). Other conserved motifs of class A enzymes are Ser-Xxx-Asn and Lys/Arg-Ser/Thr-Gly (Ambler, 1980; Paetzel *et al.*, 2000). Primary substrates of this class of enzymes are penicillins but point mutations can lead to an extended spectrum of activity involving broad-spectrum cephalosporins and monobactams (Bonnet, 2004; Bradford, 2001; Livermore, 1995). Such enzymes are called extended-spectrum β-lactamases (ESBLs). Common functional features of ESBLs are inhibition by clavulanic acid and susceptibility to cephamycins and carbapenems.

The commonest ESBLs are TEM, SHV and CTX-M derivatives found mainly in *E. coli*, *K. pneumoniae* and other *Enterobacteriaceae* (Bonnet, 2004; Livermore, 1995). Less common ESBLs include PER, VEB, and GES derivatives (Bradford, 2001; Naas *et al.*, 2006a). Detection of ESBLs can be carried out in the diagnostic laboratory using clinical microbiology phenotypic techniques or molecular detection methods (see section 1-3-10) (Bradford, 2001; Livermore, 1995). Some TEM variants (such as TEM-30 and TEM-76) are resistant to certain β-lactamase-inhibitors such as

clavulanic acid and sulbactam, and are called inhibitor-resistant TEM (IRT) enzymes (Bradford, 2001). They are found mainly in *E. coli*, but also in *Klebsiella* and *Proteus* spp. Point mutations that lead to the inhibitor-resistant phenotype occur at a few specific amino acid residues within the structural gene of the TEM enzyme, such as Met-69, Arg-244 and Asn-276 (Bradford, 2001).

More recently, class A β-lactamases which possess carbapenemase activity, such as KPC, GES and SME enzymes, are increasingly reported (Queenan & Bush, 2007). They can hydrolyse various classes of β-lactams efficiently, including penicillins, cephalosporins, aztreonam and carbapenems (Bratu *et al.*, 2005; Queenan & Bush, 2007). Among the class A carbapenemases, the KPC enzymes probably have the greatest potential for spread due to their plasmid locations and the fact that most are found in *K. pneumoniae*, which is known for its ability to accumulate and exchange resistance determinants. The epidemics caused by the spread of *K. pneumoniae* carrying plasmid-mediated KPC enzymes in the US and elsewhere highlight the therapeutic and infection control challenges that can be posed by class A carbapenemases (Bratu *et al.*, 2005; Pournaras *et al.*, 2009; Queenan & Bush, 2007).

1.3.3.1.2 Ambler class B β-lactamases

These enzymes are also known as metallo-β-lactamases (MBLs) as they each possess divalent metal ion (usually zinc) linked to a histidine or cysteine residue at the active site (Wang *et al.*, 1999). They can hydrolyse most β-lactams including carbapenems and cephalosporins but have no activity against aztreonam (Queenan & Bush, 2007).

They are also inhibited by EDTA but not by clavulanic acid. Their presence is suggested by a significant (≥ 4-fold) difference in carbapenem MICs in the presence and absence of EDTA respectively, although other enzymes, notably the OXA carbapenemases, may produce false-positive results (Boo *et al.*, 2006; Segal & Elisha, 2005).

Some environmental and opportunistic pathogens possess chromosomally mediated MBLs, such as *Bacillus cereus*, *Chryseobacterium* spp., *Stenotrophomonas maltophilia* and *Aeromonas* spp. (Queenan & Bush, 2007; Wang *et al.*, 1999). More worryingly, plasmid-mediated MBLs are increasingly reported in established pathogens. While SPM, GIM and SIM MBLs have not spread beyond their countries of origin (Brazil, Germany and Korea respectively), the VIM- and IMP-type MBLs have been reported in Asia, Europe, America and Australia (Livermore, 1995; Poirel & Nordmann, 2006b; Queenan & Bush, 2007). They are primarily detected in *P. aeruginosa*, but have been increasingly reported in *Acinetobacter* spp. and *Enterobacteriaceae* (Lee *et al.*, 2004; Poirel & Nordmann, 2006b). In particular, carbapenem resistance mediated by IMP-type MBLs is now a serious problem in Asian countries around the Pacific rim such as Korea, Japan & Taiwan (Lee *et al.*, 2004; Queenan & Bush, 2007; Yum *et al.*, 2002).

1.3.3.1.3 Ambler class C β-lactamases

These enzymes are characterised by conserved motifs of Ser-Xxx-Xxx-Lys, Tyr-Ala/Ser-Asn and Lys-Thr/Ser-Gly (Paetzel *et al.*, 2000). They are mainly

cephalosporinases with high levels of hydrolysis of cephalosporins and are not inhibited by clavulanic acid or EDTA. Examples are the various chromosomally derived AmpC enzymes found in the various species of the *Enterobacteriaceae* family (such as *Enterobacter cloacae*, *Citrobacter freundii* and *Serratia marcescens*) but also in some non-fermenters such as *P. aeruginosa*. Such enzymes usually exist in the inducible state, whereby the level of β-lactamase synthesis is normally at a low rate but can be increased by several hundred times in the presence of inducible β-lactams (Livermore & Brown, 2001).

However, mutations can result in constitutive high-level expression of such β-lactamase genes in *Enterobacteriaceae*, and such organisms are called derepressed AmpC mutants. Hyperproduction of AmpC enzymes in *Enterobacteriaceae* confers resistance to a wide range of β-lactams such as third generation cephalosporins and β-lactam/β-lactamase inhibitor combinations but not carbapenems or fourth generation cephalosporins (Livermore, 1995; Livermore & Brown, 2001). Moreover, AmpC hyperproduction, along with other mechanisms, also plays an important role in conferring carbapenem resistance in *P. aeruginosa* and *A. baumannii* (Quale *et al.*, 2006; Quale *et al.*, 2003). Detection of AmpC enzyme can be performed using double-disc approximation method or agar dilution assay with cloxacillin as the inhibitor of the enzyme (see section 1-3-10) (Tan *et al.*, 2009).

1.3.3.1.4 Ambler class D β-lactamases

These β-lactamases are serine peptidases with several conserved elements in common (Afzal-Shah *et al.*, 2001; Paetzel *et al.*, 2000; Walther-Rasmussen & Høiby, 2006). The first conserved element is Ser^{70}-Xxx-Xxx-Lys (Ser^{70} according to class D β-lactamase numbering), which is also found in class A and C β-lactamases. The second element, Ser^{118}-Xxx-Val/Ile, is equivalent to the invariable Ser-Asp-Asn motif in class A β-lactamases and Tyr-Ala/Ser-Asn motif in class C β-lactamases. The third conserved of element Lys^{216}-Thr/Ser-Gly is common to the vast majority of serine β-lactamases. Other conserved motifs that have no analogues in class A or C β-lactamases are the triad Tyr/Phe^{144}-Gly-Asn and the tetrad Trp^{232}-Xxx-Xxx-Gly elements (Afzal-Shah *et al.*, 2001; Paetzel *et al.*, 2000; Walther-Rasmussen & Høiby, 2006).

Ambler class D β-lactamases are also known as oxacillinases or OXA enzymes to denote the ability of most of these enzymes to hydrolyse oxacillin at a rate of $\geq$ 50% of those of classical penicillins such as benzylpenicillin, which is in contrast to enzymes of classes A and C for which oxacillin is a poor substrate (Bush *et al.*, 1995). Over 120 OXA β-lactamases have been identified and over 50 of these have carbapenem-hydrolysing activities (Poirel & Nordmann, 2006b; Walther-Rasmussen & Høiby, 2006). Among the OXA enzymes with no carbapenemase activity, they are largely divided into four groups (I to IV). The prototypes, which are narrow-spectrum β-lactamases, of groups I, II, III and IV are OXA-10, OXA-2, OXA-1 and OXA-9, respectively (Bradford, 2001). OXA-1 is fairly common among *E. coli* isolates, and like other early OXA enzymes (OXA-2 to OXA-10), the primary substrates are the

penicillins. Other OXA enzymes such as OXA-2 and OXA-10 are only occaionsally found in *E. coli* and *P. aerugionosa*. Some derivatives of these prototypes, such as OXA-11, -16, -18 and -28, are ESBLs found mainly in *P. aeruginosa* (Bradford, 2001). Most OXA enzymes are not inhibited by clavulanic acid, although a minority (such as OXA-18 and OXA-20) are inhibited by it (Naas *et al.*, 1998; Philippon *et al.*, 1997). Most of the OXA enzymes (such as OXA-1) are plasmid-mediated, while some OXA ESBLs such as OXA-20 are also carried on integrons (Naas *et al.*, 1998).

Over the last decade, OXA enzymes with carbapenemase activity have been discovered and characterised (Queenan & Bush, 2007; Walther-Rasmussen & Høiby, 2006). The majority of these OXA carbapenemases are reported in *Acinetobacter* spp., while some are also found in *K. pneumoniae*, *P. aeruginosa*, *Ralstonia* spp. and *Shewanella* spp. (Walther-Rasmussen & Høiby, 2006).

The OXA carbapenemases can be further classified into eight different clusters. Sequence homologies between members of the same cluster are > 90% while sequence homologies between different subgroups are < 70% (Walther-Rasmussen & Høiby, 2006). The first four clusters are the OXA-23 group, OXA-24 group, OXA-51 group and OXA-58 carbapenemases, respectively. They are the most prevalent of OXA carpbapenemases (Afzal-Shah *et al.*, 2001; Poirel & Nordmann, 2006b; Walther-Rasmussen & Høiby, 2006).

The OXA-23 group consists mainly of OXA-23, OXA-27 and OXA-49 enzymes. They are found mainly in *A. baumannii* and differed from one another by two to five

amino acids. The OXA-23 group of genes are usually plasmid-mediated and OXA-23 has become one of the most prevalent OXA carbapenemase in MDR *Acinetobacter* spp. worldwide, being reported in the various continents of Europe, America, Asia and Australasia (Boo *et al.*, 2006; Coelho *et al.*, 2006; Corvec *et al.*, 2007; Lee *et al.*, 2009; Valenzuela *et al.*, 2007; Villegas *et al.*, 2007). More recently, the group was further expanded by the discovery of $bla_{\text{OXA-23-like}}$ genes, $bla_{\text{OXA-102}}$, $bla_{\text{OXA-103}}$ and $bla_{\text{OXA-105}}$, in *Acinetobacter radioresistens* isolates. These $bla_{\text{OXA-23-like}}$ genes had up to six amino acid substitutions compared to the sequence of $bla_{\text{OXA-23}}$ (Poirel *et al.*, 2008b).

The OXA-24 group consists of OXA-24 (or its synonym OXA-40), OXA-25, OXA-26 and OXA-72 enzymes. The members of this group vary from one to five amino acids in sequence identity. They are mainly found in *Acinetobacter* isolates from Spain and Portugal, but have recently been reported in the US and Taiwan as well (Afzal-Shah *et al.*, 2001; Lolans *et al.*, 2006; Lu *et al.*, 2009). The OXA-51 group encompasses a cluster of closely related members of between one to fifteen amino acids' difference in sequence homology (Brown & Amyes, 2005). These enzymes are chromosomally located and ubiquitous in *A. baumannii* and are thus considered as naturally occurring oxacillinases in this species (Brown & Amyes, 2005; Héritier *et al.*, 2005a). The fourth cluster consists of a single enzyme of OXA-58 found mainly in carbapenem-resistant *A. baumannii* (Peleg *et al.*, 2006a; Poirel *et al.*, 2006).

The fifth cluster consists of OXA-55 and related enzymes isolated from *Shewanella* spp., while the sixth cluster comprises OXA-48 from *K. pneumoniae* and OXA-54

from *Shewanella oneidensis* (Poirel *et al.*, 2004). The seventh and eighth clusters are represented by OXA-50 enzymes in *P. aeruginosa* and OXA-60 enzymes from *Ralstonia pickettii*, respectively (Poirel & Nordmann, 2006b; Walther-Rasmussen & Høiby, 2006). The OXA carpbanemeases are carried on chromosomes (such as OXA-24 group, OXA-51 group, OXA-54 and OXA-55) or on plasmids (such as OXA-23 group, OXA-48 and OXA-58); however, they are not associated with integrons ((Afzal-Shah *et al.*, 2001; Brown & Amyes, 2005; Poirel *et al.*, 2004; Poirel & Nordmann, 2006b; Walther-Rasmussen & Høiby, 2006).

The regulation of these OXA carbapenemases has been shown to be influenced by insertion sequences (IS) that promote the expression of the bla_{OXA} genes (Corvec *et al.*, 2007; Naas *et al.*, 2007; Poirel & Nordmann, 2006a; Segal *et al.*, 2005; Turton *et al.*, 2006; Walther-Rasmussen & Høiby, 2006). A variety of IS elements have been found upstream of bla_{OXA} genes such as bla_{OXA-23}, bla_{OXA-48} and bla_{OXA-58}. These genetic elements have also been discussed in section 1.3.2.

The origins of the bla_{OXA} carbapenemase genes are the subjects of speculation and investigation among researchers (Héritier *et al.*, 2005a; Poirel *et al.*, 2004; Poirel *et al.*, 2008b). There is strong circumstantial evidence to suggest that the OXA-51 group of genes are naturally occurring genes in *A. baumannii*. They are ubiquitous in the isolates of this species, irrespective of the carbapenem susceptibility (Brown & Amyes, 2005; Héritier *et al.*, 2005a; Turton *et al.*, 2006). Moreover, this group of genes are found to be chromosomally located. Another class D carbapenemase, the plasmid-mediated OXA-48 from *K. pneumoniae*, has an amino acid homology of 92%

with the chromosomally-located OXA-54 of *Shewanella oneidensis* (Poirel *et al.*, 2004). Furthermore, genome shotgun sequencing of an environmental microbe *Shewanella* SAR-2 (CH004480) found in the Sargasso Sea has identified a putative bla_{OXA} gene which, along with flanking nucleotide sequences, is 99.2% identical to bla_{OXA-48} (Venter *et al.*, 2004). These findings strongly suggests that certain bla_{OXA} genes (such as $bla_{OXA-54-like}$ and bla_{OXA-55} genes) may be normal constituents of the genome of some *Shewanella* spp. and that the bla_{OXA-48} in *K. pneumoniae* has most likely descended from a $bla_{OXA-54-like}$ gene of the genus *Shewanella* (Poirel *et al.*, 2004; Venter *et al.*, 2004).

Recently, the possible progenitor of the OXA-23 group of genes has also been elucidated. An environmental organism, *A. radioresistens*, has been found to possess chromosomally located bla_{OXA-23} or closely related $bla_{OXA-102}$, $bla_{OXA-103}$ and $bla_{OXA-105}$ genes respectively (Poirel *et al.*, 2008b). This strongly suggests that one of the most prevalent groups of carbapenemase genes have originated from this species.

Substrate specificities of the OXA carbapenemases are diverse, but they generally hydrolyse penicillins (such as benzylpenicillin, ampicillin, ticarcillin and piperacillin) and narrow-spectrum cephalosporins (such as cephalothin and cephaloridine) efficiently but hydrolyse the extended-spectrum cephalosporins (such as cefotaxime, ceftazidime and cefepime) and aztreonam poorly (Poirel & Nordmann, 2006b; Walther-Rasmussen & Høiby, 2006). Consistent with the profiles of most Ambler class D enzymes, OXA carbapenemases are poorly inhibited by clavulanic acid. Most of them have relatively low hydrolytic activities against imipenem and even lower

activities against meropenem although OXA-48 and OXA-54 have higher hydrolytic activities against imipenem than other the OXA carbapenemases (Walther-Rasmussen & Høiby, 2006).

No specific phenotypic tests are available for the detection of OXA carbapenemases, but the carbapenem cloverleaf test can indicate the presence of carbapenemases (see section 1.3.10.1) (Queenan & Bush, 2007). OXA carbapenemases can also give false-positive results with EDTA-based carbapenem MIC tests intended for detecting metallo-β-lactamases (Segal & Elisha, 2005).

The clinical impact and epidemiology of OXA carbapenemases are further discussed in section 1.4.

1.3.4 Aminoglycosides

The aminoglycosides comprise a large group of naturally occurring or semi-synthetic polar compounds. The first aminoglycoside, streptomycin, was isolated from *Streptomyces griseus* in 1944. Neomycin, kanamycin and tobramycin are derived from other *Streptomyces* spp. while gentamicin and sisomicin are derived from *Micromonospora* spp. Netilmicin and amikacin are semi-synthetic derivatives of sisomicin and kanamycin respectively (Begg & Barclay, 1995). Most aminoglycosides in clinical use are glycosidic derivatives of streptamine and can be

divided into 4 groups based on different biochemical substitutions (Boehr *et al.*, 2003):

Neomycin group: neomycin, paramomycin.

Kanamycin group: kanamycin, amikacin, tobramycin.

Gentamicin group: gentamicin, netilmicin, sisomycin.

Other aminoglycosides: spectinomycin, streptomycin.

The chemical differences are important in determining susceptibility of the compounds to inactivation by bacterial aminoglycoside resistance enzymes (see below). As polar molecules with multiple positive charges, aminoglycosides are attracted by negatively charged lipopolysaccharides of Gram-negative outer membranes and teichoic acids of Gram-positive peptidoglycan and as a result, concentrate in the immediate bacterial environment. In GNB, penetration of the outer membrane is achieved via porins and penetration of the cytoplasmic membrane is via active transport systems involving bacterial respiratory enzymes, such as catalases, oxidases and cytochrome enzymes. Bacteria lacking such enzymes, such as strict anaerobes, are resistant to aminoglycosides. Aminoglycosides bind to bacterial 16S rRNA, a constituent of 30S ribosomal subunit, and cause abnormal protein synthesis and resultant loss of cytoplasmic membrane integrity (Begg & Barclay, 1995). Aminoglycosides are rapidly bactericidal and exhibit concentration-dependent killing. As a group, they are widely active against *Enterobacteriaceae* and other aerobic GNB such as *P. aearuginosa* and *Acinetobacter* spp (Begg & Barclay, 1995; Boehr *et al.*, 2003).

Resistance to aminoglycosides is due mainly to the acquisition of plasmid-borne aminoglycoside-modifying enzymes, which result in enzymatic inactivation of the aminoglycoside agents via the processes of acetylation, adenylylation and phosphorylation respectively (Shaw *et al.*, 1993). Other mechanisms which contribute to aminoglycoside resistance are 16S rRNA methylation or efflux pump activity (Doi & Arakawa, 2007; Magnet *et al.*, 2001).

In *Acinetobacter*, various classes of aminoglycoside-modifying enzymes have been reported, including acetyltransferases (eg. AAC(3)-Ia, AAC(6')-Ib), phosphotransferases (eg. APH(3')-VI, APH(3')-Ia) and nucleotidyltransferases (eg. ANT(2")-Ia) (Devaud *et al.*, 1982; Lambert *et al.*, 1988; Seward *et al.*, 1998; Vila *et al.*, 1999) . These enzymes are plasmid-mediated and often associated with class 1 integrons and MDR bacterial strains (Levesque *et al.*, 1995; Nemec *et al.*, 2004; Seward *et al.*, 1998; Turton *et al.*, 2005). More recently, plasmid-encoded methylase gene *armA* was found to reduce aminoglycoside affinity through methylation of the bacterial 16S rRNA and conferred high-level resistance to various aminoglycosides including gentamicin, tobramycin and amikacin (Doi & Arakawa, 2007; Lee *et al.*, 2006). Upregulation of various efflux pumps such as AdeABC and AbeM in *A. baumannii* were also associated with resistance to aminoglycosides as well as to other antimicrobial agents (Magnet *et al.*, 2001; Su *et al.*, 2005).

1.3.5 Quinolones

The quinolones comprise of a group of synthetic compounds based on the 4-quinolone nucleus. The dual-ring structure characterises all such compounds. Antibacterial spectrum is altered by alterations to this basic structure, for example, the addition of a fluorine atom at position C-6 enhances activity against staphylococci while the addition of a piperazine group at C-7 results in improved activity against aerobic Gram-negative bacteria and staphylococci (Andriole, 2003). Like cephalosporins, quinolones can also be broadly classified into four groups:

Group 1: older compounds such as nalidixic acid with spectra of activity largely confined to *E. coli* and other members of *Enterobacteriaceae.*

Group 2: compounds with much improved activity against *Enterobacteriaceae* and an enhanced spectrum including *P. aeruginosa* and Gram-positive cocci. They are known as fluoroquinolones since they are characterised by 6-fluoro and 7-piperazinyl groups and include ciprofloxacin, ofloxacin and levofloxacin.

Group 3: later compounds with further improvements in spectrum including useful activity against *Streptococcus pneumoniae* and some other Gram-positive cocci. They include gatifloxacin and sparfloxacin.

Group 4: most recent compounds with enhanced activity against Gram-positive cocci and anaerobes, such as moxifloxacin and trovafloxacin.

In Gram-negative organisms, fluoroquinolones exhibit potent activity against *Enterobacteriaceae*, *Haemophilus influenzae*, *Moraxella catarrhalis* and non-

fermenters such as *P. aeruginosa* and *Acinetobacter* spp. (Andriole, 2003). The fluoroquinolones are rapidly bactericidal against most susceptible species, through selective inhibition of bacterial DNA gyrase and topoisomerase IV by trapping topoisomerase-DNA complexes, resulting in interference with chromosomal DNA replication, transcription and segregation.

Quinolone resistance may occur as a result of alteration within the target enzymes DNA gyrase or topoisomerase IV. Quinolone resistance is generally mediated by chromosomal mutations in specific domains of the genes encoding the subunits, especially in *gyrA* (DNA gyrase) and *parC* (topoisomerase IV). These regions of the enzymes are called quinolone resistance determining regions. Such mutations lead to reduced affinity of DNA gyrase and/or topoisomerase IV. Single-step mutations generally lead to two- to eight-fold increases in MICs, and species most likely to become resistant to fluoroquinolones are those of borderline susceptibility. In *Acinetobacter* spp., mutations in *gyrA* and *parC* genes have also been well reported (Hamouda & Amyes, 2004; Seward & Towner, 1998; Vila *et al.*, 1995). As with the aminoglycosides, resistance to quinolones may also be due to increased efflux pump activity. In *A. baumannii*, AdeABC and AbeM efflux pumps are also associated with quinolone resistance (Magnet *et al.*, 2001; Su *et al.*, 2005).

In recent years, plasmid-mediated quinolone resistance genes such as *qnrA*, *qnrB* and *qnrS* have been increasingly reported in Gram-negative bacilli, particularly in *Enterobacteriaceae* (Poirel *et al.*, 2008a; Robicsek *et al.*, 2006). The Qnr proteins can protect DNA gyrase from the quinolones and confer low-level quinolone resistance

(Robicsek *et al.*, 2006). To date, *qnr* genes have not been reported in *Acinetobacter* spp.

1.3.6 Trimethoprim-sulphamethoxazole

Trimethoprim belongs to a class of agents known as the diaminopyrimidines. They are inhibitors of an enzyme involved in bacterial folate synthesis, dihydrofolate reductase (Dfr). It has broad-spectrum activity against Gram-positive organisms (including *S. aureus*) and Gram-negative bacteria including *Enterobacteriaceae*, *Haemophilus* spp., and *Pasteurella* spp. *P. aeruginosa* and most anaerobes are resistant to trimethoprim (Then, 2003).

Sulphamethoxazole belongs to a group of compounds called sulphonamides, which are inhibitors of another enzyme involved in the folate synthesis pathway, dihydropteroate synthase. Sulphonamides exhibit activity against common Gram-positive and Gram-negative pathogens but resistance is common in *Enterobacteriaceae* and usual in *P. aeruginosa*. Hypersensitivity reactions can occur in the form of rash, drug fever or serum-sickness-like reaction. Consequently, many sulphonamides have fallen into disuse as safer and more active agents are preferred. A few are still in use (for example, sulphamethoxazole), usually in combination with diaminopyrimidines.

The antimicrobial spectrum of trimethoprim-sulphamethoxazole covers pathogens susceptible to the individual agents and is expanded by synergistic interaction (Then, 2003; Wormser *et al.*, 1982). Some organisms that are refractory to many other agents may remain susceptible to it, including *S. maltophilia*, *Acinetobacter* spp. and *Burkholderia cepacia* complex. For other organisms such as *S. aureus* and *Enterobacteriaceae*, resistance rates vary widely in different parts of the world (Wormser *et al.*, 1982).

Resistance to trimethoprim may be mediated by the acquisition of trimethoprim-resistant *dfr* genes (eg. *dfrA* and *dfrB*) or hyperproduction of the target enzyme (Michael *et al.*, 2006). Similarly, sulphamethoxazole resistance may be mediated either by hyperproduction of the target enzyme, or by acquisition of mutant sulphonamide-resistant target genes (eg. *sul1*, *sul2* and *sul3*) resulting in reduced affinity of the drug for the target (Michael *et al.*, 2006). Sulphonamide and trimethoprim resistance genes are usually carried on plasmids but can often also be part of class 1 integrons found in various GNB (see section 1.3.2).

1.3.7 Polymyxins

Polymyxins are cyclic, positively charged peptide antibiotics derived from *Paenibacillus* (*Bacillus*) *polymyxa*. Of the five (polymyxins A to E) that were originally described in the 1950s, two remained in clinical use: colistin (polymyxin B)

and polymyxin E. Their use was significantly reduced in the 1970s due to concern for their toxicity, (Li *et al.*, 2006a), but with the rising prevalence of multidrug-resistant organisms such as *P. aeruginosa*, *K. pneumonia* and *A. baumannii*, clinicians are increasingly turning to colistin for treatment of such infections (Falagas & Kasiakou, 2005; Livermore, 2004). The incidences of major side-effects of nephrotoxicity and neurotoxicity appear to be lower than those observed decades earlier (Li *et al.*, 2006a). Polymyxins bind to the anionic bacterial outer membrane, resulting in a detergent effect and disrupting membrane integrity. They have a high affinity for lipid A of the LPS and prefentially displace Mg^{2+} and Ca^{2+} from the cationic binding sites (Landman *et al.*, 2008). Polymyxins are active against a wide range of nosocomial Gram-negative pathogens. The majority of isolates of *E. coli*, *Klebsiella* spp., *Enterobacter* spp., *P. aeruginosa* and *A. baumannii* are susceptible to the polymyxins (Falagas & Kasiakou, 2005; Gales *et al.*, 2006; Landman *et al.*, 2008).

Some organisms are intrinsically resistant to polymyxins. They include *Proteus* spp., *Providencia* spp., *Chromobacterium* spp., *Burkholderia* spp. and most isolates of *Serratia* spp. Organisms with intrinsic resistance to polymyxins have alterations in lipid A which result in decreased binding by polymyxins (Landman *et al.*, 2008). Acquired resistance was also observed in *Escherichia coli*, *Salmonella typhimurium* and *Klebsiella pneumoniae*, where substitution of phosphate groups in LPS led to reduced susceptibility to polymyxins (Landman *et al.*, 2008; Petersen *et al.*, 1987). Changes in outer membrane composition, such as the conversion of acidic phospholipids to neutral lipids, also led to the development of resistance in *P. aeruginosa* (Champlin *et al.*, 1983; Young *et al.*, 1992). There were also recent

reports of *in vitro* polymyxin hetero-resistance in *A. baumannii*, but the mechanism of resistance is as yet unknown (Gales *et al.*, 2006; Li *et al.*, 2006b, Lo-Ten-Foe *et al.*, 2007).

1.3.8 Glycylcyclines

Tigecycline is the first of a novel class of antimicrobial agents known as the glycylcyclines. They are related to the tetracycline class of antibiotics and possess the same four-ring carbocyclic skeleton structure. Tigecycline is a derivative of minocycline through the addition of a 9-tert-butyl-glycylamido moiety to the D ring at the 9th position. As a result of this modification, it is able to overcome efflux (from the added bulk of the side chain) and ribosomal-type (from improved affinity to ribosomal mutations) resistance mechanisms which lead to tetracycline resistance (Peterson, 2008). It inhibits bacterial protein synthesis by binding to the 30S ribosomal subunit. Tigecycline has a broad spectrum against a wide variety of pathogens. It is active against Gram-positive organisms such as staphylococci (including MRSA), enterococci (including VRE) and *S. pneumoniae*, and Gram-negative organisms including ESBL-producing *Enterobacteriaceae* and MDR-*A. baumannii* (Karageorgopoulos *et al.*, 2008; Peterson, 2008; Sader *et al.*, 2005). In a large surveillance study involving over 25,000 bloodstream isolates, the MIC_{90} values for *E. coli* and *Klebsiella* spp. isolates were 0.25 and 1 mg/L respectively. *P. aeruginosa* and members of the *Proteae* family were significantly less susceptible to

tigecycline, with MIC_{90} values of $\geq$ 8 mg/L (Sader *et al.*, 2005). More than 90% of MDR *Acinetobacter* isolates were susceptible to tigecycline in a recent review of 22 microbiological studies (Karageorgopoulos *et al.*, 2008).

Intrinsic non-susceptibility to tigecycline in *P. aeruginosa* and *Morganella morganii* (a member of the *Proteae* family) has been attributed to upregulated expression of MexXY-OprM and AcrAB multidrug efflux pumps respectively (Dean *et al.*, 2003; Ruzin *et al.*, 2005). More worrying is the development of resistance in previously susceptible organisms following exposure to tigecycline. Overproduction of AcrAB efflux pump has been associated with acquired decreased susceptibility to tigecycline in *E. coli* isolates (Keeney *et al.*, 2008). In *A. baumannii*, decreased susceptibility has also been reported following therapy with tigecycline (Peleg *et al.*, 2007b; Reid *et al.*, 2007). Hence caution should be exercised when treating MDR *A. baumannii* infections with tigecycline. The mechanism of tigecycline resistance in *A. baumannii* has been associated with the overexpression of the AdeABC multidrug efflux pump (Peleg *et al.*, 2007a; Ruzin *et al.*, 2007).

1.3.9 Antimicrobial susceptibility testing for *Acinetobacter* species

Antimicrobial susceptibility testing (AST) is usually carried out in diagnostic laboratories using methodologies recommended by internationally recognised

institutions such as the Clinical and Laboratory Standards Institute (CLSI) and the British Society of Antimicrobial Chemotherapy (BSAC) (BSAC, 2009a; BSAC, 2009b; CLSI, 2009). Reference methods for AST recommended by CLSI are the disc diffusion, agar dilution and microbroth dilution methods (CLSI, 2009). Other methods of AST have been studied and the susceptibility results compared with those obtained using the reference methods. In general, the Etest gradient MIC method was found to produce results which bear very good correlation to results from reference methods (Hope *et al.*, 2007; Lo-Ten-Foe *et al.*, 2007). Automated susceptibility test systems such as VITEK 2 (bioMerieux, Durham, North Carolina, US) and MicroScan WalkAway (Dade Microscan Inc., West Sacramento, California, US) also produced results that correlated closely to those from reference methods for most antimicrobial agents (O'Hara, 2005; Pfaller *et al.*, 2006; Shetty *et al.*, 1998). However, certain issues of AST in *Acinetobacter* spp. remain problematic or unresolved.

An area of discrepancy is the establishment of susceptibility breakpoints for the carbapenems (meropenem and imipenem). The AST medium recommended by CLSI is Mueller-Hinton agar (BBL, Sparks, Maryland, US), while the AST medium recommended by BSAC is generally Iso-Sensitest agar (Oxoid, Basingstoke, Hampshire, UK). However, in the case of carbapenems, BSAC has recommended the use of Mueller-Hinton agar for AST (BSAC, 2009b). This recommendation brings the AST methodology of BSAC in line with that of CLSI as far as carbapenem AST medium is concerned.

However, the carbapenem susceptibility breakpoints are different for BSAC and CLSI. Meropenem and imipenem MIC breakpoints as recommended by BSAC are as follow: ≤ 2 μg/mL as susceptible, 4 – 8 μg/mL as intermediate susceptibility, and ≥ 16 μg/mL as resistant, respectively (BSAC, 2009a). For CLSI, the meropenem and imipenem MIC breakpoints are as follow: ≤ 4 μg/mL as susceptible, 8 μg/mL as intermediate susceptibility, and ≥ 16 μg/mL as resistant, respectively (CLSI, 2009). As such, an isolate with meropenem or imipenem MIC of 4 μg/mL may fall either into the susceptible category (CLSI) or the category of intermediate susceptibility (BSAC), depending on the set of breakpoints selected. Therefore, caution needs to be exercised when interpreting carbapenem susceptibility results of isolates with borderline MICs.

In addition, there are currently no CLSI recommendations on disc diffusion or MIC susceptibility breakpoints for two newer antimicrobial agents, tigecycline and doripenem (CLSI, 2009). However, BSAC has established disc and MIC breakpoints for the above agents based on their recommended methodologies (BSAC, 2009a; BSAC, 2009b). For tigecycline, Etest MIC results were also found to have good correlation to those obtained with reference methods (Hope *et al.*, 2007; Bolmström *et al.*, 2007). However the BSAC breakpoints cannot necessarily be applied to MIC results obtained with CLSI reference methods due to discrepancies in tigecycline MIC results obtained with the respective methods (Casal *et al.*, 2009).

Some issues surrounding colistin susceptibility testing have recently been resolved. The latest versions of CLSI and BSAC recommendations have both established the

same susceptibility breakpoints for colistin, with MIC of ≤ 2 μg/ml as being susceptible and MIC of ≥ 4 μg/ml as being resistant (BSAC, 2009a; CLSI, 2009). Both organisations are also no longer recommending the use of disc diffusion method for colistin susceptibility testing due to an unacceptably high percentage of false susceptible results with this method (Gales *et al.*, 2001; Lo-Ten-Foe *et al.*, 2007). Agar dilution and broth microdilution methods are the recommended reference methods. Several studies of colistin susceptibility testing with the Etest method were also carried out. Most of them found close correlation of Etest MIC results with reference methods such as broth and agar dilution methods, although Etest MICs of ≥ 4 μg/ml require confirmatory testing with a reference method (Arroyo *et al.*, 2005; Landman *et al.*, 2008).

Caution should be exercised when interpreting AST results generated by automated commercial susceptibility testing systems such as VITEK 2 and MicroScan WalkAway. Significant discrepancies between MIC results of automated systems and those of other methods were found for Gram-negative organisms such as *E. cloacae*, *P. aeruginosa* and *A. baumannii* (Doern *et al.*, 1997; Joyanes *et al.*, 2001; Juretschko *et al.*, 2007; Sader *et al.*, 2006). VITEK 2 system, when compared with a reference method, had unacceptably low levels of categorical agreement for *A. baumannii* for cefepime, imipenem and ampicillin-sulbactam (Joyanes *et al.*, 2001). In *P. aeruginosa*, unacceptably high levels of very major errors (VME) (ie. false-susceptibility) were found in susceptibility results from automated systems such as BD Phoenix (BD Diagnostic Systems, Sparks, Maryland, US), VITEK, VITEK 2 and

MicroScan WalkAway for piperacillin-tazobactam as well as imipenem (Juretschko *et al.*, 2007; Sader *et al.*, 2006).

To date, neither CLSI nor BSAC has provided susceptibility breakpoints for amoxicillin-clavulanic acid for *Acinetobacter* spp., in spite of the availability of breakpoints for other β-lactam/β-lactamase-inhibitor combinations ticarcillin-clavulanic acid, piperacillin-tazobactam and ampicillin-sulbactam (BSAC, 2009a; CLSI, 2009).

1.3.10 Laboratory investigation of resistance mechanisms

In addition to AST, various laboratory techniques have been utilised for the investigation into the underlying mechanisms of antimicrobial resistance. They can be broadly divided into phenotypic microbiological tests, biochemical tests and molecular techniques. Molecular techniques can be further divided into DNA, RNA and protein studies.

1.3.10.1 Phenotypic microbiological tests

In phenotypic tests, a frequent approach to the investigation of resistance mechanisms is the employment of an inhibitor substance of a particular mechanism (such as a particular class of β-lactamase), with the presence of such mechanism inferred if there

is a significant difference in the substrate susceptibility with the addition of the inhibitor. β-lactamase inhibitors are frequently used for this purpose. Clavulanic acid, an inhibitor of Ambler class A β-lactamases, is used in combination with an oxyimino-cephalosporin such as ceftazidime or cefotaxime for the detection of ESBLs. Several variations of this test are available, such as the double-disc approximation test and the ESBL Etest (Bradford, 2001). Similarly, inhibitors of other Ambler classes of β-lactamases are also available, and tests designed using the same principles can hence infer the presence of the respective β-lactamases. EDTA and 2-mercaptoproprionic acid are inhibitors of Ambler class B β-lactamases while cloxacillin is an inhibitor of Ambler class C enzymes (Queenan & Bush, 2007; Tan *et al.*, 2009). The cloverleaf test or modified Hodge plate is an assay of β-lactamase (eg. carbapenemase) activity where suspension of the isolate is tested against the β-lactam on an agar plate. Altered growth of an indicator strain around the β-lactam disc is indicative of a positive result (Queenan & Bush, 2007).

More recently, inhibitors of bacterial efflux mechanisms are increasingly utilised in research to investigate the role of efflux pumps in the development of resistance to a class or multiple classes of antibiotics (Levy, 2002; Li & Nikaido, 2004; Pannek *et al.*, 2006; Quale *et al.*, 2006). Currently, there are several efflux pump inhibitors (EPIs) used in laboratory research but none is in clinical usage as yet. Compounds such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) abolish efflux totally by dissipating the proton-motive force of the membrane and causing cell death (Mahamoud *et al.*, 2007). However, it is uncertain whether it is the effect on the efflux pump or the resulting alteration of cell envelope itself that resulted in an increase in

antimicrobial penetration and subsequent cell death. Other agents result in more specific alteration of pump function, and are also known as flux inhibitors. They include general efflux inhibitors, peptidomimetics, quinoline derivatives, arylpiperazines and various analogues of known antibiotics (Mahamoud *et al.*, 2007). General inhibitors of efflux pumps such as verapamil or reserpine are also inhibitors of Gram-positive efflux pumps such as Bmr and NorA (Li & Nikaido, 2004). Peptidomimetics are a group of compounds that can inhibit Gram-negative efflux pumps in organisms such as *A. baumannii*, *P. aeruginosa*, *K. pnemoniae*, *E. coli* and *E. aerogenes* (Pannek *et al.*, 2006; Mahamoud *et al.*, 2007). The most commonly used peptidomimetic compound is phenylalanine arginyl β-naphthylamide (PAβN). Activities of various antibiotic classes have been reported to be restored by PAβN, including quinolones, macrolides, tetracyclines and chloramphenicol. Hence, peptidomimetics are widely used for the evaluation of Gram-negative efflux mechanisms in research settings (Pannek *et al.*, 2006; Mahamoud *et al.*, 2007). Quinoline derivatives are novel compounds which are also broad-spectrum EPIs active against *K. pneumoniae* and *E. aerogenes* efflux pumps for antibiotics such as quinolones, tetracyclines and chloramphenicol. However, they have poor activity against MexAB-OprM efflux pump of *P. aeruginosa* (Mahamoud *et al.*, 2007). Arylpiperazines are EPIs that can reverse MDR phenotypes caused by RND-type efflux pumps. A potent member of this group is N-methylpyrrolidone (NMP) whose antibiotic substrates include quinolones, linezolid, chloramphenicol and tetracyclines (Pannek *et al.*, 2006). It is active against *E. coli* and *A. baumannii* but not in *Serratia* spp. or *P. aeruginosa* (Mahamoud *et al.*, 2007).

1.3.10.2 Biochemical tests

β-lactamase activity can also be studied biochemically. Separation of bacterial proteins by charge can be performed using isoelectric focusing (IEF). This is a method of separation in which proteins align themselves as sharp bands at their isoelectric points (pI) in an electrophoretically-produced pH gradient (Matthew *et al.*, 1975). The presence of β-lactamase activity can subsequently be detected using a chromogenic substrate such as the cephalosporin nitrocefin (Bou *et al.*, 2000; Fernández-Cuenca *et al.*, 2003; Matthew *et al.*, 1975; Quale *et al.*, 2003). Although IEF results cannot identify a specific β-lactamase, information about the isoelectric points can be particularly helpful for the detection of multiple β-lactamases present in an isolate. Characterisation of a β-lactamase can also be carried out through the functional analysis of the hydrolysis and inhibition profiles of various antimicrobial substrates using purified protein molecules (Bou *et al.*, 2000; Queenan & Bush, 2007; Walther-Rasmussen & Høiby, 2006).

1.3.10.3 Molecular techniques

1.3.10.3.1 Methods based on DNA analysis

Polymerase chain reaction (PCR) utilising *Taq* polymerase is the most commonly used method for the detection of a gene or family of genes. It is specific, sensitive and relatively easy to perform. Methods using similar underlying principles, such as ligase

chain reaction, have also been used for the same purposes. Alternatively, DNA probes can also be used, although this method is more labour-intensive. More recently, the development of DNA microarrays allows the incorporation of numerous probes into a small microchip and facilitates the simultaneous detection of multiple genes (Weile & Knabbe, 2009). Genetic variants may be detected by oligotyping (requiring specific oligonucleotide probes) or PCR-RFLP (requiring nucleotide changes to result in altered restriction site for detection). However, the gold standard for detecting gene mutations or polymorphism is nucleotide sequencing along with alignment and comparison of gene sequences (Corvec *et al.*, 2007; Afzal-Shah *et al.*, 2001; Weile & Knabbe, 2009). Gene manipulation experiments can also be elucidating gene sequence and function. Cloning experiments of unknown genes into known vectors allow subsequent sequencing and characterisation of the genes, while knockout experiments can be used to analyse the causal relationship between genes and phenotypic characteristics (Corvec *et al.*, 2007; Segal *et al.*, 2005). Analysis of whole-cell genomic DNA using various typing methods such as PFGE has been mentioned in section 1.1.4. Genome analysis using methods such as shotgun sequencing or pyrosequencing can provide further insights into the genetic composition of pathogens such as pathogenicity islands and virulence factors (Fournier *et al.*, 2006).

1.3.10.3.2 Methods based on RNA analysis

The level of expression of a gene can be inferred through the quantitative analysis of the level of mRNA synthesis. This procedure requires initial reverse transcription of mRNA into complementary DNA (cDNA), followed by real-time quantitative PCR

using newly-converted cDNA as the template. The expression of efflux pumps and β-lactamases have been analysed using quantitative reverse-transcriptase real-time PCR (Huang *et al.*, 2008; Quale *et al.*, 2006). In addition, using similar working principles as DNA microarrays, RNA microarrays (also known as expression arrays) can also be used to analyse gene expression and have the advantages of speed of analysis and the ability to investigate multiple targets simultaneously (Weile & Knabbe, 2009).

1.3.10.3.3 Protein studies

The structures, masses, charges and functions of proteins can be systemically analysed following protein extraction and purification processes. Proteins can be separated and identified based on mass and charge using methods such as polyacrylamide gel electrophoresis (PAGE) and IEF. Further molecular characterisation of individual proteins can be performed using Western blotting and mass spectrometry. Tertiary and quarternary structures of purified forms of proteins can be analysed using x-ray crystallography while the functions of the proteins can be elucidated based on studies of enzyme kinetics (Bou *et al.*, 2000; Huang *et al.*, 2008; Paetzel *et al.*, 2000; Sauvage *et al.*, 2008; Wang *et al.*, 1999; Weile & Knabbe, 2009).

In the future, advances in new technologies such as microfluidics and nanotechnology can have a significant impact of microbiological research into antimicrobial resistance (Weile & Knabbe, 2009).

1.4 Carbapenem resistance in *Acinetobacter* species

1.4.1 Global epidemiology

Acinetobacter infections and outbreaks have been reported worldwide, and throughout the last few decades, *Acinetobacter* spp. have also become increasingly resistant to antibiotics (Bergogne-Bérézin & Towner, 1996; Gaynes & Edwards, 2005; Reinert *et al.*, 2007; Traub & Spohr, 1989; Wisplinghoff *et al.*, 2000). Table 1- 3 provides an overview of antimicrobial resistance in *A. baumannii* over the years. It shows an overall trend of decreased susceptibility to various antibiotics, particularly to carbapenems.

MDR isolates have now emerged as a significant clinical issue in numerous parts of the world. Transmission of epidemic strains is not only confined within individual hospitals but may extend to involve various healthcare facilities within the locality (Coelho *et al.*, 2006; Go *et al.*, 1994; Naas *et al.*, 2006b; Traub & Bauer, 2000). Furthermore, dissemination of resistant strains have also been reported at national, international and inter-continental levels (Jones *et al.*, 2004a; Lolans *et al.*, 2006; Naas *et al.*, 2006a; Peleg *et al.*, 2006a; Schulte *et al.*, 2005; Turton *et al.*, 2005). Examples include the dissemination of VEB-1 ESBL-producing *A. baumannii* clone in Europe and the spread of an amikacin-resistant *A. baumannii* clone in various regions of Spain (Naas *et al.*, 2006a; Vila *et al.*, 1999).

Table 1-3. Overview of antimicrobial resistance of *A. baumannii* from 1990 to 2006.

Antimicrobial Agent	Percentage of susceptible isolates[a]		
	1990-1992[1]	1995-1998[2]	2004-2006[3]
Ceftazidime	68-85%	64%	7.5-54%
Cefotaxime / Ceftriaxone	68-85%	35%	2.1-35%
Cefepime	N.D.	72%	16.5-56.2%
Amikacin	36-85%	96%	23.4-85%
Ciprofloxacin / Levofloxacin	6-92%	58-67%	7.5-55.3%
Imipenem	100%	100%	60.6-88.6%

[a]based on NCCLS/CLSI criteria; N.D., no data.

[1]Seifert *et al.*, 1993; [2]Wisplinghoff *et al.*, 2000; [3]Reinert *et al.*, 2007.

Most recently and of particular concern is a trend of increasing prevalence of carbapenem-resistance in *Acinetobacter* spp., which has emerged in the last decade (www.mystic-data.org; Perez *et al.*, 2007; Poirel & Nordmann, 2006b; Walther-Rasmussen & Høiby, 2006). Countries from various continents have reported the detection of such MDR isolates, including US, Brazil, UK, France, Italy, Spain, Turkey, Australia, Japan and Korea (Giamarellou *et al.*, 2008; Lee *et al.*, 2009; Lolans *et al.*, 2006; Poirel & Nordmann, 2006b; Quale *et al.*, 2003; Valenzuela *et al.*, 2007). In Europe, carbapenem-resistant *Acinetobacter* spp. was particularly prevalent in countries such as Turkey, Greece, England, Italy and Spain (Bou *et al.*, 2000; Coelho *et al.*, 2006; Poirel *et al.*, 2006; Walther-Rasmussen & Høiby, 2006). In the MYSTIC surveillance report of 48 European hospitals from 2002 to 2004, less than 75% and 70% of *Acinetobacter* isolates were susceptible to meropenem and imipenem, respectively (Unal & Garcia-Rodriguez, 2005). In North America, the data from the National Nosocomial Infection Surveillance System (NNISS) during the period of 1986 through 2003 involving many US hospitals showed a rise of carbapenem resistance in *Acinetobacter* isolates from 0% to 20% over the 18-year period (Gaynes & Edwards, 2005). In the MYSTIC surveillance programme from 2002 to 2004 Latin American isolates demonstrated a carbapenem resistance rate of nearly 30% in *Acinetobacter* (Unal & Garcia-Rodriguez, 2005), while in Asia, SENTRY isolates from 2001 through 2004 revealed that resistance rates for meropenem and imipenem exceeded 25% each (Gales *et al.*, 2006).

While most of the reported isolates were *A. baumannii*, other species were occasionally reported as well, such as *Acinetobacter* gensospecies 3, *A. calcoaceticus*,

A. junii and *A. baylyi* (Boo *et al.*, 2006; Lee *et al.*, 2009; Peleg *et al.*, 2006b; Yum *et al.*, 2002). Among such isolates, resistance to other classes of antimicrobial agents such as quinolones and aminoglycosides was also common.

1.4.2 Mechanisms of carbapenem resistance

Several mechanisms of carbapenem resistance have been characterised to date. Carbapenemases were the predominant mechanisms reported in many studies (Poirel & Nordmann, 2006b; Queenan & Bush, 2007; Walther-Rasmussen & Høiby, 2006). Ambler class D β-lactamases (ie. OXA carbapenemases) had become the most prevalent mechanism in *Acinetobacter* spp. for conferring carbapenem resistance, especially in European countries such as the UK, France, Greece, Spain and Turkey (Poirel & Nordmann, 2006b; Poirel *et al.*, 2006; Walther-Rasmussen & Høiby, 2006). Among the OXA carbapenemases, plasmid-mediated OXA-23 group and OXA-58 enzymes were most frequently reported and had a worldwide distribution (Coelho *et al.*, 2006; Peleg *et al.*, 2006a; Peleg *et al.*, 2006b; Perez *et al.*, 2007; Poirel *et al.*, 2006; Valenzuela *et al.*, 2007). Carbapenem resistance was also found to be mediated by chromosomally-mediated OXA carbapenemases such as OXA-24 and OXA-51 groups. OXA-24 group carbapenemases were found predominantly in Spain and Portugal but were also reported in the US (Lolans *et al.*, 2006; Poirel & Nordmann, 2006b). Many of the OXA carbapenemases were also associated with several insertion sequences such as IS*Aba1* and IS*Aba4*, which have been shown to

upregulate the expression of the β-lactamase genes through the provision of promoter sites for gene expression (Corvec *et al.*, 2007; Turton *et al.*, 2006).

Another group of β-lactamases reported in carbapenem-resistant *Acinetobacter* isolates were the Ambler class B enzymes (MBLs). In addition to conferring resistance to the carbapenems, they were often carried on integrons, thereby enhancing their transmissibility (Queenan & Bush, 2007; Yum *et al.*, 2002). MBLs especially of the IMP-types have been reported in *A. baumannii* predominantly in Asian countries such as Korea and Japan (Lee *et al.*, 2004; Poirel & Nordmann, 2006b; Queenan & Bush, 2007). Carbapenem resistance mediated by IMP-type MBLs is now a serious problem in parts of Asia such as Korea (Lee *et al.*, 2004).

Other mechanisms were probably involved as well, since some carbapenem-resistant isolates were found to possess neither of the above two classes of carbapenemases (Quale *et al.*, 2003). In such isolates, as well as isolates carrying class B and/or class D β-lactamases, other mechanisms were found that could have contributed to carbapenem resistance. Increased expression of Ambler class C cephalosporinase was found in *A. baumannii* strains in the US (Quale *et al.*, 2003). Decreased or loss of expression of certain outer membrane porins (such as 22 kDa, 33 kDa, 37 kDa and CarO porins) were also associated with carbapenem resistance (Bou *et al.*, 2000; Fernández-Cuenca *et al.*, 2003; Mussi *et al.*, 2005; Quale *et al.*, 2003). Changes in PBP profiles (such as a decrease in PBP-2 expression) or in efflux pump activities (such as an increase in AdeABC efflux pump expression) may also have contributed

to carbapenem resistance in *Acinetobacter* spp. (Fernández-Cuenca *et al.*, 2003; Héritier *et al.*, 2005; Huang *et al.*, 2008).

1.4.3 Clinical implications of carbapenem resistance

The emergence of carbapenem resistance in *Acinetobacter* spp. is an ominous development in antimicrobial resistance. Carbapenem resistance in *Acinetobacter* infections was associated with increased attributable mortality and prolonged hospitalisation (Kwon *et al.*, 2007; Playford *et al.*, 2007). Empirical treatment of *Acinetobacter* infections with carbapenems may also no longer be appropriate depending on the local epidemiology of antimicrobial resistance (Kwon *et al.*, 2007). Therapeutically, there are very few options for infections caused by such isolates, as they are often resistant to other commonly used classes of antimicrobial agents such as aminoglycosides and quinolones (Dijkshoorn *et al.*, 2007; Giamarellou *et al.*, 2008; Perez *et al.*, 2007).

Treatment options may be limited to polymyxins, tigecycline, or combination of antibiotics (Coelho *et al.*, 2006; Perez *et al.*, 2007). For HAIs by MDR *Acinetobacter* strains, colistin was used mainly for treatment of pneumonia, particularly VAP. Most studies investigating efficacy of colistin had confounding factors such as poorly defined protocols, lack of control group, concomitant use of other antibiotics (Giamarellou *et al.*, 2008). However, three studies comparing the efficacy of colistin

(for carbapenem-resistant isolates) versus carbapenem (for carbapenem-susceptible isolates) in the treatment of VAP caused by MDR *A. baumannii* or MDR *P. aeruginosa* found no significant differences in the clinical cure rates and/or mortality rates between the two groups (Garnacho-Montero *et al.*, 2003; Kallel *et al.*, 2007; Rios *et al.*, 2007).

Clinical data on efficacy of tigecycline for infections caused by MDR *A. baumannii* is limited. A recent review of eight retrospective clinical studies on a total of 42 patients reported clinical efficacy of tigecycline in 32 of 42 patients, although 28 patients also received other antibiotics (Karageorgopoulos *et al.*, 2008). The largest study included in the review on 25 patients reported clinical resolution in 21 patients (84%) with tigecycline monotherapy or in combination with imipenem for VAP and/or bacteraemia caused by MDR-*A. baumannii*. However, one patient with VAP and bacteraemia developed resistance to tigecycline during therapy (Schafer *et al.*, 2007). Other studies also reported the development of resistance following tigecycline therapy (Peleg *et al.*, 2007b; Reid *et al.*, 2007). In addition, an *in vitro* study demonstrated significant regrowth of an *A. baumannii* strain at 24 hours at tigecycline concentrations near the MIC, which were consistent with the mean maximum serum steady state concentrations achieved with standard dosing (Scheetz *et al.*, 2007). Hence, the current data does not support the use of tigecycline for BSIs caused by organisms with MICs of $\geq$ 1 μg/mL (Giamarellou *et al.*, 2008; Peleg *et al.*, 2007b). Caution should also be exercised when using tigecycline given the limited clinical data as well as the risk of development of resistance during therapy.

Doripenem has excellent activity against a variety of micro-organisms (see section 1-3-3) but may not be a suitable therapeutic option for infections by imipenem- and/or meropenem-resistant *Acinetobacter* spp. *In vitro* susceptibility results have demonstrated rather high doripenem MICs ranging from 8 to 64 μg/mL in carbapenem-non-susceptible strains. This would imply that the use of doripenem is unlikely to be clinically effective in the treatment of infections caused by such strains (Mushtaq *et al.*, 2004; Pillar *et al.*, 2008).

In view of the extent of resistance in *Acinetobacter*, the use of combination therapy has become an area of great interest. *In vitro* and animal studies have mostly concentrated on combinations involving rifampicin with either a polymyxin or a β-lactam (usually a carbapenem) (Perez *et al.* 2007). In the context of carbapenem resistance, the most logical combination is rifampicin and polymyxin. Results of *in vitro* studies on the synergy between rifampicin and polymyxin for carbapenem-resistant strains were conflicting, while in a mouse pneumonia model, the results were more favourable for colistin plus rifampicin than for colistin alone (Montero *et al.*, 2004; Wareham & Bean, 2006; Yoon *et al.*, 2004). For tigecycline, conflicting results were obtained in combination with other antibiotics including rifampicin, polymyxins, carbapenems and quinolones (Principe *et al.*, 2009; Scheetz *et al.*, 2007). But results from *in vitro* and animal studies may not guide or predict the outcome of antimicrobial therapy. In four retrospective and uncontrolled clinical studies, the clinical response rates of the combination of colistin and rifampicin in the treatment of patients with MDR *A. baumannii* infections varied from 50% to 100% (Bassetti *et al.*, 2008; Motaouakkil *et al.*, 2006; Petrosillo *et al.*, 2005; Song *et al.*, 2008).

Given the abilities of *Acinetobacter* spp. to acquire and disseminate resistance mechanisms as well as to adapt to hospital environments, MDR strains are liable to cause outbreaks in areas such as ICUs. Control of these outbreaks has proven be very challenging (Coelho *et al.*, 2006; Poirel *et al.*, 2006). Hence, infection control measures should be pursued aggressively. Measures such as meticulous environmental decontamination, contact precautions, handwashing, and alcohol hand decomtamination are all required to control the spread of MDR *Acinetobacter* spp. (Perez *et al.*, 2007). Antimicrobial stewardship programmes including the restriction of antimicrobial usage, the monitoring of antimicrobial resistance and its relationship to antimicrobial use, can also have an impact on MDR *Acinetobacter* spp. (Owens & Rice, 2006).

1.4.4 Molecular typing of *Acinetobacter* species

The molecular typing of *Acinetobacter* strains at the subspecies level is very important for various clinical and epidemiological purposes such as the investigation of suspected outbreaks or the identification potential reservoirs of the organism. Phenotypic typing methods such as antibiogram typing, serotyping and phage typing have been replaced largely by molecular typing methods (Bergogne-Bérézin & Towner, 1996). These molecular methods include pulsed-field gel electrophoresis (PFGE), ribotyping, AFLP analysis and MLST.

The PFGE typing method remains the reference method of choice for *Acinetobacter* spp. (Gouby *et al.*, 1992; Seifert & Gerner-Smidt, 1995; Seifert *et al.*, 2005). Although it is still a laborious method requiring several days to generate typing results, the method has the advantage of relative ubiquity of the necessary equipment in most reference and hospital-based diagnostic laboratories. *Apa*I or *Sma*I is usually the endonuclease used for the restriction of *Acinetobacter* chromosomal DNA. Chromosomal fragments are separated by gel electrophoresis and fingerprinting profiles compared either visually or using specialised software programmes such as Fingerprinting II (BioRad, Hercules, California, US) or BioNumerics software (Applied Maths NV, Sint-Martens-Latem, Belgium). While inter-laboratory comparison of fingerprinting results can be problematic, a study has demonstrated that inter-laboratory reproducibility can be achieved with adequate standardisation of protocol (Seifert *et al.*, 2005). Such protocol standardisation would allow early recognition of epidemic strains as well as rapid detection of outbreaks occurring at inter-hospital, regional, national or even international level.

Ribotyping was developed primarily to identify *Acinetobacter* isolates to the species level (Gerner-Smidt, 1992). It involves the enzymatic restriction of chromosomal DNA using endonucleases such as *Eco*RI, *Cla*I or *Sal*I. It is followed by electrophoresis, blotting and hybridisation with digoxigenin-11-UTP-labeled cDNA probes. Although it has good reproducibility, the method has limited discriminatory power and is laborious; and other methods such as PFGE are more commonly used as

they are less labour-intensive and more discriminatory (Seifert & Gerner-Smidt, 1995; Silbert *et al.*, 2004).

Typing by AFLP is a highly sensitive method of analysis. The methodology has been briefly reported in section 1.1.3. It is a laborious and expensive method, and the resulting complex profiles usually require analysis with appropriate software. While it is a relatively robust method for characterisation of *Acinetobacter* strains at the subspecies level and for outbreak investigation, it requires a high level of standardisation and extensive experience in interpretation of banding patterns even with the aid of sophisticated computer programmes (Janssen *et al.*, 1997; Koeleman *et al.*, 1998). As a result, this method is restricted to reference laboratories. Moreover, inter-laboratory comparison of results is problematic due to a lack of reproducibility when different detection platforms are used (Peleg *et al.*, 2008).

Multi-locus sequence typing (MLST) of *A. baumannii* has recently been developed as a DNA-sequencing method based on conserved regions of seven housekeeping genes: *gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi* and *rpoD* (Bartual *et al.*, 2005). The available MLST data achieved good concordance with typing results generated by PFGE and AFLP analyses, but the method requires further evaluation as only a limited number of *A. baumannii* and genospecies 13TU strains were used (Bartual *et al.*, 2005; Wisplinghoff *et al.*, 2008). It also remains to be determined if this typing scheme is appropriate for other *Acinetobacter* spp. as well. While the discriminatory power of the proposed MLST system is comparable to those of PFGE and AFLP analyses, it is

an expensive and labour-intensive method, and hence may not be suited for routine outbreak analysis or other small-scale epidemiological analysis.

1.4.5 Carbapenem-resistant *Acinetobacter* in Ireland

Little has been reported in Ireland regarding the epidemiology of carbapenem-resistant *Acinetobacter* isolates with the exception of a report on an outbreak involving the clonal spread of an *A. baumannii* strain among four patients in the Intensive Care Unit (ICU) of St. James's Hospital in Dublin in 2001 (Doyle *et al.*, personal communication). There is no data available for the national or regional prevalence rates of carbapenem-resistant *Acinetobacter* spp. over the last few years. In 2006, I reported the emergence of carbapenem resistance in two clinical isolates of *Acinetobacter* genospecies 3 (Boo *et al.*, 2006). Carbapenem resistance in that species was not observed elsewhere before the latter report. Furthermore, those isolates were also the first carbapenem-resistant *Acinetobacter* isolates encountered in the microbiology laboratory of St. James's Hospital, Dublin, since the ICU outbreak in 2001.

1.5 Aims of the study

The investigative work in this study is divided into three chapters: chapters 2, 3 and 4, respectively. In chapter 2, the aims of the study were:

- to obtain accurate epidemiological data of *Acinetobacter* isolates in St. James's Hospital using identification and typing methods,
- to determine the prevalence rates of resistance to antimicrobial agents using different AST methods,
- to correlate clinical and epidemiological data with microbiological and molecular typing data of the isolates.

In chapter 3, the study aimed to achieve the following:

- to characterise the prevalence and types of β-lactamase genes and other transferable resistance elements in the *Acinetobacter* isolates using phenotypic and molecular methods,
- to evaluate the prevalence of carbapenemase genes, the regulation of their expression and the modes of genetic dissemination,
- to compare the data on resistance genes in *Acinetobacter* isolates collected in St. James's Hospital with data from other centres.

In chapter 4, the aims of the study were:

- to investigate the role of efflux mechanism in carbapenem resistance in *Acinetobacter* isolates through the use of EPI and quantitative analysis of efflux gene expression,
- to investigate the role of permeability mechanism in carbapenem resistance in *Acinetobacter* isolates through the evaluation of OMP profiles of isolates and PCR of OMP genes.

Chapter 2

Identification, antimicrobial susceptibility and epidemiological typing of *Acinetobacter* isolates

2.1 Introduction

Acinetobacter spp. have emerged as a particularly formidable group of Gram-negative nosocomial pathogens worldwide, causing outbreaks and severe infections by virtue of their tenacious persistence in the clinical environments and their propensity for the rapid development of resistance to a wide range of antimicrobial agents (Dijkshoorn *et al.*, 2007; Giamarellou *et al.*, 2008; Perez *et al.*, 2007). The range and clinical impact of infections caused by *Acinetobacter* spp. and their resistance to different classes of antibiotics have been discussed in sections 1.2.3, 1.2.4 and 1.3, respectively. The most worrying development in recent years is the emergence of carbapenem resistance in *Acinetobacter* spp. on a worldwide scale (see section 1.4.1). Carbapenem resistance has significant implications on clinical therapeutic options and on infection control in healthcare settings; it can also have a significant impact on patient mortality, morbidity as well as healthcare expenditure. These issues have been discussed in section 1.4.3.

Phenotypic methods have been shown to have poor accuracy in the identification of *Acinetobacter* spp., while molecular methods such as ARDRA and *rpoB* sequencing have produced much more accurate results (see section 1.1.3). Nonetheless, molecular methods still remain too specialised and/or laborious for routine application in most diagnostic microbiology laboratories. Consequently, accurate epidemiological data on *Acinetobacter* spp. may not be obtained in diagnostic laboratories. In the microbiology laboratory of St. James's Hospital, *Acinetobacter* isolates are

presumptively identified by commercial phenotypic test systems such as VITEK 2 and API 20NE (bioMerieux). As such, there is a paucity of accurate data on the local epidemiology of *Acinetobacter* spp. This is compounded by the paucity of published data on *Acinetobacter* epidemiology on a national level.

Published data on antimicrobial resistance patterns and trends in *Acinetobacter* spp. in Ireland is also lacking. In particular, there is little published data on carbapenem resistance in *Acinetobacter* spp. in Ireland (Boo *et al.*, 2006). With the emergence and spread of carbapenem resistance in *Acinetobacter* on a global scale, antimicrobial resistance data both locally and nationally, along with clinical and epidemiological data, are urgently required to inform decisions pertaining to infection control, antibiotic stewardship and prescribing guidelines.

Like many diagnostic laboratories, the microbiology laboratory in St. James's Hospital is increasingly relying on automated test systems such as VITEK 2 for susceptibility testing. The potential pitfalls and problems associated with susceptibility testing by automated systems have been discussed in section 1.3.9. In particular, very major errors in piperacillin-tazobactam and carbapenem susceptibility results in *P. aeruginosa* were produced by such systems (Juretschko *et al.*, 2007; Sader *et al.*, 2006). The accuracy of automated susceptibility testing on *Acinetobacter* spp., especially pertaining to the above antibiotics, also needs to be further evaluated.

In this chapter, the study aimed to determine the epidemiology of *Acinetobacter* spp. in St. James's Hospital through the identification of isolates using phenotypic and

molecular methods as well as molecular typing of strains using PFGE. The study also aimed to establish detailed antimicrobial susceptibility profiles of the *Acinetobacter* isolates using three different methods of susceptibility testing (disc diffusion, VITEK 2 and Etest methods) and determine the prevalence rates of resistance to various antibiotics. Finally, this chapter aimed to correlate the epidemiological and susceptibility data with relevant clinical data.

2.2 Materials and methods

2.2.1 Identification and speciation of *Acinetobacter* species

Bacterial isolates presumptively identified as *Acinetobacter* species were prospectively collected in St. James's Hospital microbiology laboratory over a 32-month period from May 2005 to December 2007 inclusive.

Where the same *Acinetobacter* spp. was isolated from multiple sites in a patient, only the first isolate was collected if the antimicrobial susceptibility patterns were similar in the various isolates. Where isolates from the same patient presented with differences in susceptibility to three or more antibiotics, an isolate representative of each susceptibility pattern was collected.

2.2.1.1 Presumptive identification of *Acinetobacter* spp.: VITEK 2 GN identification system

Presumptive identification of *Acinetobacter* isolates based on phenotypic tests was carried out with the VITEK 2 GN version 4.03 Gram-negative bacilli identification system (bioMerieux, Basingstoke, Hampshire, UK). It is an integrated and automated system that performs the identification of Gram-negative bacilli using biochemical methods and phenotypic algorithms based on colorimetry. The inoculum was prepared from a fresh overnight culture on blood agar of the isolate to a concentration of 0.5 McFarland using the VITEK Densichek densitometer. The inoculum was transferred into a sterile tube which had been loaded on to the VITEK Smart carrier cassette. Within 15 minutes, the VITEK 2 GN identification card was inoculated with the suspension. The cassette was then loaded on to the VITEK 2 system for analysis. The data were analysed using the VITEK 2 Advanced Expert System software version 4.03 and the results were automatically transferred to the laboratory information management system via computer interface. The results were reported as the organism identified along with the probability of accuracy for the identification procedure. Isolates identified as *Acinetobacter* species by VITEK 2 to a probability of $\geq$ 90% were included for further confirmation of identification.

2.2.1.2 Molecular identification of *Acinetobacter* spp.: amplification and sequencing of *rpoB* and flanking spacer regions

The limitations of phenotypic tests in the speciation of *Acinetobacter* isolates have been discussed in section 1.1.3. A molecular method of *Acinetobacter* speciation was used to compare identification results with the VITEK 2 phenotypic method as described in section 2.2.1.1. The molecular method selected for the study was the DNA-sequence analysis of the *Acinetobacter rpoB* gene and its flanking spacer regions (La Scola *et al.*, 2006). Disadvantages of other commonly used molecular methods such as ARDRA and AFLP have been discussed in section 1.1.3. Compared to other methods, *rpoB* sequencing was relatively inexpensive, less labour-intensive, has greater simplicity in methodology. It has been validated as an accurate method in *Acinetobacter* identification and has the additional advantage of being validated for species only characterised in recent years, such as *A. schindleri*, *A. ursingii* and *A. parvus* (Gundi *et al.*, 2009; La Scola *et al.*, 2006; Vaneechoutte *et al.*, 2008). Sequencing results can also be expediently and universally compared with results elsewhere via the accession of data in sequence databases such as GenBank® via the internet.

2.2.1.2.1 PCR amplification

Two regions were selected for amplification and nucleotide sequencing: a 450-bp region of the *rpoB* and a region flanking the *rpoB* gene and the *rpoC* gene downstream (86-177 bp). Sequencing of the 450-bp *rpoB* region can reliably distinguish most species (showing < 96% sequence similarities in pairwise

comparisons) except for the pairs *A. baylyi*/genomic species 11 and *A. junii*/*A. grimontii* which demonstrated 98.8% and 99.6% similarities respectively (La Scola *et al.*, 2006). For isolates identified via *rpoB* sequencing as either *A. baylyi* or *Acinetobacter* genomic species 11, further PCR and sequencing of the *rpoB-rpoC* flanking region was performed. Sequence similarity of 83.8% was found in the *rpoB-rpoC* flanking regions of the two species (La Scola *et al.*, 2006). None of the four regions described by La Scola *et al.* could reliably distinguish between *A. junii* and *A. grimontii*. The results were consistent with the recent finding that *A. grimontii* is synonymous with *A. junii* (Vaneechoutte *et al.*, 2008).

Bacterial DNA was first extracted by boiling 200 µL of bacterial suspension in sterile water for 10 minutes. The boiled suspension was then centrifuged at >10,000 x *g* for five minutes and the supernatant containing the DNA was extracted for subsequent PCR.

The primer sequences used for the amplification of the 450-bp *rpoB* region were 5'-GTGATAARATGGCBGGTCGT-3' and 5'-CGBGCRTGCATYTTGTCRT-3' (forward and reverse primers, respectively); and those for the amplification of the *rpoB-rpoC* flanking regions were 5'-GTTCTTTAGGTATCAACATTGAA-3' (forward primer) and 5'-GACGCAAGACCAATACGRAT-3' (reverse primer), where B=G+T+C, R=A+G, and Y=C+T (La Scola *et al.*, 2006). The primers were obtained from Sigma-Aldrich Ltd. (Wicklow, Ireland).

PCR amplification was performed with the respective primers using the Qiagen PCR core kit (containing Taq polymerase) according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany). The PCR reaction was carried out in the GeneAmp 9700 PCR system thermal cycler (Applied BioSystems, Warrington, UK) under the following conditions:

Initial denaturation:	3 min at 94 °C
35 cycles of:	
Denaturation:	30 sec at 94 °C
Annealing:	30 sec at 56 °C
Extension:	1 min at 72 °C
Final extension:	10 min at 72 °C
Storage:	∞ at 4 °C

Agarose gels were prepared using 0.5 M TBE buffer (Sigma-Aldrich) and cast in a 7 x 15 cm casting tray. Five µL of the PCR product was loaded onto a 1.25% agarose gel and electrophoresed at a voltage of 90 V for 60 minutes. The gel was subsequently stained in ethidium bromide (1 µg/mL) and the DNA bands visualised under ultraviolet illumination. Photographic images were taken using Quantity One 1D-analysis software (BioRad, Hercules, California, US). Purification of the amplicons was carried out using the Qiaquick PCR purification kit according to the manufacturers' instructions (Qiagen GmbH, Hilden, Germany). Purified amplicons were eluted in a final volume of 30 µL of elution buffer. The purified products were then used as templates for nucleotide sequencing.

2.2.1.2.2 DNA sequencing

DNA concentration of the purified amplicon was estimated using the ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, Detroit, US) using a path length of 0.2 mm and an absorbance ratio of 260/280. The amplicon was then diluted to a 20ng/100bp/10μL concentration for DNA sequencing using elution buffer of the Qiaquick PCR purification kit. DNA sequencing was carried out using the BigDye terminator V3.1 cycle sequencing kit (Applied BioSystems, Warrington, UK) according to the manufacturer's instructions except the 1:8 dilution of the concentration of the BigDye terminator. Modified reaction component volumes were as follows:

Big-Dye Terminator	0.5 μL
5 X Sequencing Buffer	4 μL
Nuclease-free water	11.5 μL
Primer (10μM)	1.5 μL
Diluted amplicon	2.5 μL
Total volume per sample	20 μL

Amplification using the same set of primers as for initial PCR amplification was performed in a 96-well microtitre plate (Applied BioSystems, Warrington, UK) in the GeneAmp 9700 PCR system thermal cycler under the following conditions:

Initial denaturation:	1 min at 96 °C
Amplification: 25 cycles of:	

Denaturation:	10 sec at 96 °C
Annealing:	10 sec at 51 °C
Extension:	4 min at 60 °C
Final extension:	10 min at 60 °C
Storage:	∞ at 4 °C

Ethanol/sodium acetate was used to completely remove excess terminators from the reaction. After PCR amplification was completed, 2 µL of 3 M sodium acetate and 50 µL of 96% molecular grade ethanol was added to each well of the microtitre plate. Reaction components were mixed by pipetting up and down several times. The plate was sealed with an adhesive strip (Applied BioSystems, Warrington, UK) and incubated for 15 minutes at room temperature. The plate was then centrifuged at 1400 x *g* for 45 minutes in a Beckman Allegra 6A centrifuge (Beckman Coulter, Fullerton, California, US). The adhesive seal was then removed and the plate was inverted onto tissue paper and spun upside down up to 185 x *g* to empty the wells. A 70 µL volume of 70% ethanol was added to each well. The plate was sealed and spun again at 1400 x *g* for 15 minutes as described previously. The plate was removed from the centrifuge and inverted as previously described and spun upside-down again at 185 x *g* for one minute to thoroughly remove all ethanol. All minute traces of ethanol were finally removed by heating the plate up to 95 °C for one minute in the GeneAmp 9700 system thermal cycler. Pellets were resuspended in 20µL of the injection buffer HiDi Formamide (Applied BioSystems). Automated DNA sequencing was then performed on all samples using the ABI Prism 3130*xl* Analyzer (Applied BioSystems).

Sequence traces were analysed and a consensus sequence for each sample was obtained using the Lasergene SeqMan sequence analysis software (DNASTAR, Madison, Wisconsin, US). The consensus sequence data for the amplicons were then compared with reference sequences of the respective species using the Basic Local Alignment Search Tool (BLAST) programme of the National Center of Biotechnology Information (www.blast.ncbi.nih.gov) and the levels of concordance with the respective reference sequences were noted.

2.2.1.2.3 Reference strains used in the *rpoB* sequence analysis

GenBank accession numbers of reference strains for *rpoB* sequences for the isolates in this study are as follow (La Scola *et al.*, 2006):

DQ207474 (*A. calcoaceticus*); DQ207471 (*A. baumannii*); DQ207479 (*Acinetobacter* genosp. 3); DQ207484 (*A. haemolyticus*); DQ207485 (*A. johnsonii*); DQ207487 (*A. lwoffii*); DQ207481 (*Acinetobacter* genosp. 9); DQ207475 (*Acinetobacter* genosp. 10); DQ207476 (*Acinetobacter* genosp. 11); DQ207489 (*A. radioresistens*); DQ207478 (*Acinetobacter* genosp. 13BJ); DQ207490 (*A. schindleri*); DQ231239 (*A. ursingii*); DQ207492 (*A. tjernbergiae*).

2.2.2 Antimicrobial susceptibility testing (AST)

AST was performed using three different laboratory methods of CLSI disc diffusion method, Etest gradient MIC method and VITEK 2 automated susceptibility method for the following antimicrobial agents: cefotaxime, ceftazidime, cefepime, piperacillin-tazobactam, meropenem, gentamicin, amikacin, ciprofloxacin and

trimethoprim-sulphamethoxazole. In addition, AST was also performed using the Etest method for the following antibiotics: ticarcillin-clavulanic acid, amoxicillin-clavulanic acid, imipenem, doripenem, colistin and tigecycline. Ampicillin-sulbactam susceptibility testing was not performed as the Etest strip for the above antibiotic was not available to the laboratory at the time of the study; moreover, ampicillin-sulbactam was unavailable for clinical usage in Ireland.

2.2.2.1 Disc diffusion method

The disc diffusion method is a standardised methodology recommended by the CLSI Subcommittee on Antimicrobial Susceptibility Testing (CLSI, 2009). Following a fresh overnight culture of the organism on sheep blood agar, a bacterial suspension in sterile saline was made to a turbidity of 0.5 McFarland as measured by a nephelometer. The suspension was then inoculated within 15 minutes onto Mueller-Hinton II agar (BBL, Sparks, Maryland, US) using a sterile cotton swab. Antibiotic discs were then applied onto the agar using an antibiotic disc dispenser. The antimicrobial agents and the disc strengths used in this study were shown in Table 2-1 in accordance to the guidelines provided by CLSI for antimicrobial susceptibility testing of *Acinetobacter* spp. (CLSI, 2009). The plates were incubated at 37 ^{o}C in ambient air for 20 hours and the inhibitory zone diameter for each antibiotic was measured and compared with interpretative breakpoints stipulated by CLSI as shown in Table 2-2 (CLSI, 2009).

Table 2-1. Disc content and MIC ranges of the methods used in antimicrobial susceptibility testing.

Antimicrobial agent	Disc diffusion method antibiotic concentration (µg)[a]	E-test gradient method MIC range (µg/mL)	VITEK 2 AST method MIC range (µg/mL)
Piperacillin-tazobactam	100/10	0.016/4 – 256/4	4 - 128
Cefotaxime	30	0.016 – 256	1 - 64
Ceftazidime	30	0.016 – 256	1 - 64
Cefepime	30	0.016 – 256	1 - 64
Ciprofloxacin	5	0.002 – 32	0.25 – 4
Gentamicin	10	0.016 – 256	1 - 16
Amikacin	30	0.016 – 256	2 - 32
Trimethoprim-sulphamethoxazole	1.25/23.75	0.002/0.038 – 32/608	20 – 320[b]
Meropenem	10	0.002 – 32	0.25 – 16
Imipenem	ND	0.002 – 32	ND
Doripenem	ND	0.002 – 32	ND
Tigecycline	ND	0.016 – 256	ND
Colistin	ND	0.064 – 1024	ND
Ticarcillin-clavulanic acid	ND	0.016/0.008 – 256/128	ND
Amoxicillin-clavulanic acid	ND	0.016/0.008 – 256/128	ND

[a]according to CLSI recommendations; [b]sulphamethoxazole component.

ND, not done in the study.

Table 2-2. CLSI zone diameter and MIC interpretative standards for *Acinetobacter* spp. (CLSI M100-S19, Jan 2009).

Antimicrobial Agent	Zone Diameter Breakpoints (mm)			MIC Interpretative Standard (μg/mL)		
	S	I	R	S	I	R
Cefotaxime	≥ 23	15-22	≤ 14	≤ 8	16-32	≥ 64
Ceftazidime	≥ 18	15-17	≤ 14	≤ 8	16	≥ 32
Cefepime	≥ 18	15-17	≤ 14	≤ 8	16	≥ 32
Piperacillin-tazobactam	≥ 21	18-20	≤ 17	≤ 16/4	32/4 – 64/4	≥ 128/4
Ticarcillin-clavulanic acid	≥ 20	15-19	≤ 14	≤ 16/2	32/2– 64/2	≥ 128/2
Meropenem	≥ 16	14-15	≤ 13	≤ 4	8	≥ 16
Imipenem	≥ 16	14-15	≤ 13	≤ 4	8	≥ 16
Ciprofloxacin	≥ 21	16-20	≤ 15	≤ 1	2	≥ 4
Gentamicin	≥ 15	13-14	≤ 12	≤ 4	8	≥ 16
Amikacin	≥ 17	15-16	≤ 14	≤ 16	32	≥ 64
Trimethoprim-sulphamethoxazole	≥ 16	11-15	≤ 10	≤ 2/38	-	≥ 4/76
Colistin	-	-	-	≤ 2	-	≥ 4

S, susceptible; I, intermediate; R, resistant.

2.2.2.2 VITEK 2 antimicrobial susceptibility testing system

In tandem with organism identification, VITEK 2 (bioMerieux) has also developed an automated and integrated system of antimicrobial susceptibility testing (AST) based on kinetic analysis of growth data. The method is based on the MIC technique, whereby the VITEK 2 Gram-negative AST-N020 card is essentially a miniaturized version of the doubling dilution technique for MIC determination by the microdilution method. Each card contains 64 microwells including a control well. The remaining wells contain pre-measured amounts of specific antibiotic combined with culture media. The procedure is similar to the above VITEK 2 method for organism identification. An inoculum of 0.5 McFarland was made from a fresh overnight culture of the isolate and transferred into a sterile tube which was loaded onto VITEK 2 Smart carrier cassette. The VITEK 2 Gram-negative AST-N020 card was then inoculated with the bacterial suspension and the cassette was then loaded onto the VITEK 2 automated system. The MICs for the respective antimicrobial agents were analysed using the VITEK 2 Advanced Expert System software version 4.03 and the results were transferred to the laboratory information management system. The antimicrobial agents and the MIC ranges for the VITEK 2 system were also shown in Table 2-1. Interpretation of MIC results was based on MIC interpretative standards laid down by CLSI as shown in Table 2-2 (CLSI, 2009).

2.2.2.3 Etest gradient MIC method

Etest (AB Biodisk, Solna, Sweden) is a quantitative MIC method which comprises of the application of an antimicrobial-impregnated strip with a predefined concentration gradient of the antimicrobial agent to agar media inoculated with the organism. The MIC value is the lowest concentration of the antimicrobial agent to inhibit growth of the organism.

A bacterial suspension of 0.5 McFarland turbidity was made from an overnight fresh culture of the isolate. The bacterial suspension was inoculated onto the test agar media using a sterile cotton swab. The choice of media depended on the methodology employed. For the methodology recommended by CLSI, the medium used was Mueller-Hinton II agar (BBL, Sparks, Maryland, US) (CLSI, 2009). For the BSAC methodology, the recommended medium varied depending on the antimicrobial agent tested. For tigecycline, the medium was Iso-Sensitest agar (Oxoid, Basingstoke, Hampshire, UK), while the medium for carbapenems such as doripenem was Mueller-Hinton II agar (BBL, Sparks, Maryland, US) (BSAC, 2009b). In this study, Etest MIC method for tigecycline was also performed on Mueller-Hinton agar for comparison with the results obtained with Iso-Sensitest agar. Following the inoculation of the agar plate, the agar surface was allowed to dry for 10-15 minutes before the application of the E-test strip. The agar plate was then incubated at 37 ^{o}C in ambient air for 20 hours and the determination of MIC was performed in accordance to the manufacturer's instructions. Etest strip MIC ranges for the respective antimicrobial agents are shown in Table 2-1. Interpretation of MIC values for the respective antibiotics was carried out according to CLSI interpretative standards as shown in Table 2-2 except for

doripenem, tigecycline, and amoxicillin-clavulanic acid. CLSI did not have interpretative criteria for the three agents at the time of the study (CLSI, 2009). Interpretation of MIC values for doripenem and tigecycline was performed in accordance to the BSAC interpretative criteria as shown in Table 2-3 (BSAC, 2009a). As with CLSI, no interpretative standards were available for amoxicillin-clavulanic acid from BSAC.

2.2.2.4 Analysis of antimicrobial susceptibility results.

Antimicrobial susceptibility results from the three methods were categorised into susceptible, intermediate or resistant categories according to the appropriate interpretative standards. Categorical agreement, defined as test results within the same susceptibility category, was compared between the three methods of susceptibility testing. The acceptable categorical agreement between methods is $\geq 90\%$ as recommended by CLSI (CLSI, 2007). Using the CLSI disc diffusion as the reference method, error rates for the Etest and VITEK 2 methods were calculated for each antimicrobial agent.

Errors were categorised into very major errors (VME), major errors (ME) and minor errors (mE). A VME is defined as a false-susceptible result; an ME is defined as a false-resistant result and an mE is defined as a susceptible or resistant result from one method, and an intermediate result from another method (CLSI, 2007). Unacceptable levels of errors are $\geq 1.5\%$ for VME, $\geq 3\%$ for ME and $\geq 10\%$ for mE as recommended by CLSI document M23-A3 (CLSI, 2007).

Table 2-3. BSAC MIC interpretative standards for tigecycline and doripenem for *Acinetobacter* spp. (BSAC Methods for Antimicrobial Susceptibility Testing version 8, Jan 2009).

Antimicrobial agent	MIC Interpretative Standard (μg/mL)		
	S	I	R
Doripenem	≤ 1	2-4	≥ 8
Tigecycline	≤ 1	2	≥ 4

S, susceptible; I, intermediate; R, resistant.

Non-susceptibility rates, defined as the combination of intermediate and resistant results, were also derived for the following groups of isolates: all *Acinetobacter* isolates, *A. calcoaceticus-baumannii* complex (Acbc) isolates, non-Acbc isolates, *A. baumannii* isolates and *Acinetobacter* genospecies 3 isolates. Statistical comparison of non-susceptibility rates of the respective groups of isolates was carried out using the Fisher's exact test for categorical data.

2.2.3 Molecular typing of *Acinetobacter* isolates

Molecular typing of *Acinetobacter* isolates was performed using the current gold standard method of PFGE. PFGE was the most widely used method of typing for *Acinetobacter* isolates and it was carried out in accordance to the method proposed by Seifert *et al.* for the purpose of standardisation of PFGE methodology for the analysis of *Acinetobacter* fingerprinting results (Seifert *et al.*, 2005).

2.2.3.1 Plug preparation

Isolates were inoculated onto sheep blood agar and incubated for 20 hours at 37 °C in ambient air. A loopful of the organism was taken from the agar and suspended in 2.5 mL of suspension buffer comprising of 100 mM of Tris and 100mM of EDTA adjusted to a pH of 8.0. The bacterial suspension was then adjusted to give a cell density of approximately 10^9 cells/mL (3.5 McFarland standard) using a

nephelometer. The bacterial suspension was incubated at 55 °C for 10 minutes in a waterbath, followed by the addition of 25 µL of proteinase K (from 20mg/mL stock solution in ultrapure water) and mixed gently by inverting the tube three to four times. 2.5 mL of molten 1% SeaKem Gold agarose (Biozym Diagnostics GmbH, Oldendorf, Germany) plus 1% sodium dodecyl-sulphate (SDS) was prepared with TE Buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The molten agarose was added to the bacterial suspension and the agarose/bacterial suspension combination was mixed by pipetting up and down several times. The mixture was then immediately dispensed into the wells of disposable plug moulds (BioRad, Hercules, California, US). The agarose plugs were left to solidify at room temperature for five minutes followed by another five minutes at 4 °C.

2.2.3.2 Cell lysis and washing of plugs

Plugs were transferred to disposable screw-cap 50-mL tubes containing five mL of cell lysis buffer (50 mM Tris, 50 mM EDTA, pH 8.0, 1% sarcosine) and 25 µL of proteinase K (from 20 mg/mL stock solution). The tubes are incubated in a shaking waterbath for two hours with constant and vigorous agitation at 150-200 rpm to achieve effective lysis of cells. After lysis, the buffer was removed and the plugs were washed five times (15 min/wash) at 55 °C (twice with sterile ultrapure water and thrice with TE buffer; 10 mL for each washing step) in the shaking waterbath. The water and TE buffer were preheated to 50 - 55 °C before each washing step. After the last wash, the TE buffer was poured off and 5 - 10 mL of fresh TE buffer was added

to each tube. The plugs was used on the same day or stored for later use (within several weeks) at 4 °C.

2.2.3.3 Restriction digestion and gel loading

A slice measuring about 4 mm by 5.5 mm was cut from each plug using a scalpel, and transferred to a microcentrifuge tube containing 200 µL of Digestion Buffer SA (Sigma-Aldrich, Wicklow, Ireland) plus 100 µg/mL of purified bovine serum albumin (New England Biolabs, Hitchin, Herts, UK). The plug slices were incubated in this buffer for 15 minutes at 25 °C. The digestion buffer was then removed and replaced with 200 µL of fresh digestion buffer containing 30 units of restriction endonuclease Apa I (Sigma-Aldrich, Wicklow, Ireland). The plug slices were incubated at 25 °C for two hours. Prior to casting of the gel, the restriction mixture was removed from each tube and replaced with 200 µL of 0.5 X TBE (from 10 X TBE stock solution comprising of 0.89 M Tris, 0.89M boric acid and 20 mM EDTA, pH 8.3).

The plug slices were allowed to stand at room temperature for five minutes, after which they were loaded into appropriate wells of a 1% SeaKem Gold agarose gel (Biozym Diagnostics GmbH, Oldendorf, Germany). PFGE Pulse Marker lambda DNA marker of 50-1000 kb (Sigma-Aldrich, Wicklow, Ireland) was loaded into lanes 1, 8 and 15 to allow subsequent normalisation of electrophoretic patterns across the gel, and plug slices of isolates were loaded into lanes 2 to 7 and lanes 9 to 14.

2.2.3.4 Electrophoresis

Electrophoresis was performed in a contour-clamped homogenous electric field (CHEF) using the CHEF Mapper apparatus (BioRad, Hercules, California, US). Running temperature was set at 14 ^{o}C and the duration of the run time was 18 hours. The voltage was 6 V/cm, and switch times ranged from 2.16 seconds to 54.17 seconds, with linear ramping. Following the completion of electrophoresis, the gel was stained with 400 mL of ethidium bromide solution (1 µg/mL of ethidium bromide) for 30 minutes followed by 45 minutes to an hour of de-staining in distilled water. The DNA bands on the gel were visualised with ultraviolet transillumination and digital photographic images were taken using Quantity One 1D-analysis software (BioRad, Hercules, California, US) and stored electronically as TIFF files.

2.2.3.5 Data analysis

Analysis of relatedness of PFGE patterns was performed using the Fingerprinting II software version 3 (BioRad, Hercules, California, US). DNA relatedness of PFGE patterns were calculated using the band-based Dice coefficient with an optimisation setting of 1% for the whole profile and a tolerance setting of 1-2% band tolerance. Only bands larger than 50 kb were analysed.

2.2.4 Demographic and clinical data of isolates

Clinical, epidemiological and demographic data of the isolates and patients were obtained from the laboratory information management system, hospital patient information database as well as clinical case notes.

2.3 Results

2.3.1 Identification of *Acinetobacter* species

A total of 121 isolates from 120 patients were collected in the microbiology laboratory of St. James's Hospital for further analysis over the 32-month period. Two isolates (27616 and 27458) from one patient were collected as the isolates demonstrated significant differences in susceptibility to five antibiotics (piperacillin-tazobactam, ticarcillin-clavulanic acid, meropenem, imipenem and doripenem).

All 121 isolates were identified as *Acinetobacter* spp. by VITEK 2 GNI to probability levels of > 90%. 117 isolates were confirmed to be *Acinetobacter* isolates by *rpoB* sequencing. Four isolates presumptively identified as *A. lwoffii* by VITEK 2 were subsequently identified by *rpoB* sequencing as *Pseudomonas fluorescens* (two isolates), *Psychrobacter* sp. (one isolate) and *Photorhabdus* sp. (one isolate). The accuracy of VITEK 2 in the identification of *Acinetobacter* isolates to the genus level

was 96.7% (117/121). The above four isolates were subsequently excluded from further analysis in the study.

Speciation results of the remaining 117 *Acinetobacter* isolates by VITEK 2 and *rpoB* sequencing methods were shown in Table 2-4. VITEK 2 identified most isolates to probability levels of ≥ 95% except for eight isolates that were identified either as *A. junii* or *A. lwoffii* to probability levels ranging from 91.3% to 93.7%. All eight isolates were subsequently confirmed as *A. ursingii* by *rpoB* sequencing. Sequencing results of PCR of *rpoB* gene as well as *rpoB-rpoC* spacer achieved high levels of sequence concordance with reference strains' sequences. For *rpoB*, there was 98.4% to 100% intra-species relatedness, with 93.1% of isolates achieved sequence concordance of over 99% compared to reference strains, while all isolates achieved concordance of > 98% compared to reference strains (see Table 2-4).

A total of 78, 30, seven and two isolates were identified by VITEK 2 as *A. baumannii*, *A. lwoffii*, *A. junii* and *A. haemolyticus* respectively. Of the 117 isolates, in comparison with *rpoB* sequencing results, only 30 isolates were correctly speciated by VITEK 2. The overall error rate of speciation of *Acinetobacter* isolates by VITEK 2 GNI system was 74.4% (87/117).

Of the 78 *A. baumannii* isolates presumptively identified by VITEK 2, *rpoB* sequencing confirmed 27 isolates to be *A. baumannii*, while 45 and six isolates were

Table 2-4. Comparison of speciation results of *Acinetobacter* spp. using VITEK 2 GNI identification system and *rpoB* sequencing methods.

Isolate Identifier	Identification by VITEK 2 (confidence level)	Identification by *rpoB* sequencing (sequence concordance with reference strains)
229437	*A. baumannii* (99.0%)	*A. baumannii* (99.8%)
11648	*A. baumannii* (99.0%)	*A. baumannii* (99.8%)
26756	*A. baumannii* (99.0%)	*A. baumannii* (100%)
101049	*A. baumannii* (99.0%)	*A. baumannii* (99.8%)
20802	*A. baumannii* (99.0%)	*A. baumannii* (99.8%)
217068	*A. baumannii* (99.0%)	*A. baumannii* (100%)
12937	*A. baumannii* (99.0%)	*A. baumannii* (99.3%)
89848	*A. baumannii* (99.0%)	*A. baumannii* (99.6%)
26750	*A. baumannii* (99.0%)	*A. baumannii* (99.6%)
181329	*A. baumannii* (99.0%)	*A. baumannii* (99.8%)
27623	*A. baumannii* (99.0%)	*A. baumannii* (100%)
102450	*A. baumannii* (99.0%)	*A. baumannii* (98.9%)
29419	*A. baumannii* (99.0%)	*A. baumannii* (99.8%)
14495	*A. baumannii* (99.0%)	*A. baumannii* (99.6%)
13207	*A. baumannii* (99.0%)	*A. baumannii* (99.3%)
100983	*A. baumannii* (99.0%)	*A. baumannii* (99.8%)
14773	*A. baumannii* (99.0%)	*A. baumannii* (99.6%)
33047	*A. baumannii* (99.0%)	*A. baumannii* (99.1%)
107228	*A. baumannii* (99.0%)	*A. baumannii* (99.8%)
31364	*A. baumannii* (99.0%)	*A. baumannii* (99.3%)
107532	*A. baumannii* (99.0%)	*A. baumannii* (99.8%)
107245	*A. baumannii* (99.0%)	*A. baumannii* (99.8%)
27021	*A. baumannii* (99.0%)	*A. baumannii* (100%)
233651	*A. baumannii* (99.0%)	*A. baumannii* (99.6%)
232982	*A. baumannii* (99.0%)	*A. baumannii* (99.8%)
218722	*A. baumannii* (99.0%)	*A. baumannii* (99.8%)
220165	*A. baumannii* (99.0%)	*A. baumannii* (99.3%)
29540	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (100%)
12659	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.6%)
23269	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.6%)
14448	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.8%)
14748	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.8%)
28594	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.6%)
27616	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.6%)
32357	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (100%)
36277	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (100%)
103165	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.8%)
206479	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (100%)

Table 2-4 (Continued)

Isolate Identifier	Identification by VITEK 2 (% probability)	Identification by *rpoB* sequencing (sequence concordance with reference strains)
106770	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.8%)
27458	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.6%)
88276	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.6%)
105621	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.8%)
86096	*A. baumannii* (95.0%)	*Acinetobacter* genosp. 3 (98.4%)
107012	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.3%)
14027	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.8%)
30077	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.8%)
22344	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (100%)
12193	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.8%)
93743	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.1%)
35272	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.8%)
86661	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.8%)
12253	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.1%)
28933	*A. baumannii* (97.6%)	*Acinetobacter* genosp. 3 (99.8%)
20949	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.6%)
31479	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.6%)
106498	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.8%)
106008	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.8%)
183436	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.8%)
29420	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.8%)
14331	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (100%)
27035	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.8%)
25904	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.3%)
16198	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.6%)
82991	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (98.9%)
181882	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.6%)
13320	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (100%)
101327	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.8%)
91135	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.6%)
106449	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.8%)
35168	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (98.4%)
232787	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (99.8%)
225991	*A. baumannii* (99.0%)	*Acinetobacter* genosp. 3 (100%)
33241	*A. baumannii* (99.0%)	*A. calcoaceticus* (99.8%)
92328	*A. baumannii* (99.0%)	*A. calcoaceticus* (99.6%)
183262	*A. baumannii* (99.0%)	*A. calcoaceticus* (100%)
20304	*A. baumannii* (99.0%)	*A. calcoaceticus* (99.6%)
28263	*A. baumannii* (99.0%)	*A. calcoaceticus* (100%)
100558	*A. baumannii* (99.0%)	*A. calcoaceticus* (99.3%)

Table 2-4 (Continued)

Isolate Identifier	Identification by VITEK 2 (% probability)	Identification by *rpoB* sequencing (sequence concordance with reference strains)
28001	*A. lwoffii* (99.0%)	*A. johnsonii* (99.6%)
100441	*A. lwoffii* (99.0%)	*A. johnsonii* (99.8%)
181441	*A. lwoffii* (99.0%)	*A. johnsonii* (99.6%)
26612	*A. lwoffii* (99.0%)	*A. johnsonii* (99.6%)
215194	*A. lwoffii* (98.3%)	*A. johnsonii* (99.3%)
226419	*A. lwoffii* (99.0%)	*A. johnsonii* (99.8%)
182952	*A. lwoffii* (99.0%)	*A. johnsonii* (100%)
215759	*A. lwoffii* (99.0%)	*A. johnsonii* (99.1%)
215687	*A. lwoffii* (99.0%)	*A. johnsonii* (99.3%)
214737	*A. lwoffii* (99.0%)	*A. johnsonii* (99.6%)
25868	*A. lwoffii* (99.0%)	*A. johnsonii* (100%)
221358	*A. lwoffii* (99.0%)	*A. johnsonii* (99.6%)
88389	*A. lwoffii* (91.3%)	*A. ursingii* (99.6%)
183357	*A. junii* (93.7%)	*A. ursingii* (98.9%)
89302	*A. junii* (93.7%)	*A. ursingii* (99.6%)
90772	*A. junii* (93.7%)	*A. ursingii* (99.8%)
217818	*A. lwoffii* (92.5%)	*A. ursingii* (100%)
91411	*A. junii* (93.7%)	*A. ursingii* (99.8%)
30431	*A. lwoffii* (91.3%)	*A. ursingii* (99.3%)
91352	*A. junii* (93.7%)	*A. ursingii* (99.8%)
203910	*A. lwoffii* (99.0%)	*Acinetobacter* genosp. 9 (99.8%)
22104	*A. lwoffii* (99.0%)	*Acinetobacter* genosp. 9 (99.6%)
180361	*A. lwoffii* (99.0%)	*Acinetobacter* genosp. 9 (99.8%)
190152	*A. lwoffii* (99.0%)	*Acinetobacter* genosp. 9 (99.3%)
184576	*A. lwoffii* (99.0%)	*Acinetobacter* genosp. 9 (99.3%)
107690	*A. lwoffii* (99.0%)	*A. lwoffii* (100%)
23063	*A. lwoffii* (99.0%)	*A. lwoffii* (100%)
101559	*A. lwoffii* (98.1%)	*A. baylyi*/ genosp. 11 (99.6%-99.8%)
27611	*A. junii* (98.3%)	*A. baylyi*/ genosp. 11 (99.3%-99.6%)
219537	*A. junii* (98.0%)	*A. baylyi*/ genosp. 11 (99.8%-100%)
105947	*A. lwoffii* (98.1%)	*A. baylyi*/ genosp. 11 (99.6%-99.8%)
87374	*A. lwoffii* (99.0%)	*A. radioresistens* (99.6%)
88427	*A. lwoffii* (99.0%)	*A. radioresistens* (99.3%)
20715	*A. lwoffii* (99.0%)	*Acinetobacter* genosp. 13BJ (98.7%)
30293	*A. lwoffii* (99.0%)	*Acinetobacter* genosp. 13BJ (98.9%)
101055	*A. lwoffii* (99.0%)	*Acinetobacter* genosp. 10 (99.6%)
89182	*A. haemolyticus* (98.4%)	*A. haemolyticus* (99.8%)
227313	*A. lwoffii* (99.0%)	*A. schindleri* (98.9%)
103727	*A. haemolyticus* (98.6%)	*A. tjernbergiae* (99.1%)

identified to be *Acinetobacter* genospecies 3 and *A. calcoaceticus*, respectively. The error rate of speciation within the *Acinetobacter calcoaceticus-baumannii* complex (Acbc) was 65.4% (51/78).

Among the 39 non-Acbc isolates presumptively identified by VITEK 2, four isolates (identifier numbers 101559, 27611, 219537 and 105947) were identified by *rpoB* sequencing as either *Acinetobacter* genospecies 11 or *A. baylyi* (see Table 2-4). Further PCR and sequencing of the *rpoB-rpoC* spacer of the four isolates yielded *circa* 150-bp amplicons with sequence concordance of 98.7% to 100% with the corresponding spacer region of the *Acinetobacter* genospecies 11 reference strain (GenBank accession no. DQ207499), thereby confirming the four isolates to be *Acinetobacter* genospecies 11 instead of *A. baylyi*.

Of the 30 isolates presumptively identified by VITEK 2 as *A. lwoffii*, the following species were identified by *rpoB* and *rpoB-rpoC* sequencing (number of isolates): *A. johnsonii* (12); *Acinetobacter* genospecies 9 (5); *A. lwoffii* (2); *A. ursingii* (3); *Acinetobacter* genospecies 11 (2); *A. radioresistens* (2); *Acinetobacter* genospecies 13BJ (2); *Acinetobacter* genospecies 10 (1) and *A. schindleri* (1). Of the seven *A. junii* isolates presumptively identified by VITEK 2, five were confirmed to be *A. ursingii* and the remaining two identified as *Acinetobacter* genospecies 11 by *rpoB* and *rpoB-rpoC* sequencing. The two *A. haemolyticus* isolates identified by VITEK 2 were confirmed to be *A. haemolyticus* and *A. tjernbergiae* respectively by *rpoB* sequencing.

Among the 39 non-Acbc isolates, only three of the isolates were correctly speciated by VITEK 2. The error rate of speciation of non-Acbc isolates by VITEK 2 was therefore 92.3% (36/39).

All *A. baumannii*, *Acinetobacter* genospecies 3 and *A. calcoaceticus* isolates (according to *rpoB* sequencing) were correctly identified to the Acbc level by VITEK 2 (78/78), while none of the other species (including non-*Acinetobacter* genera) was identified as Acbc isolate (0/43). The sensitivity and specificity of VITEK 2 GNI in identifying isolates to the Acbc level were therefore 100% and 100%, respectively.

2.3.2 Antimicrobial susceptibility testing

Individual disc diffusion zone diameter, Etest MIC and VITEK 2 MIC results of the 117 isolates and the corresponding susceptibility interpretation results are shown in Appendix 1.

2.3.2.1 Comparison of antimicrobial susceptibility results of disc diffusion, Etest and VITEK 2 methods

Category agreement of antimicrobial susceptibility interpretation results of the three AST methods is shown in Table 2-5 for the following antimicrobial agents:

cefotaxime, ceftazidime, cefepime, piperacillin-tazobactam, meropenem, gentamicin, amikacin, ciprofloxacin and trimethoprim-sulphamethoxazole.

There was a high level of category agreement between disc diffusion and Etest methods (97.4% - 100%) for all the nine antimicrobial agents. When disc diffusion and VITEK 2 methods were compared, category agreement varied from 87.2% to 100%. Category agreement was high (98.3% - 100%) for meropenem, aminoglycosides, ciprofloxacin and trimethoprim-sulphamethoxazole. The level of agreement was lower for the cephalosporins (91.5% - 94%) and the category agreement between disc diffusion and VITEK 2 for piperacillin-tazobactam was unacceptably low (87.2%). The pattern of category agreement between Etest and VITEK 2 methods was similar to that between disc diffusion and VITEK 2, with the category agreement for piperacillin-tazobactam being unacceptably low at 86.3%.

Using CLSI disc diffusion method as the reference method, the error types and rates in susceptibility interpretation by Etest and VITEK 2 methods are shown in Table 2-6. Etest MIC method did not record any VME or ME for the nine antimicrobial agents. Acceptably low mE rates (0.9% - 2.6%) were found for seven agents (cephalosporins, piperacillin-tazobactam, aminoglycosides and ciprofloxacin). VITEK 2 method recorded an unacceptably high rate of VME (1.7%) for piperacillin-tazobactam, while the other agents produced no VME or low VME rate (0% - 0.9%). VITEK 2 produced

Table 2-5. Category agreement of susceptibility results based on disc diffusion, Etest and VITEK 2 methods.

Antimicrobial Agent	Category agreement (no. of isolates out of 117 isolates)		
	Disc diffusion vs Etest	Disc diffusion vs VITEK 2	Etest vs VITEK 2
Cefotaxime	99.1% (116)	94.0% (110)	93.1% (109)
Ceftazidime	97.4% (114)	93.1% (109)	92.3% (108)
Cefepime	97.4% (114)	91.5% (107)	90.6% (106)
Piperacillin-tazobactam	99.1% (116)	87.2% (102)	86.3% (101)
Meropenem	100% (117)	100% (117)	100% (117)
Gentamicin	99.1% (116)	98.3% (115)	97.4% (114)
Amikacin	99.1% (116)	99.1% (116)	100% (117)
Ciprofloxacin	99.1% (116)	98.3% (115)	99.1% (116)
Trimethoprim-sulphamethoxazole	100% (117)	100% (117)	100% (117)

Table 2-6. Error rates in susceptibility interpretation by Etest MIC and VITEK 2 AST methods.[a]

Antimicrobial Agent	Etest MIC method (n=117)			VITEK 2 AST method (n=117)		
	VME (%)	ME (%)	mE (%)	VME (%)	ME (%)	mE (%)
Cefotaxime	0	0	1 (0.9%)	0	0	7 (6.0%)
Ceftazidime	0	0	3 (2.6%)	0	0	8 (6.8%)
Cefepime	0	0	3 (2.6%)	0	0	10 (8.5%)
Piperacillin-tazobactam	0	0	1 (0.9%)	2 (1.7%)	0	13 (11.1%)
Meropenem	0	0	0	0	0	0
Gentamicin	0	0	1 (0.9%)	1 (0.9%)	0	1 (0.9%)
Amikacin	0	0	1 (0.9%)	0	0	1 (0.9%)
Ciprofloxacin	0	0	1 (0.9%)	0	0	2 (1.7%)
Trimethoprim-sulphamethoxazole	0	0	NA	0	0	NA

[a]using CLSI disc diffusion as reference method and based on CLSI susceptibility breakpoints.

VME, very major errors; ME, major errors; mE, minor errors; NA, not applicable.

mE rates for the cephalosporins that ranged from 6% to 8.5 %. As with the VME rate, the VITEK 2 mE rate for piperacillin-tazobactam was unacceptably high (11.1%).

The results of tigecycline Etest MICs on Iso-Sensitest agar and on Mueller-Hinton agar were also compared. 19 and 70 isolates had two- and one-dilution higher MICs on Iso-Sensitest agar than on Mueller-Hinton agar, respectively ie. 76% of isolates had higher MICs on Iso-Sensitest agar (see Appendix 1). Only two isolates had one-dilution lower MICs on Iso-Sensitest agar while the remaining isolates (26) had the same MICs on the two media. Of the 29 isolates with tigecycline Etest MICs of ≥ 1 μg/mL on Iso-Sensitest agar, 26 had higher MICs when tested on Iso-Sensitest agar than on Mueller-Hinton agar, with 17 of the 26 isolates showing a 2-dilution difference in results (see Appendix 1).

2.3.2.2 MIC and susceptibility results of *Acinetobacter* isolates

The MIC values based on the Etest MIC method and corresponding susceptibility interpretation of the results for the *Acinetobacter* isolates are shown in Tables 2-7 through 2-21 for 15 antimicrobial agents. With the exception of tigecycline, doripenem, and amoxicillin-clavulanic acid, susceptibility interpretation of the MIC results are based on CLSI breakpoints (CLSI, 2009). Tigecycline and doripenem susceptibility interpretation are based on BSAC breakpoints (BSAC, 2009a). No breakpoints for amoxicillin-clavulanic acid have been established by either CLSI or BSAC (BSAC, 2009a; CLSI, 2009).

Susceptibility data for extended-spectrum cephalosporins are shown in Tables 2-7 to 2-9. Table 2-7 shows the cefotaxime MIC values and susceptibility interpretation results of the *Acinetobacter* isolates. Overall, only 28 of 117 isolates (23.9%) were within the susceptible category. 7 of 78 isolates (9.0%) of *Acinetobacter calcoaceticus-baumannii* complex (Acbc) were susceptible while 21 of 39 (53.8%) of non-Acbc isolates were susceptible to cefotaxime. Table 2-8 shows the susceptibility data for ceftazidime. 76 of 117 *Acinetobacter* isolates (65.0%) were susceptible to ceftazidime but within this category, 61 of the 76 isolates (80.3%) had reduced ceftazidime susceptibility with MIC values of 4 to 8 μg/mL. 45 of 78 Acbc isolates (57.7%) and 31 of 39 non-Acbc isolates (79.5%) were within the susceptible category. Cefepime susceptibility data is shown in Table 2-9. 88 of 117 isolates (75.2%) were susceptible to cefepime but 53 of the 88 isolates (60.2%) had reduced susceptibility (MIC values of 4 to 8 μg/mL). 54 of 78 Acbc isolates (69.2%) and 34 of 39 non-Acbc isolates (87.1%) were within the susceptible category for cefepime.

Susceptibility data for the β-lactam/β-lactamase inhibitor combinations of ticarcillin-clavulanic acid and piperacillin-tazobactam are shown in Tables 2-10 and 2-11, respectively. 102 of 117 *Acinetobacter* isolates (87.2%) were within the susceptible category for ticarcillin-clavulanic acid. 65 of 78 Acbc isolates (83.3%) were susceptible to ticarcillin-clavulanic acid while 37 of 39 non-Acbc isolates (94.9%) were within the same category. For piperacillin-tazobactam, 89 of 117 isolates (76.1%) were within the susceptible category although 27 of the 89 isolates (30.3%)

Table 2-7. Cefotaxime MIC and susceptibility data of *Acinetobacter* isolates.[a]

Acinetobacter spp.	Cefotaxime MICs (μg/mL)								
	S				I		R		
	≤1	2	4	8	16	32	64	128	≥ 256
All isolates (117)	1	6	9	12	25	41	14	6	3
Acbc isolates (78)	-	-	1	6	15	35	13	5	3
A. baumannii (27)	-	-	1	2	8	11	-	3	2
Acinetobacter genosp. 3 (45)	-	-	-	4	6	21	11	2	1
A. calcoaceticus (6)	-	-	-	-	1	3	2	-	-
Non-Acbc isolates (39)	1	6	8	6	10	6	1	1	-
A. johnsonii (12)	-	3	4	3	2	-	-	-	-
A. ursingii (8)	-	1	-	-	2	3	1	1	-
Other species (19)	1	2	4	3	6	3	-	-	-

[a]MICs using Etest method and susceptibility interpretation based on CLSI breakpoints; Acbc, *Acinetobacter calcoaceticus-baumannii* complex.

Table 2-8. Ceftazidime MIC and susceptibility data of *Acinetobacter* isolates.[a]

Acinetobacter spp.	Ceftazidime MICs (µg/mL)								
	S				I	R			
	≤1	2	4	8	16	32	64	128	≥256
All isolates (117)	6	9	37	24	33	5	1	-	2
Acbc isolates (78)	1	2	25	17	28	3	-	-	2
A. baumannii (27)	1	1	12	6	4	1	-	-	2
Acinetobacter genosp. 3 (45)	-	1	12	11	19	2	-	-	-
A. calcoaceticus (6)	-	-	1	-	5	-	-	-	-
Non-Acbc isolates (39)	5	7	12	7	5	2	1	-	-
A. johnsonii (12)	2	3	6	1	-	-	-	-	-
A. ursingii (8)	-	1	-	1	3	2	1	-	-
Other species (19)	3	3	6	5	2	-	-	-	-

[a]MICs using Etest method and susceptibility interpretation based on CLSI breakpoints; Acbc, *Acinetobacter calcoaceticus-baumannii* complex.

Table 2-9. Cefepime MIC and susceptibility data of *Acinetobacter* isolates.[a]

Acinetobacter spp.	Cefepime MICs (µg/mL)								
	S				I	R			
	≤1	2	4	8	16	32	64	128	≥256
All isolates (117)	7	28	28	25	15	9	3	1	1
Acbc isolates (78)	1	16	15	22	13	6	3	1	1
A. baumannii (27)	1	10	6	5	2	2	-	-	1
Acinetobacter genosp. 3 (45)	-	5	9	12	11	4	3	1	-
A. calcoaceticus (6)	-	1	-	5	-	-	-	-	-
Non-Acbc isolates (39)	6	12	13	3	2	3	-	-	-
A. johnsonii (12)	-	1	3	3	2	3	-	-	-
A. ursingii (8)	1	2	5	-	-	-	-	-	-
Other species (19)	5	9	5	-	-	-	-	-	-

[a]MICs using Etest method and susceptibility interpretation based on CLSI breakpoints; Acbc, *Acinetobacter calcoaceticus-baumannii* complex.

Table 2-10. Ticarcillin-clavulanic acid MIC and susceptibility data of *Acinetobacter* isolates.[a]

Acinetobacter spp.	Ticarcillin-clavulanic acid MICs (µg/mL)								
	S					I		R	
	≤ 1/2	2/2	4/2	8/2	16/2	32/2	64/2	128/2	≥ 256/2
All isolates (117)	28	13	31	26	4	2	1	-	12
Acbc isolates (78)	4	7	27	23	4	1	1	-	11
A. baumannii (27)	2	2	12	8	-	1	1	-	1
Acinetobacter genosp. 3 (45)	2	4	14	12	3	-	-	-	10
A. calcoaceticus (6)	-	1	1	3	1	-	-	-	-
Non-Acbc isolates (39)	24	6	4	3	-	1	-	-	1
A. johnsonii (12)	9	2	-	-	-	-	-	-	1
A. ursingii (8)	5	3	-	-	-	-	-	-	-
Other species (19)	10	1	4	3	-	1	-	-	-

[a]MICs using Etest method and susceptibility interpretation based on CLSI breakpoints; Acbc, *Acinetobacter calcoaceticus-baumannii* complex.

Table 2-11. Piperacillin-tazobactam MIC and susceptibility data of *Acinetobacter* isolates.[a]

Acinetobacter spp.	Piperacillin-tazobactam MICs (µg/mL)								
	S					I		R	
	≤ 1/4	2/4	4/4	8/4	16/4	32/4	64/4	128/4	≥ 256/4
All isolates (117)	51	3	8	17	10	9	5	9	5
Acbc isolates (78)	28	1	6	10	9	8	3	8	5
A. baumannii (27)	13	-	2	-	2	5	2	2	1
Acinetobacter genosp. 3 (45)	15	-	3	8	7	2	-	6	4
A. calcoaceticus (6)	-	1	1	2	-	1	1	-	-
Non-Acbc isolates (39)	23	2	2	7	1	1	2	1	-
A. johnsonii (12)	11	-	-	-	-	-	-	1	-
A. ursingii (8)	3	-	-	4	1	-	-	-	-
Other species (19)	9	2	2	3	-	1	2	-	-

[a]MICs using Etest method and susceptibility interpretation based on CLSI breakpoints; Acbc, *Acinetobacter calcoaceticus-baumannii* complex.

within this category had reduced susceptibility to the agent (MIC values of 8/4 to 16/4 μg/mL). 54 of 78 Acbc isolates (69.2%) and 35 of 39 non-Acbc isolates (89.7%) were susceptible to piperacillin-tazobactam.

Susceptibility data for the three carbapenems are shown in Tables 2-12 to 2-14. Meropenem susceptibility results are shown in Table 2-12. 105 of 117 isolates (89.7%) were susceptible to meropenem and two of the 105 isolates (both Acbc isolates) had reduced meropenem susceptibility (MIC values of 2 to 4 μg/mL). Of the Acbc isolates, 67 of 78 isolates (85.9%) were susceptible to meropenem while 38 of 39 non-Acbc isolates (97.4%) were within the same category. For imipenem, 105 of 117 isolates (89.7%) were in the susceptible category, with eight of the 105 isolates (7.6%) having reduced susceptibility of MIC 2 to 4 μg/mL. As with meropenem, 85.9% of Acbc isolates and 97.4% of non-Acbc isolates were susceptible to imipenem. Doripenem susceptibility results (based on BSAC breakpoints) are shown in Table 2-14. 104 of 117 isolates (88.9%) were within the susceptible category, of which four Acbc isolates had MICs of 1 μg/mL. 66 of 78 Acbc isolates (84.6%) and 38 of 39 non-Acbc isolates (97.4%) were susceptible to doripenem.

Tables 2-15 and 2-16 show the susceptibility data of the *Acinetobacter* isolates to gentamicin and amikacin, respectively. 108 of 117 isolates (92.3%) were susceptible to gentamicin. Acbc and non-Acbc isolates had the same proportions of gentamicin-susceptible isolates, with 72 of 78 Acbc isolates (92.3%) and 36 of 39 non-Acbc isolates (92.3%) being in the susceptible category. 116 of 117 isolates (99.1%) were susceptible to amikacin. Only one *A. baumannii* isolate (229437) was resistant; while

Table 2-12. Meropenem MIC and susceptibility data of *Acinetobacter* isolates.[a]

Acinetobacter spp.	Meropenem MICs (µg/mL)								
	S						I	R	
	≤ 0.125	0.25	0.5	1	2	4	8	16	≥ 32
All isolates (117)	29	47	17	10	1	1	-	-	12
Acbc isolates (78)	11	32	14	8	1	1	-	-	11
A. baumannii (27)	6	10	6	2	1	1	-	-	1
Acinetobacter genosp. 3 (45)	3	18	8	6	-	-	-	-	10
A. calcoaceticus (6)	2	4	-	-	-	-	-	-	-
Non-Acbc isolates (39)	18	15	3	2	-	-	-	-	1
A. johnsonii (12)	8	3	-	-	-	-	-	-	1
A. ursingii (8)	2	6	-	-	-	-	-	-	-
Other species (19)	8	6	3	2	-	-	-	-	-

[a]MICs using Etest method and susceptibility interpretation based on CLSI breakpoints; Acbc, *Acinetobacter calcoaceticus-baumannii* complex.

Table 2-13. Imipenem MIC and susceptibility data of *Acinetobacter* isolates.[a]

Acinetobacter spp.	Imipenem MICs (µg/mL)								
	S						I	R	
	≤ 0.125	0.25	0.5	1	2	4	8	16	≥ 32
All isolates (117)	13	37	34	13	7	1	-	-	12
Acbc isolates (78)	6	18	26	10	6	1	-	-	11
A. baumannii (27)	3	8	8	3	3	1	-	-	1
Acinetobacter genosp. 3 (45)	2	6	17	7	3	-	-	-	10
A. calcoaceticus (6)	1	4	1	-	-	-	-	-	-
Non-Acbc isolates (39)	7	19	8	3	1	-	-	-	1
A. johnsonii (12)	3	7	1	-	-	-	-	-	1
A. ursingii (8)	1	5	2	-	-	-	-	-	-
Other species (19)	3	7	5	3	1	-	-	-	-

[a]MICs using Etest method and susceptibility interpretation based on CLSI breakpoints; Acbc, *Acinetobacter calcoaceticus-baumannii* complex.

Table 2-14. Doripenem MIC and susceptibility data of *Acinetobacter* isolates.[a]

Acinetobacter spp.	Doripenem MICs (μg/mL)								
	S				I		R		
	≤ 0.125	0.25	0.5	1	2	4	8	16	≥ 32
All isolates (117)	58	33	9	4	1	-	-	7	5
Acbc isolates (78)	30	24	8	4	1	-	-	7	4
A. baumannii (27)	12	7	4	2	1	-	-	-	1
Acinetobacter genosp. 3 (45)	14	15	4	2	-	-	-	7	3
A. calcoaceticus (6)	4	2	-	-	-	-	-	-	-
Non-Acbc isolates (39)	28	9	1	-	-	-	-	-	1
A. johnsonii (12)	11	-	-	-	-	-	-	-	1
A. ursingii (8)	6	2	-	-	-	-	-	-	-
Other species (19)	11	7	1	-	-	-	-	-	-

[a]MICs using Etest method and susceptibility interpretation based on BSAC breakpoints; Acbc, *Acinetobacter calcoaceticus-baumannii* complex.

Table 2-15. Gentamicin MIC and susceptibility data of *Acinetobacter* isolates.[a]

Acinetobacter spp.	Gentamicin MICs (μg/mL)								
	S					I	R		
	≤ 0.25	0.5	1	2	4	8	16	32	≥ 64
All isolates (117)	12	38	56	2	-	1	1	4	3
Acbc isolates (78)	6	25	41	-	-	1	-	3	2
A. baumannii (27)	4	11	10	-	-	-	-	-	2
Acinetobacter genosp. 3 (45)	2	12	27	-	-	1	-	3	-
A. calcoaceticus (6)	-	2	4	-	-	-	-	-	-
Non-Acbc isolates (39)	6	13	15	2	-	-	1	1	1
A. johnsonii (12)	2	4	5	-	-	-	-	-	1
A. ursingii (8)	1	-	6	-	-	-	-	1	-
Other species (19)	3	9	4	2	-	-	1	-	-

[a]MICs using Etest method and susceptibility interpretation based on CLSI breakpoints; Acbc, *Acinetobacter calcoaceticus-baumannii* complex.

Table 2-16. Amikacin MIC and susceptibility data of *Acinetobacter* isolates.[a]

Acinetobacter spp.	Amikacin MICs (µg/mL)								
	S						I	R	
	≤ 0.5	1	2	4	8	16	32	64	≥ 128
All isolates (117)	46	47	18	3	1	1	-	-	1
Acbc isolates (78)	37	26	12	1	1	-	-	-	1
A. baumannii (27)	11	13	2	-	-	-	-	-	1
Acinetobacter genosp. 3 (45)	26	10	7	1	1	-	-	-	-
A. calcoaceticus (6)	-	3	3	-	-	-	-	-	-
Non-Acbc isolates (39)	9	21	6	2	-	1	-	-	-
A. johnsonii (12)	5	6	1	-	-	-	-	-	-
A. ursingii (8)	1	3	4	-	-	-	-	-	-
Other species (19)	3	12	1	2	-	1	-	-	-

[a]MICs using Etest method and susceptibility interpretation based on CLSI breakpoints; Acbc, *Acinetobacter calcoaceticus-baumannii* complex.

one non-Acbc isolate (*A. haemolyticus* isolate 89182) had reduced amikacin susceptibility with an MIC of 16 µg/mL. Ciprofloxacin susceptibility results of the *Acinetobacter* isolates are shown in Table 2-17. 93 of 117 isolates (79.5%) were susceptible to ciprofloxacin, of which 21 isolates (22.6%) had reduced susceptibility with MIC values of 0.5 to 1 µg/mL. 58 of 78 Acbc isolates (74.4%) and 35 of 39 non-Acbc isolates (89.7%) were susceptible to ciprofloxacin. Susceptibility results for trimethoprim-sulphamethoxazole are shown in Table 2-18. 110 of 117 isolates (94.0%) were susceptible to the agent. Of the 78 Acbc isolates, 73 isolates (93.6%) were susceptible to trimethoprim-sulphamethoxazole, while a similar proportion of non-Acbc isolates (37 of 39 isolates; 94.9%) were also in the susceptible category. Table 2-19 shows the susceptibility results of the isolates for colistin. All *Acinetobacter* isolates were susceptible to colistin.

Table 2-20 shows the susceptibility results for tigecycline (based on BSAC breakpoints and test methodology). 97 of 117 isolates (82.9%) were susceptible to tigecycline, of which nine isolates (9.3%) had reduced susceptibility of MICs of 1 µg/mL. All tigecycline non-susceptible isolates were *A. baumannii* (seven) and *Acinetobacter* genospecies 3 isolates (13).

Table 2-21 shows the MIC results of the *Acinetobacter* isolates for amoxicillin-clavulanic acid. 12 of 117 isolates (10.3%) had MIC values of ≥ 256/128 µg/mL; they were the same isolates with full resistance to all three carbapenems (see Appendix 1). 11 of 117 isolates (9.4%) had MIC values of 16/8 µg/mL, of which 10 were Acbc

Table 2-17. Ciprofloxacin MIC and susceptibility data of *Acinetobacter* isolates.[a]

Acinetobacter spp.	Ciprofloxacin MICs (µg/mL)								
	S				I	R			
	≤ 0.125	0.25	0.5	1	2	4	8	16	≥ 32
All isolates (117)	36	36	12	9	1	-	3	5	15
Acbc isolates (78)	22	20	8	8	1	-	3	5	11
A. baumannii (27)	8	6	4	3	1	-	-	-	5
Acinetobacter genosp. 3 (45)	10	12	4	5	-	-	3	5	6
A. calcoaceticus (6)	4	2	-	-	-	-	-	-	-
Non-Acbc isolates (39)	14	16	4	1	-	-	-	-	4
A. johnsonii (12)	6	4	-	-	-	-	-	-	2
A. ursingii (8)	1	5	1	1	-	-	-	-	-
Other species (19)	7	7	3	-	-	-	-	-	2

[a]MICs using Etest method and susceptibility interpretation based on CLSI breakpoints; Acbc, *Acinetobacter calcoaceticus-baumannii* complex.

Table 2-18. Trimethoprim-sulphamethoxazole MIC and susceptibility data of *Acinetobacter* isolates.[a]

Acinetobacter spp.	Trimethoprim-sulphamethoxazole MICs (μg/mL)					
	S		R			
	≤ 1/19	2/38	4/76	8/152	16/304	≥ 32/608
All isolates (117)	109	1	-	-	-	7
Acbc isolates (78)	72	1	-	-	-	5
A. baumannii (27)	25	-	-	-	-	2
Acinetobacter genosp. 3 (45)	41	1	-	-	-	3
A. calcoaceticus (6)	6	-	-	-	-	-
Non-Acbc isolates (39)	37	-	-	-	-	2
A. johnsonii (12)	11	-	-	-	-	1
A. ursingii (8)	8	-	-	-	-	-
Other species (19)	18	-	-	-	-	1

[a]MICs using Etest method and susceptibility interpretation based on CLSI breakpoints; Acbc, *Acinetobacter calcoaceticus-baumannii* complex.

Table 2-19. Colistin MIC and susceptibility data of *Acinetobacter* isolates.[a]

Acinetobacter spp.	Colistin MICs (µg/mL)								
	S					R			
	≤ 0.125	0.25	0.5	1	2	4	8	16	≥ 32
All isolates (117)	26	46	42	3	-	-	-	-	-
Acbc isolates (78)	15	29	31	3	-	-	-	-	-
A. baumannii (27)	6	11	7	3	-	-	-	-	-
Acinetobacter genosp. 3 (45)	7	15	23	-	-	-	-	-	-
A. calcoaceticus (6)	2	3	1	-	-	-	-	-	-
Non-Acbc isolates (39)	11	17	11	-	-	-	-	-	-
A. johnsonii (12)	2	7	3	-	-	-	-	-	-
A. ursingii (8)	6	1	1	-	-	-	-	-	-
Other species (19)	3	9	7	-	-	-	-	-	-

[a]MICs using Etest method and susceptibility interpretation based on CLSI breakpoints; Acbc, *Acinetobacter calcoaceticus-baumannii* complex.

Table 2-20. Tigecycline MIC and susceptibility data of *Acinetobacter* isolates.[a]

Acinetobacter spp.	Tigecycline MICs (μg/mL)								
	S				I	R			
	≤ 0.125	0.25	0.5	1	2	4	8	16	≥ 32
All isolates (117)	17	29	42	9	17	1	2	-	-
Acbc isolates (78)	3	20	30	5	17	1	2	-	-
A. baumannii (27)	1	1	14	4	5	-	2	-	-
Acinetobacter genosp. 3 (45)	1	16	14	1	12	1	-	-	-
A. calcoaceticus (6)	1	3	2	-	-	-	-	-	-
Non-Acbc isolates (39)	14	9	12	4	-	-	-	-	-
A. johnsonii (12)	9	2	1	-	-	-	-	-	-
A. ursingii (8)	-	1	5	2	-	-	-	-	-
Other species (19)	5	6	6	2	-	-	-	-	-

[a]MICs using Etest method and susceptibility interpretation based on BSAC breakpoints; Acbc, *Acinetobacter calcoaceticus-baumannii* complex.

Table 2-21. Amoxicillin-clavulanic acid MIC data of *Acinetobacter* isolates.[a]

Acinetobacter spp.	Amoxicillin-clavulanic acid MICs (μg/mL)[b]								
	≤ 1	2	4	8	16	32	64	128	≥ 256
All isolates (117)	9	21	22	42	11	-	-	-	12
Acbc isolates (78)	-	2	19	36	10	-	-	-	11
A. baumannii (27)	-	2	8	13	3	-	-	-	1
Acinetobacter genosp. 3 (45)	-	-	10	20	5	-	-	-	10
A. calcoaceticus (6)	-	-	1	3	2	-	-	-	-
Non-Acbc isolates (39)	9	19	3	6	1	-	-	-	1
A. johnsonii (12)	5	5	1	-	-	-	-	-	1
A. ursingii (8)	2	6	-	-	-	-	-	-	-
Other species (19)	2	8	2	6	1	-	-	-	-

[a]MICs using Etest method; [b]amoxicillin component with the amoxicillin:clavulanic acid ratio of 2:1; Acbc, *Acinetobacter calcoaceticus-baumannii* complex.

isolates. 42 of 117 isolates (35.9%) had MIC values of 8/4 μg/mL, of which 36 were Acbc isolates.

Table 2-22 summarises the non-susceptibility rates of all isolates, Acbc isolates and non-Acbc isolates, respectively. There were high non-susceptibility rates to cefotaxime especially amongst the Acbc isolates (91%), while the corresponding rates for ceftazidime, cefepime, piperacillin-tazobactam and ciprofloxacin were all above 20%. The tigecycline non-susceptibility rate was 17.1%, while those for carbapenems and ticarcillin-clavulanic acid ranged from 10.3% to 12.8%. Non-susceptibility rates were lowest for colistin and amikacin at 0% and 0.9%, respectively. 80.3% (94 isolates) of all *Acinetobacter* isolates had amoxicillin-clavulanic acid MICs of ≤ 8/4 μg/mL.

With the exception of colistin, gentamicin, amikacin and trimethoprim-sulphamethoxazole, non-susceptibility rates of Acbc isolates were statistically significantly higher than those of non-Acbc isolates (p values of ≤ 0.05) for most β-lactams, ciprofloxacin and tigecycline. The difference in non-susceptibility rates for ticarcillin-clavulanic acid between Acbc and non-Acbc isolates showed a trend towards statistical significance with a p value of 0.07.

For Acbc isolates, cefotaxime non-susceptibility rate was above 90% while those for ceftazidime and cefepime were above 40% and 30%, respectively. Both ciprofloxacin and tigecycline non-susceptibility rates were above 25% and carbapenem non-

Table 2-22. Antimicrobial susceptibility of *Acinetobacter* isolates based on Etest MIC results.[a]

Antimicrobial Agent	Non-susceptibility rates in *Acinetobacter* spp. (number of isolates)			
	All isolates (117)	Acbc isolates (78)	Non-Acbc isolates (39)	*p* value[c] (Acbc vs non-Acbc)
Cefotaxime	76.1% (89)	91.0% (71)	46.2% (18)	< 0.001
Ceftazidime	35.0% (41)	42.3% (33)	20.5% (8)	0.02
Cefepime	24.8% (29)	30.8% (24)	12.8% (5)	0.03
Piperacillin-tazobactam	23.9% (28)	30.8% (24)	10.3% (4)	0.01
Ticarcillin-clavulanic acid	12.8% (15)	16.7% (13)	5.1% (2)	0.07
Meropenem	10.3% (12)	14.1% (11)	2.6% (1)	0.05
Imipenem	10.3% (12)	14.1% (11)	2.6% (1)	0.05
Doripenem[b]	11.1% (13)	15.4% (12)	2.6% (1)	0.03
Gentamicin	7.7% (9)	7.7% (6)	7.7% (3)	0.65
Amikacin	0.9% (1)	1.3% (1)	0% (0)	0.67
Ciprofloxacin	20.5% (24)	25.6% (20)	10.3% (4)	0.04
Trimethoprim-sulphamethoxazole	6.0% (7)	6.4% (5)	5.1% (2)	0.57
Tigecycline[b]	17.1% (20)	25.6% (20)	0% (0)	< 0.001
Colistin	0% (0)	0% (0)	0% (0)	1.00

[a]CLSI susceptibility breakpoints unless stated otherwise; [b]based on BSAC susceptibility breakpoints; [c]statistical analysis using Fisher's Exact Test; Acbc, *Acinetobacter calcoaceticus-baumannii* complex.

susceptibility rates ranged from 14.1% to 15.4%. Rates for non-susceptibility to colistin and amikacin were lowest at 0% and 1.3%, respectively.

2.3.2.3 Susceptibility pattern of different *Acinetobacter* species

Differences in antimicrobial susceptibility patterns were observed for individual *Acinetobacter* species, particularly for the cephalosporins ceftazidime and cefepime. The majority of isolates from most species were susceptible to ceftazidime and cefepime, but the majority of *A. calcoaceticus* and *A. ursingii* isolates were not susceptible to ceftazidime (see Table 2-8 and Appendix 1). On the other hand, some species had relatively higher cefepime MICs compared to ceftazidime MICs. Most notably, *Acinetobacter* genospecies 3 and *A. johnsonii* isolates produced overall higher MICs and non-susceptibility rates for cefepime compared to ceftazidime (see Table 2-9 and Appendix 1). Tigecycline non-susceptibility was present only in isolates of *A. baumannii* and *Acinetobacter* genospecies 3.

Non-susceptibility rates of the 14 antimicrobial agents for *A. baumannii* and *Acinetobacter* genospecies 3 are shown in Table 2-23. Non-susceptibility rates for meropenem, imipenem and cefepime were statistically significantly higher for *Acinetobacter* genospecies 3 compared to *A. baumannii* (p value < 0.05). Ceftazidime and doripenem non-susceptibility rates showed a trend towards higher levels in *Acinetobacter* genospecies 3 compared to *A. baumannii*, with p values of 0.07 and 0.09, respectively.

Table 2-23. Comparison of Etest MIC results between *Acinetobacter* genospecies 3 and *Acinetobacter baumannii* isolates.[a]

Antimicrobial Agent	Non-susceptibility rates in *Acinetobacter* spp. (number of isolates)		
	A. baumannii (27)	*Acinetobacter* genosp. 3 (45)	*p* value[c]
Cefotaxime	88.9% (24)	91.1% (41)	0.53
Ceftazidime	25.9% (7)	46.7% (21)	0.07
Cefepime	18.5% (5)	42.2% (19)	0.03
Piperacillin-tazobactam	37.0% (10)	26.7% (12)	0.25
Ticarcillin-clavulanic acid	11.1% (3)	22.2% (10)	0.19
Meropenem	3.7% (1)	22.2% (10)	0.03
Imipenem	3.7% (1)	22.2% (10)	0.03
Doripenem[b]	7.4% (2)	22.2% (10)	0.09
Gentamicin	7.4% (2)	8.9% (4)	0.60
Amikacin	3.7% (1)	0% (0)	0.38
Ciprofloxacin	22.2% (6)	31.1% (14)	0.30
Trimethoprim-sulphamethoxazole	7.4% (2)	6.7% (3)	0.63
Tigecycline[b]	25.9% (7)	28.9% (13)	0.50
Colistin	0% (0)	0% (0)	1.00

[a]CLSI susceptibility breakpoints unless stated otherwise; [b]based on BSAC susceptibility breakpoints; [c]statistical analysis using Fisher's Exact Test.

2.3.2.4 Susceptibility patterns of carbapenem-resistant *Acinetobacter* species

Table 2-24 shows the different antibiogram patterns of the carbapenem-resistant *Acinetobacter* spp. All 12 isolates are resistant to the carbapenems, ciprofloxacin and β-lactam/β-lactamase-inhibitor combinations.

Seven isolates of *Acinetobacter* genospecies 3 had a pattern of susceptibility to several non-β-lactam antimicrobial agents such as the aminoglycosides, trimethoprim-sulphamethoxazole and colistin (see antibiogram pattern 1). *A. johnsonii* isolate 25868 had a more resistant phenotype (antibiogram pattern 2), being resistant to gentamicin and trimethoprim-sulphamethoxazole but remaining susceptible to tigecycline, amikacin and colistin, while three *Acinetobacter* genospecies 3 isolates had a similar antibiogram pattern with the exception of higher cephalosporin MICs and non-susceptibility to tigeycline (antibiogram pattern 3). As observed in other isolates of *Acinetobacter* genospecies 3 and *A. johnsonii*, cefepime MICs were higher than ceftazidime MICs in the carbapenem-resistant isolates as well (see Appendix 1). Finally, the *A. baumannii* isolate 229437 had the most resistant phenotype, being resistant to all antimicrobial agents except colistin (antibiogram pattern 4).

Table 2-24. Antimicrobial susceptibility patterns of pan-carbapenem-resistant *Acinetobacter* spp.

Antimicrobial agent	Antibiogram[a] (Etest MIC μg/mL)			
	***Acinetobacter* genosp. 3** (12659, 23629, 27616, 28594, 14448, 14748, 103165)	***A. johnsonii*** (25868)	***Acinetobacter* genosp. 3** (29540, 32357, 36277)	***A. baumannii*** (229437)
	Pattern 1	**Pattern 2**	**Pattern 3**	**Pattern 4**
Cefotaxime	**I/R** (32-64)	**I** (16)	**R** (≥ 128)	**R** (≥ 256)
Ceftazidime	**S/I** (4-16)	**S** (8)	**I/R** (16-32)	**R** (≥ 256)
Cefepime	**I/R** (16-64)	**R** (32)	**R** (64-128)	**R** (≥ 256)
Piperacillin-tazobactam	**R** (≥ 128/4)	**R** (128/4)	**R** (≥ 256/4)	**R** (≥ 256/4)
Ticarcillin-clavulanic acid	**R** (≥ 256/2)	**R** (≥ 256/2)	**R** (≥ 256/2)	**R** (≥ 256/2)
Meropenem	**R** (≥ 32)	**R** (≥ 32)	**R** (≥ 32)	**R** (≥ 32)
Imipenem	**R** (≥ 32)	**R** (≥ 32)	**R** (≥ 32)	**R** (≥ 32)
Doripenem[b]	**R** (≥ 32)	**R** (≥ 32)	**R** (≥ 32)	**R** (≥ 32)
Trimethoprim-sulphamethoxazole	**S** (≤ 1/19)	**R** (≥ 32/608)	**R** (≥ 32/608)	**R** (≥ 32/608)
Ciprofloxacin	**R** (8-≥ 32)	**R** (≥ 32)	**R** (≥ 32)	**R** (≥ 32)
Tigecycline[b]	**I** (2)	**S** (0.5)	**I** (2)	**R** (8)
Gentamicin	**S** (0.5-1)	**R** (128)	**R** (32)	**R** (≥ 256)
Amikacin	**S** (0.5-4)	**S** (1)	**S** (0.5-2)	**R** (≥ 256)
Colistin	**S** (0.5)	**S** (0.5)	**S** (0.5)	**S** (0.5)

[a]susceptibility interpretation according to CLSI MIC breakpoints unless stated otherwise (CLSI, 2009); [b]susceptibility interpretation according to BSAC MIC breakpoints (BSAC, 2009); S, susceptible; I, intermediate; R, resistant. See section 2.3.2.4 for discussion of antibiogram patterns of isolates.

2.3.3 PFGE fingerprinting

PFGE was performed for the following species (number of isolates): *Acinetobacter* genospecies 3 (45); *A. baumannii* (27); *A. calcoaceticus* (6); *A. johnsonii* (12) and *A. ursingii* (8).

2.3.3.1 PFGE of *Acinetobacter* genospecies 3

PFGE patterns and analysis of banding similarity of 45 isolates are shown in Figure 2-1. Polyclonal origins of the isolates were demonstrated by the PFGE banding patterns.

However, there was some evidence of expansion of certain strains with indistinguishable or closely related banding patterns. Three isolates (106498, 106770 and 107012) exhibited ≥ 95% banding similarity with isolates 106498 and 106770 having indistinguishable PFGE patterns. Carbapenem-resistant isolates also demonstrated evidence of clonal expansion.

Four groups of banding patterns were noted for carbapenem-resistant isolates. The first group consisted of isolates 29540, 32357 and 36277, which had indistinguishable PFGE patterns. The banding pattern of the latter clone had ≤ 80% similarity with those of the other three groups. The second group, consisting of isolates 23629 and 103165, had banding similarity of > 95%. The third group consisted of isolates 14448, 14748 and 12659. Banding similarity was > 95% among these isolates and PFGE

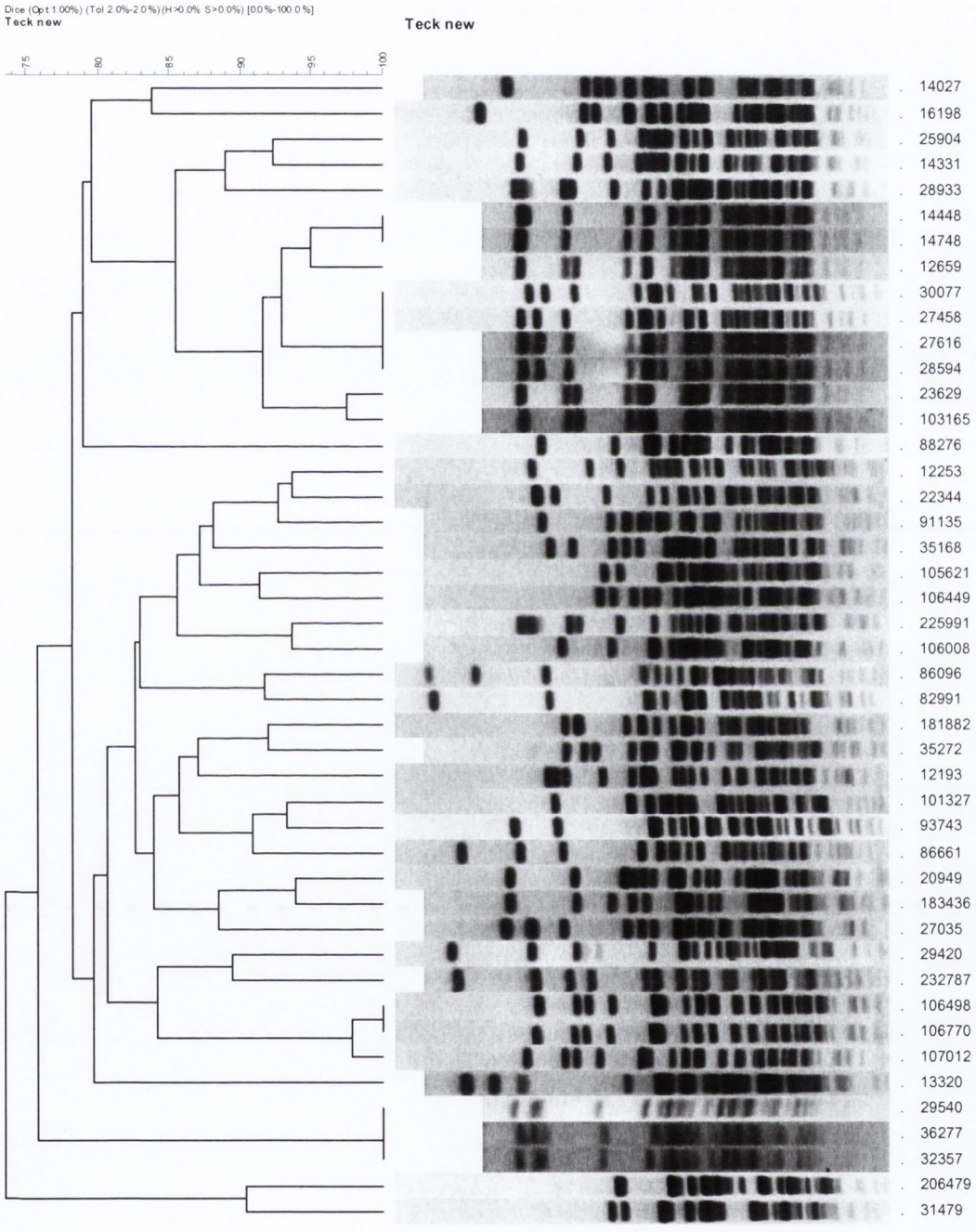


Figure 2-1. Analysis of PFGE of *Acinetobacter* genospecies 3 (45 isolates) using BioRad Fingerprinting II software version 3.

patterns of isolates 14448 and 14748 were indistinguishable. The fourth group consisted of two carbapenem-resistant isolates 27616 and 28594 as well as two carbapenem-susceptible isolates 27458 and 30077. The four isolates had indistinguishable PFGE patterns.

2.3.3.2 PFGE of *A. baumannii*

PFGE patterns of the 27 isolates are shown in Figure 2-2. PFGE again demonstrated the predominantly polyclonal origins of the *A. baumannii* isolates. Apart from three isolates (107245, 107228 and 107532) with indistinguishable PFGE banding patterns, all the other *A. baumannii* isolates shared less than 90% similarity in banding patterns.

2.3.3.3 PFGE of *A. calcoaceticus*

PFGE patterns of the six isolates are shown in Figure 2-3. The patterns revealed a mixture of polyclonal origins of isolates as well as the expansion of strains with closely related or indistinguishable banding patterns (isolates 183262, 28263 and 33241).

2.3.3.4 PFGE of *A. ursingii*

PFGE patterns of the eight isolates are shown in Figure 2-4. Four isolates (isolates 89302, 90772, 91352 and 91411) had indistinguishable PFGE banding patterns, while

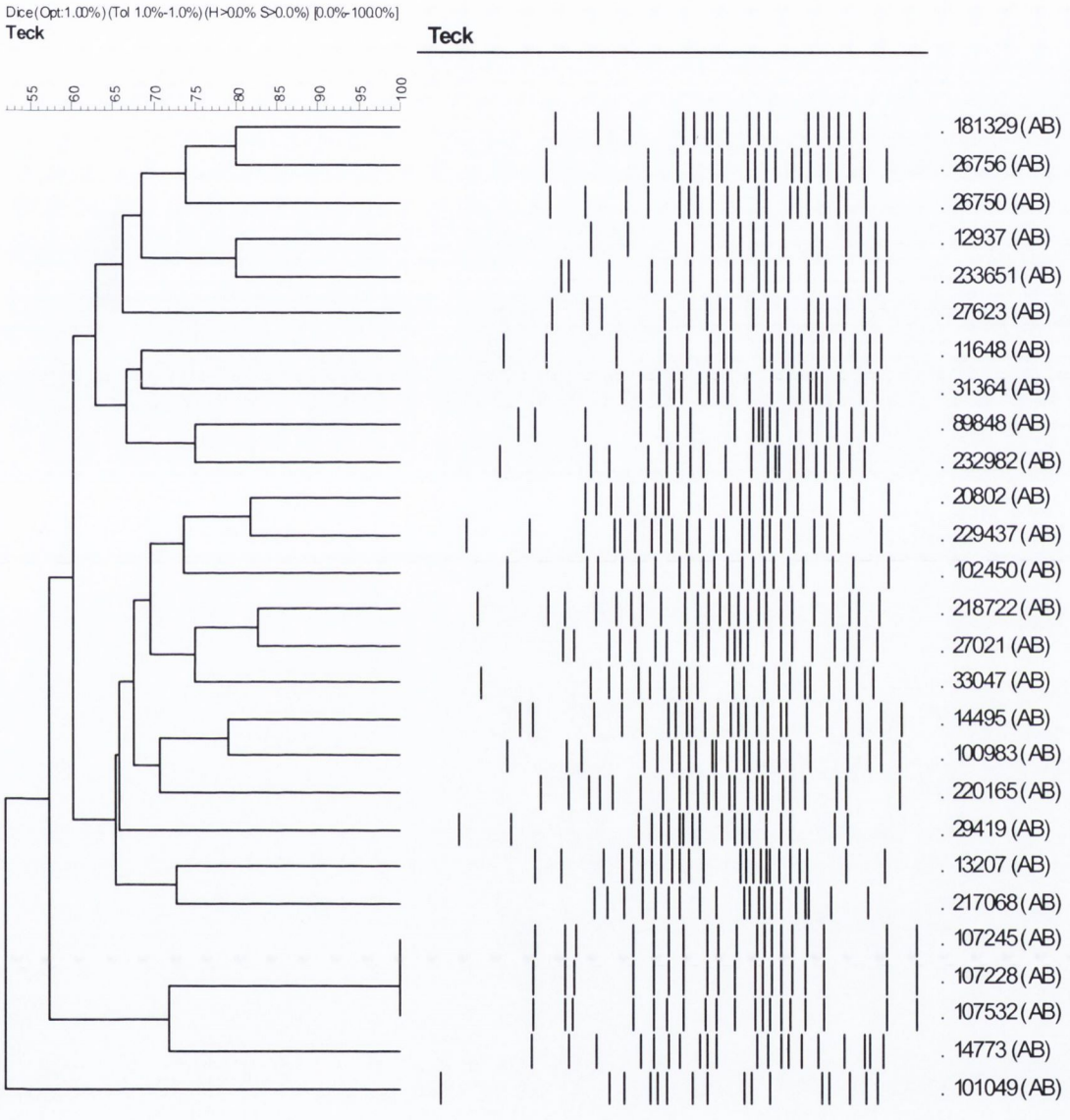


Figure 2-2. Analysis of *A. baumannii* PFGE (27 isolates) using BioRad Fingerprinting II software version 3.

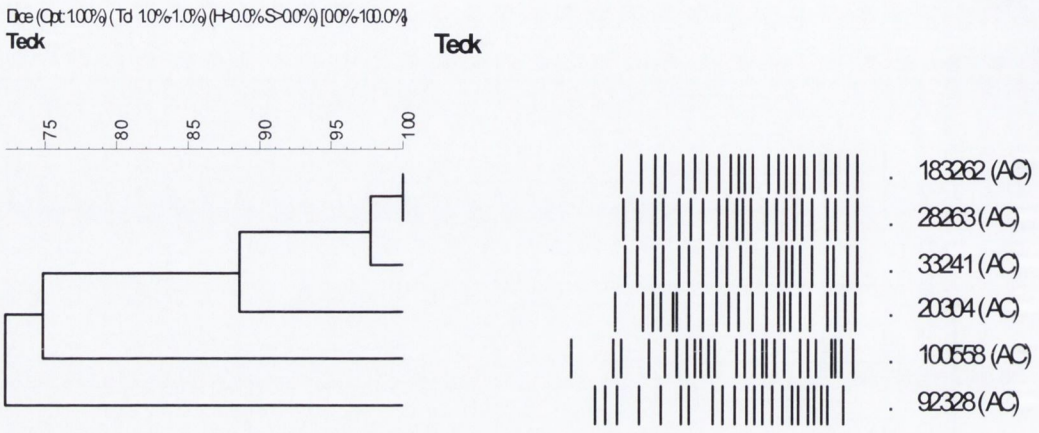


Figure 2-3. Analysis of *A. calcoaceticus* PFGE (6 isolates) using BioRad Fingerprinting II software version 3.

Figure 2-4. Analysis of *A. ursingii* PFGE (8 isolates) using BioRad Fingerprinting II software version 3.

the remaining four isolates had < 85% similarity in PFGE patterns compared to one another and to the above clone.

2.3.3.5 PFGE of *A. johnsonii*

PFGE patterns of the 12 isolates are shown in Figure 2-5. Polyclonal origins of the *A. johnsonii* isolates were demonstrated with all isolates showing less than 80% similarity in banding patterns when compared to one another.

2.3.4 Demographic and clinical data of isolates

The 117 *Acinetobacter* isolates were isolated from 116 patients. Data on gender, age and location of patient were obtained along with data on specimen type, date of sampling and the location of patient at the time of sampling.

2.3.4.1 General demographics

All 117 isolates were considered sporadic in nature as no outbreaks were identified during the 32-month period of isolate collection from May 2005 to December 2007. An outbreak in this study was defined as two or more cases with clear temporal or geographical link, although exhaustive patient and healthcare staff tracking was not possible in this study. The types of specimens from which the 117 *Acinetobacter* spp. were cultured are shown in Table 2-25. The majority of isolates were derived from

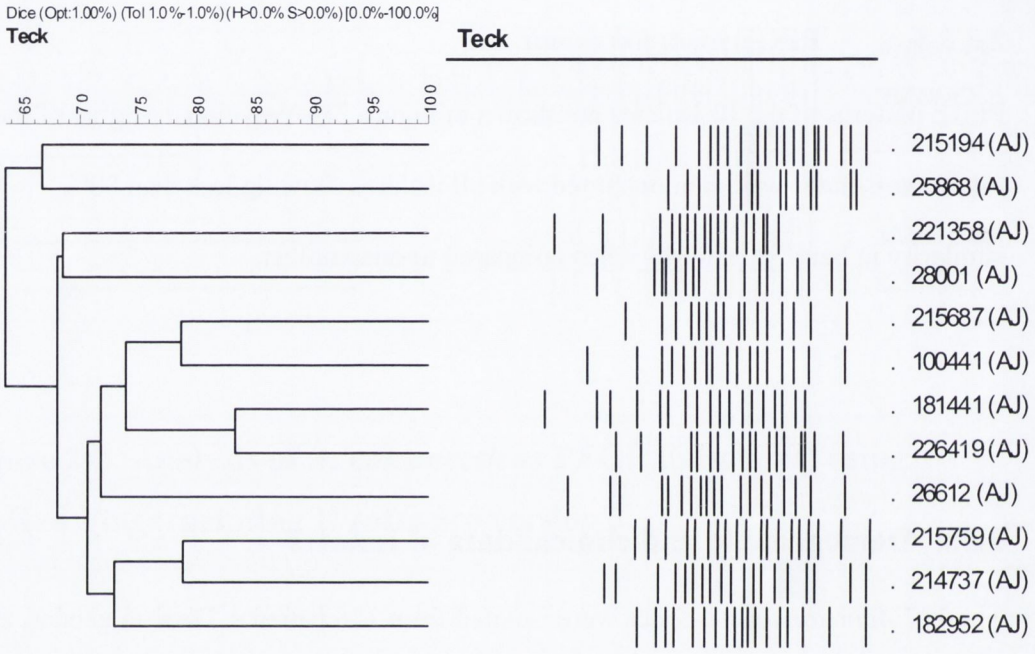


Figure 2-5. Analysis of *A. johnsonii* PFGE (12 isolates) using BioRad Fingerprinting II software version 3.

Table 2-25. Types of specimen from which *Acinetobacter* spp. were isolated in the study.

Types of specimens	Total no. of isolates (n = 117)	Acbc isolates[a] (n = 78)	Non-Acbc isolates (n = 39)	*p* value[b] (Acbc vs non-Acbc)
Blood cultures	14	8	6	0.30
Respiratory specimens	35	29	6	0.01
Specimens from sterile sites	9	5	4	0.35
Swabs	40	27	13	0.53
Urinary specimens	19	9	10	0.05

cultures of respiratory specimens and swabs (35 and 40 isolates, respectively). The different types of respiratory specimens were sputum, broncho-alveolar lavage and tracheal aspirate, while swabs were taken from superficial sites such as wounds, skin, eyes and ears. On comparing Acbc and non-Acbc isolates from different specimen types, a greater proportion of Acbc isolates were cultured from respiratory specimens than of non-Acbc isolates (29/78 vs 6/39; *p* value of 0.01, Fisher's exact test) while the converse was true for urinary specimens (9/78 vs 10/39; *p* value of 0.05, Fisher's exact test). A significant minority of isolates were cultured from blood or other sterile sites (14 and 9 isolates, respectively). Of the 14 blood culture isolates, six were *Acinetobacter* genospecies 3, while five were *A. ursingii*. The remaining three isolates were *A. baumannii*, *A. calcoaceticus* and *A. haemolyticus*, respectively.

Data on gender and age were available for 113 of the 116 patients. There was a male preponderance with a male:female ratio of 67:46. The ages of patients ranged from 1 month to 96 years. For patients with Acbc isolates, the age range was two months to 96 years, with a mean age of 56 years. For patients with non-Acbc isolates, the age range was one month to 82 years, with a mean age of 21 years.

The locations of patients at the time of sampling were shown in Table 2-26. The majority of isolates were cultured from patients residing in hospital wards or units such as the medical wards or ICUs, while a significant minority were cultured from patients in the community setting. Notably, a greater proportion of non-Acbc isolates were cultured from patients in the community setting than of Acbc isolates (16/39 vs 8/78; *p* value of < 0.01, Fisher's exact test), while a greater proportion of Acbc

Table 2-26. Locations of patients at the time of sampling.

Location of patient	Total no. of isolates (n = 117)	Acbc isolates[a] (n = 78)	Non-Acbc isolates (n = 39)	*p* value[b] (Acbc vs non-Acbc)
Community	24	8	16	< 0.01
Acccient & emergency department	5	5	0	0.17
Outpatient department	10	7	3	0.56
Medical wards	28	24	4	0.01
Haematology & oncology units	10	4	6	0.07
Surgical wards	20	16	4	0.13
Intensive care units	16	13	3	0.15
Unknown	4	1	3	0.11

[a]Acbc, *Acinetobacter calcoaceticus-baumannii* complex; [b]statistical analysis using Fisher's exact test.

isolates were derived from patients in the medical wards than of non-Acbc isolates (24/78 vs 4/39; *p* value of 0.01, Fisher's exact test).

2.3.4.2 Epidemiological data of *Acinetobacter* clones

Clonal expansion of certain strains of various *Acinetobacter* spp. was identified from the analysis of PFGE patterns. For the *A. baumannii* clone consisting of isolates 107228, 107245 and 107532, all three patients were in the ICU over a two-month period from November to December 2007. For the *Acinetobacter* genospecies 3 clone consisting of isolates 106498 and 106770, the two patients were in the same ward over a two-month period from October to November 2007. Of the four patients with the same strain of *A. ursingii*, two were located in the haematology unit while the other two patients were in medical and surgical wards respectively. The four *A. ursingii* isolates were cultured within a three-month period from August to October 2007. The epidemiology of carbapenem-resistant clones of *Acinetobacter* genospecies 3 is discussed in the next section 2.3.4.3.

2.3.4.3 Clinical data of carbapenem-resistant *Acinetobacter* spp.

Clinical and epidemiological data of carbapenem-resistant *Acinetobacter* isolates are shown in Table 2-27. Carbapenem-resistant *Acinetobacter* were isolated from a variety of specimens including sputum, urine, swabs and sterile site fluids. Patients were aged between 44 and 73 years. There was a male-to-female ratio of 2:1 and most

Table 2-27. Clinical data of patients with pan-carbapenem-resistant *Acinetobacter* isolates.

Specimen data				Patient data						
Isolate	**Organism**	**Sampling date (mth/yr)**	**Specimen Type**	**Age (yrs)**	**Sex**	**Location**	**Duration in hospital[a]**	**Infection status**	**Prior GNB antibiotics**	**Underlying conditions**
12659	*Acinetobacter* genosp. 3	05/05	Sputum	49	M	ICU	19 days	Colonised	Mer, Cip	Crohn's Disease, pancolectomy.
29540	*Acinetobacter* genosp. 3	08/05	Biliary fluid	62	F	Surgical ward A	37 days	Infected	Ptz, Cip	Pancreatic carcinoma, gastrojejunostomy.
23629	*Acinetobacter* genosp. 3	03/06	Biliary fluid	71	F	Surgical ward B	15 days	Infected	Aug, Cip	Pancreatic carcinoma, biliary tract surgery.
25868	*A. johnsonii*	05/06	Chest drain fluid	44	M	Medical ward A	61 days	Infected	Cip	Liver failure, recurrent empyema.
27616	*Acinetobacter* genosp. 3	06/06	Wound swab	44	M	ICU	29 days	Infected	Ptz, Cip	Rhabdomyolysis, hepatitis.
28594	*Acinetobacter* genosp. 3	07/06	Leg wound	49	M	Surgical ward C	22 days	Colonised	Pen, Clox	Lower limb cellulitis.
14448	*Acinetobacter* genosp. 3	08/06	Sputum	56	F	Oncology ward	58 days	Infected	Mer, Cip	Oesophageal carcinoma, diabetes.
14748	*Acinetobacter* genosp. 3	09/06	Sputum	73	M	Oncology ward	5 days[b]	Colonised	Aug, Cip	Lung carcinoma.
32357	*Acinetobacter* genosp. 3	10/06	Leg swab	62	M	ICU	24 days	Colonised	Ptz, Cip	Abdominal surgery, cardiovascular disease.
36277	*Acinetobacter* genosp. 3	12/06	Leg swab	57	M	Surgical ward D	33 days	Colonised	Cip	Major trauma and surgery.
103165	*Acinetobacter* genosp. 3	05/07	Sputum	64	M	Medical ward B	27 days	Infected	Ptz, Cip	Wegener's disease, diabetes mellitus.
229437	*A. baumannii*	08/07	MSU	71	F	Community	N/A	Infected	Data unavailable	Data unavailable.

[a]duration from time of admission to the time of organism isolation in the current patient episode; [b]transferred from another hospital.
ICU, intensive care unit; Gen, gentamicin; Cip, ciprofloxacin; Ptz, piperacillin-tazobactam; Pen, penicillin; Mer, meropenem; Aug, amoxicillin-clavulanic acid; MSU, midstream specimen of urine; N/A, not applicable. Refer to Table 2-24 for laboratory characteristics of isolates.

were hospital in-patients for longer than two weeks when the organisms were isolated. Seven of the 12 patients had clinical infections attributable to the carbapenem-resistant *Acinetobacter* isolates, while most patients had histories of significant co-morbidities and/or major surgery. Of the 11 patients with data on antimicrobial therapy prior to the isolation of the organisms, only two had prior carbapenem (meropenem) treatment. Six had prior therapy with β-lactam/β-lactamase-inhibitor combinations and 10 patients had prior therapy with ciprofloxacin.

Epidemiologically, the patients were located in a wide variety of settings, not just in 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 hospitalisation in the patient. However, the strains of *Acinetobacter* genospeices 3 showed some degree of clonal persistence within the institution. Three isolates with indistinguishable PFGE patterns (isolates 29540, 32357 and 36277) were recovered from three different locations. The latter two isolates may be epidemiologically related, as they were recovered within two months of one another. The patient carrying isolate 32357 was in ICU at the time of sampling, while the other patient carrying isolate 36277 had a history of ICU stay prior to his transfer to the orthopaedic ward, where the isolate was subsequently recovered. In another cluster, isolates 14448 and 14748 were both recovered from the oncology ward within a month and shared indistinguishable PFGE patterns. However, a closely related strain on PFGE (isolate 12659) was not epidemiologically related to the two isolates in space or time. Similarly, two closely related strains on PFGE (isolates 23629 and 103165) were recovered from different locations more than a year apart. Four isolates

(27458, 27616, 28594 and 30077) with indistinguishable PFGE patterns presented an interesting epidemiological picture. Carbapenem-susceptible isolate 27458 and carbapenem-resistant isolate 27616 were recovered within a week of each other from the same patient in ICU. Following the patient's subsequent transfer to the orthopaedic ward, carbapenem-resistant isolate 28594 and carbapenem-susceptible isolate 30077 were recovered from two patients in the ward, respectively. The two patients had acquired the isolates within two weeks and five weeks respectively of the index patient's transfer to the ward.

2.4 Discussion

Speciation of *Acinetobacter* isolates using phenotypic characteristics such as biochemical reactions has been found to be highly unreliable (Bergogne-Bérézin & Towner, 1996; Peleg *et al.*, 2008). The latter finding was further corroborated by data obtained in this study. The reason for the poor discriminatory ability of common biochemical tests in the unambiguous identification of the various *Acinetobacter* spp. is probably the result of their biochemical similarities (Bouvet & Grimont, 1986; Peleg *et al.*, 2008). Compared to the partial *rpoB* sequencing results, the VITEK 2 GNI system was only able to speciate correctly about a quarter of the *Acinetobacter* isolates, giving an overall error rate in speciation of 74.4%. Moreover, while the

VITEK 2 was reasonably accurate in correctly identifying *Acinetobacter* spp. to the genus level, four isolates belonging to the *Pseudomonas*, *Psychrobacter* and *Photorhabdus* genera were misidentified as belonging to the *Acinetobacter* spp. This highlights the limitations of phenotypic methods in accurately identifying non-fermentative GNB and the need to regard identification results for *Acinetobacter* spp. generated by these methods as preliminary data.

Molecular methods such as ARDRA, DNA-DNA hybridisation and *rpoB* sequencing are the preferred methods for the accurate speciation of *Acinetobacter*. The merits and drawbacks of the various molecular methods have been discussed in section 1.1.3. The *rpoB* sequencing method was found to be accurate in distinguishing the respective *Acinetobacter* spp. (Gundi *et al.*, 2009; La Scola *et al.*, 2006; Vaneechoutte *et al.*, 2008). In this study, *rpoB* sequencing has also been found to be highly reliable in *Acinetobacter* speciation, with over 90% of the sequencing results achieving > 99% sequence concordance with those of the reference strains. The high level of intra-species sequence concordance (98.4% to 100%) in this study concurs with the result of 98.7% to 100% sequence similarity obtained by Gundi *et al.* (Gundi *et al.*, 2009). It was noted that *rpoB* sequencing may not be able to distinguish reliably between certain pairs of species, given their high levels of sequence similarity in the particular region of the *rpoB* gene (La Scola *et al.*, 2006). Such pairs are *Acinetobacter* genospecies 11/*A. baylyi* and *A. junii*/*A. grimontii*. The former pair can be reliably distinguished through the sequencing of the *rpoB-rpoC* spacer region. *Acinetobacter* genospecies 11 produced a *circa* 150-bp amplicon while *A. baylyi* produced a *circa* 85-bp amplicon. Four isolates in this study were reliably identified as *Acinetobacter*

genospecies 11 using the above method. Distinguishing between *A. junii* and *A. grimontii* has been problematic, as very high levels of concordance (> 99%) were found for the *rpoB* gene, spacer region between *rplL* and *rpoB* as well as the spacer region between *rpoB* and *rpoC* (La Scola *et al.*, 2006). However, a recent study using AFLP and 16S rRNA sequencing confirmed that *A. grimontii* was synonymous with *A. junii*, which corroborated the above findings by La Scola *et al.* (Vaneechoutte *et al.*, 2008).

Epidemiologically, the study revealed the unusual finding that *Acinetobacter* genospecies 3 was the predominant *Acinetobacter* species encountered in this laboratory and hospital. Where validated molecular methods have been employed, most studies have reported *A. baumannii* as the predominant species, but occasionally, *Acinetobacter* genospecies 3 has been found to be more prevalent in centres in Germany and Sweden (Bouvet & Grimont, 1986; Traub & Bauer, 2000). The accurate speciation of *Acinetobacter* spp. is of clinical significance particularly in the early recognition of an outbreak occurring in the hospital setting. It may also aid in predicting the antimicrobial susceptibility pattern of the isolate based on the local antimicrobial epidemiology.

Etest gradient method has been shown to produce results with very good correlation to those obtained with CLSI reference method (disc diffusion), with very high levels of category agreement and acceptable levels of VME, ME and mE for all the tested antimicrobial agents. On the other hand, VITEK 2 AST method had lower levels of

category agreement with CLSI disc diffusion method, with testing of piperacillin-tazobactam producing unacceptable levels of category agreement, VME and mE. Therefore, interpretation of piperacillin-tazobactam susceptibility results of *Acinetobacter* isolates by VITEK 2 should be done with caution; and on the basis of data obtained in this study, it is recommended that VITEK 2 results be confirmed by CLSI disc diffusion method or Etest method.

The study also demonstrated the ambiguity in carbapenem susceptibility interpretation of isolates with borderline MIC results. Both CLSI and BSAC had recommended the testing of carbapenem susceptibility on Mueller-Hinton medium (BSAC, 2009b; CLSI, 2009). *A. baumannii* isolate 11648 had meropenem and imipenem Etest MICs of 4 μg/mL respectively on the recommended medium. Using CLSI susceptibility breakpoints, the isolate would have been deemed meropenem- and imipenem-susceptible but with BSAC susceptibility breakpoints, the isolate would have been reported as having intermediate level of resistance to both carbapenems (BSAC, 2009a; CLSI, 2009). Hence, caution is recommended in the interpretation of results of *Acinetobacter* spp. with reduced carbapenem susceptibility. The standardisation of susceptibility breakpoints by various authorities would also greatly reduce the ambiguity in the interpretation of susceptibility data.

In this study, AST of the *Acinetobacter* spp. has revealed that a significant proportion of isolates are not susceptible to third- and fourth-generation cephalosporins, ciprofloxacin and piperacillin-tazobactam. Notably, the non-susceptibility of *Acinetobacter* to cefepime was almost 25%. This is consistent with data from other

recent studies showing that non-susceptibility to cefepime is considerably higher in *Acinetobacter* spp. than in *Enterobacteriaceae* and *P. aeruginosa*. Interestingly, cefepime non-susceptibility rates were highest in *Acinetobacter* genospecies 3 and *A. johnsonii* (42.2% and 41.7% respectively) in comparision to *A. baumannii* or other species (18.5% and 0% respectively) (see Table 2-9). Ceftazidime non-susceptibility in *Acinetobacter* spp. also showed differences between various species. *Acinetobacter* genospecies 3, *A. calcoaceticus* and *A. ursingii* had higher ceftazidime non-susceptibility rates (46.7%, 83.3% and 75%, respectively) compared to *A. baumannii* or other species (25.9% and 6.5%, respectively) (see Table 2-8). Cefotaxime non-susceptibility rates amongst different *Acinetobacter* spp. were generally high (see Table 2-7).

The differences in cephalosporin susceptibility profiles of the respective species may be the result of different substrate specificities of Ambler class C cephalosporinases in the various species. However to date, only cephalosporinases from *A. baumannii*, *Acinetobacter* genospecies 3 and *A. baylyi* have been characterised. Hence, the postulation that species-related differences in cephalosporin profiles are due to genetic differences in the species' chromosomal cephalosporinases remains unconfirmed. Cephalosporins probably have a very limited role in the treatment of *Acinetobacter* infections in view of the high non-susceptibility rates *in vitro*. Moreover, data on clinical efficacy with cephalosporin treatment is also lacking.

Non-susceptibility rates of isolates to β-lactam/β-lactamase-inhibitor combinations were also quite high in this study. Isolates were least susceptible to piperacillin-

tazobactam while ticarcillin-clavulanic acid non-susceptibility rate followed those of the carbapenems quite closely (see Tables 2-10 and 2-22). In fact, the 12 isolates that were fully resistant to ticarcillin-clavulanic acid were the same isolates that were resistant to the carbapenems (see Appendix 1). Although no CLSI or BSAC susceptibility breakpoints exist for amoxicillin-clavulanic acid for *Acinetobacter* spp., presumptive breakpoints may be derived based on CLSI breakpoints for *Enterobacteriaceae* (CLSI, 2009). Since CLSI interpretative standards for amoxicillin-clavulanic acid and ampicillin-sulbactam are the same for *Enterobacteriaceae*, and in addition, there are interpretative breakpoints for ampicillin-sulbactam for *Acinetobacter* spp., it would be reasonable to adopt the following presumptive amoxicillin-clavulanic breakpoints for *Acinetobacter* spp.: ≤ 8/4 μg/mL as susceptible, 16/8 μg/mL as intermediate and ≥ 32/16 μg/mL as resistant. Based on these presumptive breakpoints, non-susceptibility rate for amoxicillin-clavulanic acid was 19.7% (23/117), which was between those for ticarcillin-clavulanic acid and piperacillin-tazobactam respectively (see Table 2-21). As with ticarcillin-clavulanic acid, the isolates with high amoxicillin-clavulanic acid MICs (≥ 256 μg/mL) were also resistant to the carbapenems.

More worryingly, in the present study, the carbapenem resistance in *Acinetobacter* isolates was over 10%, mirroring a trend in antimicrobial resistance which is also emerging in various parts of the world. The resistance rate rose to over 14% when Acbc isolates were considered separately. Apart from an ICU outbreak in 2001 originating from a patient who had acquired MDR *A. baumannii* overseas, St. James's Hospital did not encounter any carbapenem-resistant *Acinetobacter* isolates from 2002

to 2004 (Kelleher M, St. James's Hospital, personal communication). The emergence of carbapenem-resistant isolates from 2005 to 2007 is a worrying development. All 12 isolates were highly resistant to meropenem and imipenem (MICs of $\geq$ 32 μg/mL and $\geq$ 32 μg/mL, respectively). Doripenem MICs were lower than those for meropenem and imipenem for seven of the 12 isolates. Nonetheless, all isolates remained resistant to doripenem according to BSAC interpretative criteria (see Tables 2-12 to 2-14). Doripenem MICs for most of the *Acinetobacter* isolates in this study were one- to two-fold lower than those of meropenem and imipenem (see Appendix 1). This finding is in keeping with the observations of other reports (Marti *et al.*, 2009; Mushtaq *et al.*, 2004). Apart from carbapenems, the 12 isolates were also resistant to ciprofloxacin and most other β-lactams, but remained susceptible to colistin (see Table 2-24). Susceptibility to other agents such as aminoglycosides and trimethoprim-sulphamethoxazole was more variable. These agents may be considered for the treatment of infections caused by susceptible isolates. However, it should be noted that 11 of the 12 isolates had reduced susceptibility to tigecycline. The susceptibility results of these isolates suggest that non-β-lactam options for the treatment of infections by these carbapenem-resistant isolates may be significantly limited.

Speciation revealed the majority of carbapenem-resistant *Acinetobacter* isolates encountered in this laboratory to be *Acinetobacter* genospecies 3. This finding was in sharp contrast to other studies where *A. baumannii* was the overwhelmingly predominant species. The unusual finding may, at least in part, be due to the local predominance of *Acinetobacter* genospecies 3. This also suggests that carbapenem resistance in this hospital developed in endemic strains, rather than arising from

imported strains. This is supported by PFGE findings of clonally indistinguishable hospital strains of carbapenem-susceptible and carbapenem-resistant *Acinetobacter* genospecies 3. The origins of the carbapenem-resistant *A. baumannii* and *A. johnsonii* isolates were unclear. However, since carbapenem-resistant *A. johnsonii* had not been reported elsewhere, it is again possible that an endemic strain had acquired the resistance mechanism(s). Clinical and epidemiological data also suggest that carbapenems may not be the only antimicrobial agents that exerted selective pressure for the emergence of carbapenem-resistant strains, since a recent history of carbapenem exposure was not present in most of the patients harbouring the carbapenem-resistant isolates (see Table 2-27). The PFGE results also suggest that carbapenem resistance may confer a survival advantage on the *Acinetobacter* isolates, since clonal expansion of *Acinetobacter* genospecies 3 predominantly involved the carbapenem-resistant strains (see Figure 2-1). The recent emergence of carbapenem resistance in this laboratory's population of *Acinetobacter* isolates suggests that caution should be exercised when considering a carbapenem for the empirical treatment of serious nosocomial *Acinetobacter* infections, at least in this hospital.

Of particular concern is the finding that tigecycline non-susceptibility in the collection of *Acinetobacter* isolates was 25% for Acbc isolates. Since all of the patients in the study did not have previous exposure to tigecycline, the results would suggest that some isolates may be intrinsically non-susceptible to tigecycline. Much confusion and uncertainty still surrounds tigecycline susceptibility testing and its clinical implications. Several studies of tigecycline's clinical efficacy in the treatment of MDR *A. baumannii* infections had extrapolated CLSI tigecycline susceptibility

breakpoints recommended for *Enterobacteriaceae* to the interpretation of tigecycline MICs of *Acinetobacter* spp. (Anthony *et al.*, 2008; CLSI, 2009; Gallagher & Rouse, 2008; Karageorgopoulos *et al.*, 2008; Schafer *et al.*, 2007). Based on such assumptions, tigecycline MICs of ≤ 2 μg/mL would be considered susceptibible, whereas BSAC would report an MIC of 2 μg/mL as an intermediate level of susceptibility (BSAC, 2009a). Many isolates in the above studies would have to be re-classified microbiologically as having reduced susceptibility or being fully resistant to tigecycline based on BSAC interpretation criteria. Perhaps not too surprisingly, positive clinical outcomes of the above studies varied from 28% to 84% (Anthony *et al.*, 2008; Gallagher & Rouse, 2008; Schafer *et al.*, 2007). Moreover, a recent study also found that different testing methodologies could yield variable tigecycline MIC results, further complicating the interpretation of tigecycline susceptibility (Casal *et al.*, 2009). They found a divergence of results using Etest and broth microdilution methods in isolates with tigecycline Etest MICs of ≥ 2 μg/mL. In the present study, the medium used for the Etest method was found to influence the tigecycline MIC results. MICs of 76% of isolates were one- to two-dilution higher when tested on Iso-Sensitest Agar (as recommended by BSAC) than on Mueller-Hinton Agar, with the greatest divergence of results (2-dilution difference) occurring when the tigecycline Etest MICs were ≥ 1 μg/mL (see Appendix 1). However, tigecycline susceptibility results obtained by the Etest method (using Iso-Sensitest Agar) were shown to have very good correlation with those of the BSAC agar dilution reference method (Hope *et al.*, 2007). With such divergence in methodology and susceptibility interpretation criteria, it is difficult to draw definitive conclusions on the results of reported studies.

Currently, the only source with official recommendations on tigecycline testing methodology and interpretation criteria for *Acinetobacter* spp. is BSAC (BSAC, 2009a). Hence, the results of this study were based on their recommendations. However, it should be noted that the tigecycline susceptibility breakpoints provided by BSAC were tentative for a 12-month period, and may be subjected to revision in the future (BSAC, 2009a). Nonetheless, the preliminary findings in this study present a worrying prospect that tigecycline may not be a suitable option for the treatment of multidrug-resistant *Acinetobacter* spp., especially since only one of the 12 carbapenem-resistant isolates (*A. johnsonii* isolate 25868) was susceptible to tigecycline (see Table 2-24). In addition to inconsistent treatment outcomes, there were also worrying reports of the development of resistance during tigecycline therapy (Peleg *et al.*, 2007b; Reid *et al.*, 2007; Schafer *et al.*, 2007). High rate of baseline non-susceptibility to tigecycline among MDR isolates was also found in an Israeli study (78%) although Etest MIC values were read at the point of 80% inhibition (Navon-Venezia *et al.*, 2007). Numerous clinical and laboratory issues surrounding tigecycline testing and therapy remained unresolved. Caution is advised when interpretating tigecycline susceptibility results and empirical therapy with tigecycline for MDR *Acinetobacter* infections may not be appropriate.

It should be noted that colistin appears to be the most active antimicrobial agent against *Acinetobacter* spp., including carbapenem-resistant isolates. However, it is not without diagnostic and clinical uncertainties. Disc diffusion has been shown to be an unreliable method for colistin susceptibility testing (Gales *et al.*, 2001; Lo-Ten-Foe *et al.*, 2007). Divergence of results using Etest and reference methods was also observed

with higher colistin Etest MICs of ≥ 4 μg/mL (Arroyo *et al.*, 2005; Landman *et al.*, 2008). Clinically, different drug formulations and dosing regimens exist, and the drug's penetration into various body sites has yet to be defined (Landman *et al.*, 2008; Li *et al.*, 2006a).

In the analysis of antimicrobial susceptibility patterns of different *Acinetobacter* spp., Acbc isolates were found to be more resistant than non-Acbc isolates (see Table 2-22). Since most of the clinical infections due to *Acinetobacter* spp. were caused by Acbc isolates, it is plausible that the antimicrobial susceptibility patterns may be the result of the different levels of antimicrobial exposure by different species. In any case, from a clinical viewpoint, this present study showed that the more clinically important isolates were also the more drug-resistant ones. Acbc isolates were significantly more resistant to most β-lactams, ciprofloxacin and tigecycline. Fortunately, the resistance rates of both Acbc and non-Acbc isolates for aminoglycosides, trimethoprim-sulphamethoxazole and colistin have remained low (< 10%).

Interestingly and rather unusually, the *Acinetobacter* genospecies 3 isolates were significantly more resistant than the *A. baumannii* isolates in this study (see Table 2-23). *Acinetobacter* genospecies 3 isolates were significantly more resistant to meropenem, imipenem and cefepime, respectively, than the *A. baumannii* isolates (p values of < 0.05, respectively). This unusual epidemiological pattern of antimicrobial resistance has not been reported in the literature to date. The susceptibility results of the *Acinetobacter* genospecies 3 isolates in this study also suggest that antimicrobial

resistance in this species have increased over time, as shown in Table 2-28 (Ribera *et al.*, 2004; Seifert *et al.*, 1993a; Traub & Spohr, 1989). Most β-lactams showed increases in MIC values over time. Most notably, the MIC_{90} values of carbapenems and ciprofloxacin of the isolates in this study were much higher than those of isolates from earlier studies. While regional differences in antimicrobial susceptibility of *Acinetobacter* genospecies 3 isolates could have partly accounted for the observations, it was also possible that this species has become increasingly resistant over time.

The role of accurate speciation of *Acinetobacter* isolates has been discussed above. The importance of intra-species molecular typing methods such as PFGE in the further characterisation of isolates in outbreak settings has also been well documented. PFGE in this study has also revealed interesting results with regards to the epidemiology of some isolates in the study. PFGE-indistinguishable strains of *A. baumannii*, *Acinetobacter* genospecies 3 and *A. ursingii*, respectively, were recovered over a few months rather than days; and in the case of *A. ursingii*, the strains were isolated from different locations as well. Hence, the dissemination of certain strains might not be readily recognised or suspected clinically. Taking into consideration the potential of *Acinetobacter* spp. to survive and persist in the hospital environment over long periods of time, the demonstration of clonal expansion of certain strains by PFGE suggests the possibility of clinically unrecognised reservoirs. The data in the study highlight the difficulty in recognising clonal expansion of strains clinically if the cross-transmission is occurring at a slower rate and on a smaller scale, and underscore the usefulness of PFGE in the recognition of such epidemiological trends.

Table 2-28. Comparison of antimicrobial susceptibility of *Acinetobacter* genospecies 3 isolates from 1989 to present.

Antibiotic	Traub & Spohr, 1989. (n = 48)		Seifert *et al.*, 1993. (n = 17)		Ribera *et al.*, 2004. (n = 15)		This study. (n= 45)	
	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)
Ticarcillin-clavulanate	16	32	ND	ND	ND	ND	8	≥ 256
Cefotaxime	16	32	8	16	ND	ND	32	64
Ceftazidime	4	16	4	8	8	8	8	16
Cefepime	ND	ND	ND	ND	4	16	8	32
Imipenem	1	2	≤ 0.5	≤ 0.5	< 0.5	0.5	0.5	≥ 32
Meropenem	ND	ND	ND	ND	< 0.5	1	0.5	≥ 32
Cipro-floxacin	0.25	0.5	≤ 0.25	1	< 0.5	1	0.25	≥ 32
Gentamicin	1	16	≤ 1	≥ 32	< 1	4	1	1
Amikacin	2	8	≤ 4	≤ 4	1	4	≤ 0.5	2
Colistin	ND	ND	ND	ND	1	2	0.25	0.5

ND, no data.

Another interesting observation is that there was some evidence of clonal expansion among carbapenem-resistant strains (of *Acinetobacter* genospecies 3) whereas most carbapenem-susceptible strains did not have evidence of clonal dissemination. It is possible that carbapenem resistance may have played a role in the dissemination of particular strains by enhancing the survival ability of the strains in the hospital environment. This would be in keeping with reports by other researchers of nosocomial outbreaks by carbapenem-resistant *Acinetobacter* spp. (Coelho *et al.*, 2006; Lolans *et al.*, 2006; Poirel *et al.*, 2006; Schulte *et al.*, 2005; Scott *et al.*, 2007; Valenzuela *et al.*, 2007).

MDR *Acinetobacter* spp. represent the paradigm of Gram-negative resistance. They can be resistant to almost all antimicrobial agents with the exception of polymyxins, as exemplified by the carbapenem-resistant *A. baumannii* isolate 229437 in this study. Therapeutically, they present a formidable challenge to clinicians. There are also important infection control implications. In the control of carbapenem resistance in *Acinetobacter* spp., in addition to the prudent use of carbapenems, stewardship of the use of other β-lactams (such as β-lactam/β-lactamase-inhibitor) is also necessary. As PFGE revealed the expansion of carbapenem-resistant strains, such phenotypes may require further aggressive infection control measures (such as stringent isolation and disinfection measures) to be taken by infection control specialists in order to contain the dissemination of these versatile pathogens. In the laboratory, accurate speciation and molecular typing of *Acinetobacter* strains as well as comprehensive susceptibility testing are also crucial in elucidating epidemiological trends in these organisms.

Chapter 3

Molecular characterisation of β-lactamase resistance genes in *Acinetobacter* spp.

3.1 Introduction

The β-lactam class of antimicrobial agents is one of the most effective and commonly used group of antibiotics for infections caused by a variety of Gram-negative organisms (see section 1.3.3). In response, GNBs have acquired a wide array of β-lactamases of different Ambler classes, many of which have broad spectra of activity (see section 1.3.3.1).

One of the main reasons for *Acinetobacter*'s rapid ascension as a major public health threat is the propensity for the rapid development of antimicrobial resistance (Giamarellou *et al.*, 2008; Perez *et al.*, 2007). However, it is mainly within the last decade that the most significant developments in the elucidation of *Acinetobacter* resistance mechanisms such as β-lactamases have been made. Ambler class A ESBLs that are prevalent in *Enterobacteriaceae*, such as SHV and CTX-M enzymes (see section 1.3.3.1.1), are relatively uncommon in *Acinetobacter* spp. (Bonnet, 2004; Bradford, 2001, Naas *et al.*, 2007; Peleg *et al.*, 2008). Instead, class A ESBLs such as VEB and PER enzymes are increasingly reported in *A. baumannii* isolates in parts of Europe such as France and Belgium (Naas *et al.*, 2006a; Naas *et al.*, 2006b). Similarly, while AmpC enzymes are well-characterised in *Enterobacteriaceae* and *P. aeruginosa* (see section 1.3.3.1.3), *Acinetobacter* class C β-lactamases (designated *Acinetobacter*-derived cephalosporinases, ADC) have only been characterised recently in *A. baumannii*, *Acinetobacter* genospecies 3 and *A. baylyi* (Beceiro *et al.*, 2007; Héritier *et al.*, 2006; Hujer *et al.*, 2005; Beceiro *et al.*, 2009). Their functional

as well as genetic diversity are now increasingly recognised (Hujer *et al.*, 2005; Beceiro *et al.*, 2009). However, ADC enzymes in many *Acinetobacter* spp. have yet to be characterised.

The most significant development in antimicrobial resistance of *Acinetobacter* spp. over the last decade has been the increasing prevalence of carbapenem resistance (Dijkshoorn *et al.*, 2007; Peleg *et al.*, 2008). Ambler class B enzymes (MBLs) such as VIM and IMP enzymes (see sections 1.3.3.1.2 and 1.4.2) are one of two main groups of carbapenemases found in carbapenem-resistant isolates (Lee *et al.*, 2004; Poirel & Nordmann, 2006b; Queenan & Bush, 2007; Yum *et al.*, 2002). The other group, Ambler class D OXA carbapenemases (see sections 1.3.3.1.4 and 1.4.2), were only discovered in the last decade, but since then their prevalence has risen dramatically worldwide (Peleg *et al.*, 2008; Poirel & Nordmann, 2006b; Queenan & Bush, 2007; Walther-Rasmussen & Høiby, 2006). Many of these OXA carbapenemase genes are carried on plasmids, which facilitate the dissemination of these resistance genes between different *Acinetobacter* strains (Peleg *et al.*, 2008; Valenzuela *et al.*, 2007; Walther-Rasmussen & Høiby, 2006). Globally, numerous outbreaks caused by MDR *Acinetobacter* isolates carrying these class D carbapenemases have been reported in various clinical settings (Bou *et al.*, 2000; Coelho *et al.*, 2006; Lee *et al.*, 2009; Lolans *et al.*, 2006; Lu *et al.*, 2009; Peleg *et al.*, 2006a; Poirel *et al.*, 2006; Scott *et al.*, 2007; Villegas *et al.*, 2007).

In addition to β-lactamases, other transferable elements in antimicrobial resistance such as integrons (see section 1.3.2) and aminoglycoside-modifying enzymes (see

sections 1.3.2 and 1.3.4) are increasingly reported in *Acinetobacter* spp. (Koeleman *et al.*, 2001; Nemec *et al.*, 2004; Seward *et al.*, 1998; Turton *et al.*, 2005; Vila *et al.*, 1999). However, transferable quinolone resistance genes (*qnr* genes), found predominantly in *Enterobacteriaceae* (see section 1.3.5), have not been reported in *Acinetobacter* spp. to date (Poirel *et al.*, 2008a; Robicsek *et al.*, 2006).

To date, there has been a paucity of published data regarding the prevalence and epidemiology of resistance genes such as β-lactamase genes, integrons and genes encoding aminoglycoside-modifying enzymes in MDR *Acinetobacter* spp. in Ireland. This chapter aimed to characterise the β-lactamase genes and other transferable antimicrobial resistance elements in the *Acinetobacter* isolates in St. James's Hospital using phenotypic and molecular methods, with particular emphasis on the investigation of carbapenem resistance genes, the regulation of their expression as well as the modes of genetic dissemination. The study also aimed to compare the resistance epidemiology of the *Acinetobacter* isolates with data from other centres, as well as to examine the clinical implications of MDR *Acinetobacter* isolates.

3.2 Materials and methods

3.2.1 Phenotypic β-lactamase screening methods

β-lactamase phenotypic screening methods were discussed in section 1.3.10. Etest MIC methods based on the utilisation of clavulanic acid and EDTA, as respective inhibitors of Ambler class A and B β-lactamases, were performed on certain *Acinetobacter* isolates depending on their antimicrobial susceptibility results (see below).

3.2.1.1 Screening for Ambler class A β-lactamases

Screening test for Ambler class A β-lactamases was performed on *Acinetobacter* isolates with reduced or non-susceptibility (ie. MICs of ≥ 8 μg/mL) to one or more of the cephalosporins cefotaxime, ceftazidime and cefepime as per CLSI breakpoints (CLSI, 2009). The method employed was the ESBL Etest MIC method (AB Biodisk, Solna, Sweden) using incremental concentrations of the cephalosporin on one side of the strip and incremental concentrations of the cephalosporin plus clavulanic acid on the other side of the strip (see section 1.3.10). A positive screening result was taken to be a cephalosporin to cephalosporin+clavulanic acid MIC ratio of ≥ 8 according to the manufacturers' instructions. Cefotaxime and cefepime ESBL Etest methods were performed on an isolate with reduced or non-susceptibility to one or more of the three third- and fourth-generation cephalosporins: cefotaxime, ceftazidime and cefepime.

3.2.1.2 Screening for Ambler class B β-lactamases

Screening for Ambler class B β-lactamases (metallo-β-lactamases, MBL) was performed on *Acinetobacter* isolates with reduced or non-susceptibility to one or more of the carbapenems meropenem, imipenem and doripenem (ie. meropenem and/or imipenem MICs of ≥ 4 μg/mL as per CLSI breakpoints and/or doripenem MIC of ≥ 1 μg/mL as per BSAC breakpoints) using a method similar to the ESBL Etest method. In this test, EDTA was utilised as an inhibitor of class B β-lactamases. The MBL Etest MIC method (AB Biodisk, Solna, Sweden) utilises incremental concentrations of imipenem on one side of the strip and incremental concentrations of imipenem with EDTA on the other side. An imipenem to imipenem+EDTA MIC ratio of ≥ 8 was taken as a presumptive positive screening result for MBL.

3.2.2 PCR amplification of β-lactamase genes of Ambler classes A and B

β-lactamase (*bla*) genes of Ambler classes A and B were sought using PCR with appropriate primers. Amplification by PCR was carried out on all 117 *Acinetobacter* isolates irrespective of the antimicrobial susceptibility patterns. DNA extraction and the PCR method were similar to the methodologies described for *rpoB* sequencing in section 2.2.1.2.1. The primers for the respective genes are shown in Table 3-1. The following Ambler class A β-lactamase genes were sought for: bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{VEB}, bla_{PER}, bla_{GES}, bla_{KPC}, bla_{PSE} and bla_{SME}. Amplification was also performed

Table 3-1. Primers used in chapter 3 of this study.

Gene or region	Primer sequence	Reference
bla$_{TEM}$	Forward: 5'-ATTCTTGAAGACGAAAGGGC-3' Reverse: 5'-ACGCTCAGTGGAACGAAAAC-3'	Hopkins *et al.*, 2006.
bla$_{SHV}$	Forward: 5'-ATGCGTTATATTCGCCTG-3' Reverse: 5'-TTAGCGTTGCCAGTGCTC-3'	Hopkins *et al.*, 2006.
bla$_{CTX-M}$	Forward: 5'-CGATGTGCAGTACCAGTAA-3' Reverse: 5'-TTAGTGACCAGAATCAGCGG-3'	Hopkins *et al.*, 2006.
bla$_{VEB}$	Forward: 5'-CGACTTCCATTTCCCGATGC-3' Reverse: 5'-GGACTCTGCAACAAATACGC-3'	Naas *et al.*, 2006a.
bla$_{PER}$	Forward: 5'-ATGAATGTCATTATAAAAGC-3' Reverse: 5'-AATTTGGGCTTAGGGCAGAA-3'	Naas *et al.*, 2006a.
bla$_{PSE}$	Forward: 5'-AATGGCAATCAGCGCTTC-3' Reverse: 5'-GCGCGACTGTGATGTATA-3'	Lee S *et al.*, 2005.
bla$_{GES}$	Forward: 5'-TGCGTCGGAAAAATTAACCT-3' Reverse: 5'-CGTGCTCAGGATGAGTTGTG-3'	This study
bla$_{KPC}$	Forward: 5'-ATCGCCGTCTAGTTCTGCTG-3' Reverse: 5'-AGACGGCCAACACAATAGGT-3'	This study
bla$_{SME}$	Forward: 5'-AACGGCTTCATTTTTGTTTAG-3' Reverse: 5'-GCTTCCGCAATAGTTTTATCA-3'	This study
bla$_{IMP}$	Forward: 5'-GAAGGYGTTTATGTTCATAC-3' Reverse: 5'-GTAMGTTTCAAGAGTGATGC-3'	Pitout *et al.*, 2005.
bla$_{VIM}$	Forward: 5'-GTTTGGTCGCATATCGCAAC-3' Reverse: 5'-AATGCGCAGCACCAGGATAG-3'	Pitout *et al.*, 2005.
bla$_{SPM}$	Forward: 5'-CTGCTTGGATTCATGGGCGC-3' Reverse: 5'-CCTTTTCCGCGACCTTGATC-3'	Pitout *et al.*, 2005.
bla$_{GIM}$	Forward: 5'-AGAACCTTGACCGAACGCAG-3' Reverse: 5'-ACTCATGACTCCTCACGAGG-3'	Castanheira *et al.*, 2004.
bla$_{SIM}$	Forward: 5'-TACAAGGGATTCGGCATCG-3' Reverse: 5'-TAATGGCCTGTTCCCATGTG-3'	Lee K *et al.*, 2005.
bla$_{ADC}$ (set 1)	Forward: 5'-TGCGGRCAATACAYCAAAAG-3' Reverse: 5'-GCYTTAAWGCGYTCTTCATT-3'	This study
bla$_{ADC}$ (set 2)	Forward: 5'-TTGYYTACTTTTAYCYCCKCTTT-3' Reverse: 5'-ATTTCTTTATTGCMTTYARYACDR-3'	This study

bla$_{OXA}$ group III	Forward: 5'-AGCCGTTAAAATTAAGCCC-3' Reverse: 5'-CTTGATTGAAGGGTTGGGCG-3'	Lee S *et al.*, 2005.
bla$_{OXA}$ group II	Forward: 5'-TCTTCGCGATACTTTTCTCCA-3' Reverse: 5'-AGCGTCCGAGTTGACTGC-3'	Lee S *et al.*, 2005.
bla$_{OXA}$ group I	Forward: 5'-TCTTTCGAGTACGGCATTAGC-3' Reverse: 5'-CCAATGATGCCCTCACTTTCC-3'	Lee S *et al.*, 2005.
bla$_{OXA}$ group IV	Forward: 5'-TTGGCATCGGAAAGCGAACC-3' Reverse: 5'-ATTTCAACGGTTTGCGACG-3'	Philippon *et al.*, 1997
bla$_{OXA-23-like}$	Forward: 5'-AGCCGCGCAAATACAGAATA-3' Reverse: 5'-GCCTGCACTTTTTCACTAAATG-3'	This study
bla$_{OXA-24-like}$	Forward: 5'-GTACTAATCAAAGTTGTGAA-3' Reverse: 5'-TTCCCCTAACATGAATTTGT-3'	Afzal-Shah *et al.*, 2001.
bla$_{OXA-48}$	Forward: 5'-GTGGCATCGATTATCGGAAT-3' Reverse: 5'-CTTCTTTTGTGATGGCTTGG-3'	This study
bla$_{OXA-51-like}$	Forward: 5'-TTTTATTTCAGCCTGCTCACCTT-3' Reverse: 5'-CGAACAGAGCTAGGTATTCCTTTTT-3'	This study
bla$_{OXA-58}$	Forward: 5'-CGATCAGAATGTTCAAGCGC-3' Reverse: 5'-ACGATTCTCCCCTCTGCGC-3'	Poirel & Nordmann, 2006a.
IS*Aba1*	Forward: 5'-GTGCTTTGCGCTCATCATGC-3' Reverse: 5'-CATGTAAACCAATGCTCACC-3'	Poirel & Nordmann, 2006a.
IS*Aba2*	Forward: 5'-AATCCGAGATAGAGCGGTTC-3' Reverse: 5'-TGACACATAACCTAGTGCAC-3'	Poirel & Nordmann, 2006a.
IS*Aba3*	Forward: 5'-CAATCAAATGTCCAACCTGC-3' Reverse: 5'-CGTTTACCCCAAACATAAGC-3'	Poirel & Nordmann, 2006a.
IS*Aba3*-like	Forward: 5'-AGCAATATCTCGTATACCGC-3'	Poirel & Nordmann, 2006a.
pre-IS*Aba3*-like	Forward: 5'-TTTAACATAATGGCTGTTATACG-3'	This study
IS*Aba4*	Forward: 5'-ATTTGAACCCATCTATTGGC-3' Reverse: 5'-ACTCTCATATTTTTTCTTGG-3'	Corvec *et al.*, 2007
IS*18*	Forward: 5'-CACCCAACTTTCTCAAGATG-3' Reverse: 5'-ACCAGCCATAACTTCACTCG-3'	Poirel & Nordmann, 2006a.
gcp	Forward: 5'-TATGCTGGCGCCTTTACTTT-3'	This study

ATPase	Reverse: 5'-TCTGGCCTTTAATGCTGAAGA-3'	This study
est	Forward: 5'-ATCCAACCATTCATCAAACTCTGGC-3'	Poirel & Nordmann, 2006a.
araC1	Forward: 5'-ATTCATGCCCACTTCAAAGG-3' Reverse: 5'-ACATTTGTGCACGCTTTTTG-3'	This study
araC2	Reverse: 5'-CTATTTGGTTTTAAGGGGC-3'	Poirel & Nordmann, 2006a.
lysE	Reverse: 5'-AACAGCAATAGCCATCAAC-3'	Poirel & Nordmann, 2006a.
post-*lysE*	Reverse: 5'-TTCGTATAACCGCCATTATG-3'	Poirel & Nordmann, 2006a.
*intI*1	Forward: 5'-CAGTGGACATAAGCCTGTTC-3' Reverse: 5'-CCCGAGGCATAGACTGTA-3'	Koeleman *et al.*, 2001.
*intI*2	Forward: 5'-TTGCGAGTATCCATAACCTG-3' Reverse: 5'-TTACCTGCACTGGATTAAGC-3'	Koeleman *et al.*, 2001.
qnrA	Forward: 5'-ATTTCTCACGCCAGGATTTG-3' Reverse: 5'-GATCGGCAAAGGTTAGGTCA-3'	Robicsek *et al.*, 2006.
qnrB	Forward: 5'-GATCGTGAAAGCCAGAAAGG-3' Reverse: 5'-ACGATGCCTGGTAGTTGTCC-3'	Robicsek *et al.*, 2006.
qnrS	Forward: 5'-ACGACATTCGTCAACTGCAA-3' Reverse: 5'-TAAATTGGCACCCTGTAGGC-3'	Robicsek *et al.*, 2006.
aac(3)-Ia (*aacC1*)	Forward: 5'-CATCATTCGCACATGTAGGC-3' Reverse: 5'-CCCGTATGCCCAACTTTGTA-3'	This study
aac(3)-IIa (*aacC2*)	Forward: 5'-CGAAAACTCGGAGTCCAAAC-3' Reverse: 5'-CGTCGAACAGGTAGCACTGA-3'	This study
aph(3')-VIa (*aphA6*)	Forward: 5'-ATACAGAGACCACCATACAGT-3' Reverse: 5'-GGACAATCAATAATAGCAAT-3'	Vila *et al.*, 1999.
aac(6')-Ib (*aacA4*)	Forward: 5'-TGACCTTGCGATGCTCTATG-3' Reverse: 5'-CGGTACCTTGCCTCTCAAAC-3'	This study

The following letters denoted in some of the primers represent the corresponding combination of nucleotides:
D = G+A+T; M = A+C; R = A+G; W = A+T; Y = C+T.

on all isolates for the detection of MBL genes bla_{VIM}, bla_{IMP}, bla_{SPM}, bla_{GIM} and bla_{SIM}. Amplicons were purified and sent for nucleotide sequencing using the corresponding primers as used in the PCR method above (see section 3.2.6).

3.2.3 PCR amplification of Ambler class C *Acinetobacter*-derived cephalosporinase (ADC) genes

Amplification of Ambler class C bla_{ADC} was performed on all 117 *Acinetobacter* isolates using the methodology as described in section 2.2.1.2.1. The primers were designed based on conserved regions of bla_{ADC} genes of *A. baumannii* and *Acinetobacter* genospecies 3 available in GenBank®. Two sets of primers were used for the detection of bla_{ADC} genes in the isolates and are shown in Table 3-1. The amplicons of isolates with positive PCR results were subsequently sent for sequencing and the amino acid sequence deduced from the obtained nucleotide sequence. The presence of IS elements (such as IS*Aba1*) associated with bla_{ADC} was also investigated as the overexpression of ADC enzymes in *A. baumannii* has been associated with the presence of IS*Aba1* upstream of the β-lactamase gene (Heritier *et al.*, 2006; Segal *et al.*, 2004).

3.2.4 PCR amplification of Ambler class D (OXA) β-lactamase genes

Ambler class D OXA genes were sought using PCR methodology as described in section 2.2.1.2.1 with appropriate primers as listed in Table 3-1. They include *bla*$_{OXA}$ genes with narrow or extended spectrum of β-lactamase activity but with no significant carbapenemase activity: OXA group I (*bla*$_{OXA-10}$ and derivatives), OXA group II (*bla*$_{OXA-2}$ and derivatives), OXA group III (*bla*$_{OXA-1}$ and derivatives) and *bla*$_{OXA-18}$. Genes encoding OXA carbapenemases were also sought: OXA-23 group, OXA-24 group, OXA-51 group, OXA-58 and OXA-48. Amplicons were purified and sent for nucleotide sequencing was performed using the corresponding primers as used in the PCR method (see section 3.2.6).

3.2.4.1 Molecular characterisation of genetic environment of *bla*$_{OXA}$ genes

Genetic environment of *bla*$_{OXA}$ genes detected in *Acinetobacter* spp. were characterised by PCR and nucleotide sequencing. Insertion sequences (IS) and other genes associated with *bla*$_{OXA}$ genes in *Acinetobacter* spp. were sought (Corvec *et al.*, 2007; Poirel & Nordmann, 2006a; Poirel *et al.*, 2008b; Segal *et al.*, 2003; Turton *et al.*, 2006). The IS elements sought for were IS*Aba1*, IS*Aba2*, IS*Aba3*, IS*Aba3*-like, IS*Aba4* and IS*18* elements respectively and the primers used are shown in Table 3-1. The relationship of the IS to the *bla*$_{OXA}$ gene was investigated using forward primer of the IS and the reverse primer of the *bla*$_{OXA}$ gene, or conversely, the reverse primer of the IS and the forward primer of the the *bla*$_{OXA}$ gene. The amplicons were then

sequenced to confirm the genetic environment of the bla_{OXA} genes. In addition, other genes associated with bla_{OXA} genes were also investigated. PCR detection and sequencing of genes associated with $bla_{OXA\text{-}23\text{-}like}$, namely *ATPase* and O-sialoglycoprotein endopeptidase *gcp* genes, was performed (Corvec *et al.*, 2007; GenBank accession no. EU571228). Genes associated with $bla_{OXA\text{-}58}$ were also sought (Poirel & Nordmann, 2006a). They were the putative esterase *est* gene, transcriptional regulator genes *araC1* and *araC2*, as well as the threonine efflux protein *lysE* gene.

3.2.4.2 Plasmid studies

To investigate if the bla_{OXA} genes were plasmid-mediated, plasmid extraction was performed using Qiagen Midi Kit (Qiagen GmbH, Hilden, Germany). 5 µL of the plasmid preparation were used as DNA template for PCR with the appropriate bla_{OXA} primers. PCR for *rpoB* gene was also included to rule out chromosomal DNA carry-over. In the absence of chromosomal contamination, a positive PCR result with the plasmid preparation would suggest that the corresponding bla_{OXA} gene was of plasmidic origin. To locate the specific plasmids carrying those genes, DNA was isolated from individual plasmids using a modification of the method described by Blin *et al.* (Blin *et al.*, 1975). Agarose gel electrophoresis of plasmid preparations was first used to visualise individual plasmids and DNA from individual plasmid bands was subsequently eluted using Qiagen Gel Extraction Kit (Qiagen GmbH, Hilden, Germany). 2 µL of the eluate was used as DNA template for PCR with bla_{OXA} primers. PCR for *rpoB* gene was again performed to rule out chromosomal DNA

carry-over. The location of the gene from a specific plasmid was suggested by a positive PCR result with DNA eluted from the particular plasmidic band.

3.2.4.3 Isoelectric focusing (IEF)

Expression of β-lactamase genes was investigated in the 12 pan-carbapenem-resistant *Acinetobacter* isolates using isoelectric focusing (IEF) with nitrocephin (see section 1.3.10). IEF was performed using a modification of the method described by Matthew *et al.* (Matthew *et al.*, 1975). The *Acinetobacter* isolates were inoculated into 10 ml of Luria-Burtani (LB) broth and incubated for 16 hours at 37°C in a shaking incubator at 100 rpm. Bacterial cells were harvested by centrifugation at 2,500 *g* for 10 minutes at 4°C in a Beckman J-6B centrifuge (Beckman Coulter, Fullerton, CA, US), washed and resuspended in 1 ml of chilled 0.1M phosphate buffer, pH 7.0. Cell suspensions were immersed in an ice water bath and disrupted by sonication using a Branson Ultrasonicator (Branson Ultrasonics, Hayes, Middlesex, UK) for three cycles of 30 seconds on and 30 seconds off. Cellular debris was removed by centrifugation at 17,400 *g* for 30 minutes at 4°C using an Eppendorf 5417 microcentrifuge (Eppendorf GmbH, Hamburg, Germany). Aliquots of supernatant containing crude β-lactamases were stored at -70°C until IEF was performed.

β-lactamases were analysed by IEF on precast polyacrylamide gels (Ampholine PAG plate; Amersham Biosciences AB, Uppsala, Sweden) containing Pharmalyte in the pH range of 3.5 – 9.5. Samples of β-lactamase extracts were thawed and diluted in 0.1M phosphate buffer to a concentration such that a red colour developed within one to

two minutes following dilution (1 in 2) in nitrocefin solution of 50 μg/mL concentration (Oxoid, Basingstoke, Hampshire, UK). Diluted samples of 5 μL each were applied directly to the polyacrylamide gels using an applicator strip. The gels were focused at 4°C for 90 minutes at 1,500 V and 15 W in an LKB Multiphor II electrophoresis tank (LKB Instruments Inc., Rockville, US) using 1M H_3PO_4 and 1M NaOH as anode and cathode solutions respectively. *E. coli* isolate expressing TEM-1 and *A. baumannii* expressing OXA-23 were included as β-lactamase standards. Low (pH 2.5 – 6.5) and high (pH 5.0 – 10.5) isoelectric point (pI) calibration kit markers (IEF Calibration Kit, Amersham Biosciences) were included as protein standards.

3.2.5 Molecular characterisation of other transferable resistance elements/genes: integrons, genes encoding aminoglycoside-modifying enzymes, and transferable quinolone resistance (*qnr*) genes

Amplification of the *intI*1 and *intI*2 genes (class 1 and 2 integrase genes) for the detection of class 1 and 2 integrons was performed for all *Acinetobacter* isolates according to the primers and method described by Koeleman *et al.* (Koeleman *et al.*, 2001). PCR detection was also performed for genes encoding the aminoglycoside-modifying enzymes commonly found in *Acinetobacter* spp. (Nemec *et al.*, 2004; Seward *et al.*, 1998). Amplification by PCR was performed on the nine isolates with non-susceptibility to either or both of the two tested aminoglycosides, gentamicin and

amikacin, as well as the *A. haemolyticus* isolate 89182 with borderline amikacin susceptibility of MIC 16 μg/mL (see tables 2-15 and 2-16 and appendix 1). IS elements were also sought in association with genes encoding aminoglycoside-modifying enzymes. PCR detection of transferable quinolone resistance genes *qnrA*, *qnrB* and *qnrS* was also performed for all 117 *Acinetobacter* isolates using primers and PCR conditions as reported by Robicsek *et al.* (Robicsek *et al.*, 2006). All primers used in this section of the study are included in Table 3-1.

3.2.6 Nucleotide sequencing, GenBank sequence submission and accession numbers

Amplicons obtained by PCR in this chapter of the study were sent to the Genomics Core Facility, Queen's University, Belfast, for nucleotide sequencing. Novel nucleotide sequences found in this study were submitted to the genetic sequence database GenBank® of the National Center of Biotechnology Information, National Institutes of Health, US (www.ncbi.nlm.nih.gov/Genbank). Subsequent accession numbers assigned to the respective GenBank® sequence submissions were recorded and shown in section 3.3.6.

3.3 Results

3.3.1 Phenotypic β-lactamase screening methods

Of the 117 isolates, 97 isolates had MICs of ≥ 8 μg/mL for one or more of the three third- and fourth-generation cephalosporins of cefotaxime, ceftazidime and cefepime. They consisted of the following (number of isolates): *A. baumannii* (26), *Acinetobacter* genospecies 3 (45), *A. calcoaceticus* (6), *A. johnsonii* (8), *Acinetobacter* genospecies 9 (3), *A. lwoffii* (1), *Acinetobacter* genospecies 11 (4), *Acinetobacter* genospecies 13BJ (2), *A. tjernbergiae* (1) and *A. radioresistens* (1). Of these isolates, 18 gave presumptive positive class A ESBL screening results with both of the cefotaxime and cefepime Etest ESBL tests, with ESBL ratios of ≥ 64 for both cephalosporins. They consisted of the following (number of isolates of total): *A. baumannii* (1/26), *Acinetobacter* genospecies 3 (1/45), *A. calcoaceticus* (1/6), *A. johnsonii* (7/8), *Acinetobacter* genospecies 9 (3/3), *A. lwoffii* (1/1), *Acinetobacter* genospecies 11 (4/4). Ten isolates had positive ESBL screening results for cefepime with the ESBL ratios of 8 to 16, but interestingly, these isolates gave negative class A ESBL results when cefotaxime was used. They consisted of nine *Acinetobacter* genospecies 3 isolates and one *A. baumannii* isolate. The remaining 79 isolates gave negative class A ESBL screening results for both cephalosporins.

Seventeen isolates showed non-susceptibility or reduced susceptibility to meropenem, imipenem and/or doripenem (see Appendix 1). All 12 pan-carbapenem-resistant *Acinetobacter* isolates (ten *Acinetobacter* genospecies 3, one *A. baumannii* and one *A.*

johnsonii isolates) gave presumptive positive MBL screening results with imipenem:imipenem-EDTA Etest MIC ratios of 8 to 24, suggestive of MBL production. The remaining 5 *Acinetobacter* isolates (consisting of the doripenem non-susceptible *A. baumannii* isolate 11648 and four other isolates with reduced susceptibility to doripenem) gave negative MBL screening results.

3.3.2 β-lactamase genes of Ambler classes A and B

More than 99% of the isolates (116/117) were positive for $bla_{\text{TEM-1}}$. No other types of bla_{TEM} were found. No other Ambler class A β-lactamase genes (bla_{SHV}, $bla_{\text{CTX-M}}$, bla_{PER}, bla_{VEB}, bla_{GES}, bla_{KPC} and bla_{SME}) were found in the *Acinetobacter* isolates. All *Acinetobacter* isolates, including the 12 pan-carbapenem-resistant isolates which were MBL-positive on phenotypic testing, were negative for the five Ambler class B MBL genes of bla_{VIM}, bla_{IMP}, bla_{SPM}, bla_{GIM} and bla_{SIM}. The PCR results suggested that the MBL phenotypic screening test produced false-positive results.

3.3.3 Ambler class C *Acinetobacter*-derived cephalosporinase (ADC) genes

Using both sets of bla_{ADC} primers, amplicons were obtained for the 78 *A. baumannii*, *Acinetobacter* genospecies 3 and *A. calcoaceticus* isolates. Nucleotide sequencing was

also performed for the above 78 amplicons. No PCR product was obtained with the other species using either set of bla_{ADC} primers.

The bla_{ADC} genes of the 78 isolates of the *Acinetobacter calcoaceticus-baumannii* complex are shown in Table 3-2. Novel bla_{ADC} sequences were obtained in three *A. baumannii* isolates (102450, 181329 and 14773) and all six *A. calcoaceticus* isolates. These bla_{ADC} sequences of the *A. calcoaceticus* isolates had < 90% sequence homology with existing bla_{ADC} genes within the GenBank database while the bla_{ADC} genes of the three *A. baumannii* isolates had < 95% sequence homology with other *A. baumannii* bla_{ADC} genes within the GenBank database. The remaining 69 isolates had bla_{ADC} genes with >98.5% sequence concordance with reference bla_{ADC} genes as shown in Table 3-2.

In *A. baumannii* isolates, the bla_{ADC} genes were as follow (number of isolates): $bla_{ADC\text{-}39}$ (7), $bla_{ADC\text{-}29}$ (5), $bla_{ADC\text{-}32}$ (5), $bla_{ADC\text{-}6}$ (2), $bla_{ADC\text{-}33}$ (2), $bla_{ADC\text{-}3}$ (1), $bla_{ADC\text{-}7}$ (1) and $bla_{ADC\text{-}25}$ (1). The bla_{ADC} genes in *Acinetobacter* genospecies 3 isolates were as follow (number of isolates): $bla_{ADC\text{-}18}$ (25), $bla_{ADC\text{-}19}$ (6), $bla_{ADC\text{-}22}$ (5), $bla_{ADC\text{-}16}$ (4), $bla_{ADC\text{-}41}$ (2), $bla_{ADC\text{-}42}$ (2) and $bla_{ADC\text{-}43}$ (1).

Two isolates, *A. baumannii* isolates 229437 and 11648, were found to have IS*Aba1* upstream of their $bla_{ADC\text{-}25}$ and $bla_{ADC\text{-}29}$ genes, respectively. The finding suggested that there was overexpression of the respective bla_{ADC} genes in both isolates. No upstream IS elements were detected for the bla_{ADC} genes of the remaining 76 isolates.

Table 3-2. Ambler class C *bla*$_{ADC}$ genes of isolates of *Acinetobacter calcoaceticus-baumannii* complex.

Isolate Identifier	Species	*bla*$_{ADC}$-	Reference GenBank accession
229437	*A. baumannii*	IS*Aba1*/*bla*$_{ADC-25}$	EF016355
11648	*A. baumannii*	IS*Aba1*/*bla*$_{ADC-29}$	EU604835
26756	*A. baumannii*	*bla*$_{ADC-7}$	AY648950
101049	*A. baumannii*	*bla*$_{ADC-33}$	EU687478
20802	*A. baumannii*	*bla*$_{ADC-29}$	EU604835
217068	*A. baumannii*	*bla*$_{ADC-29}$	EU604835
12937	*A. baumannii*	*bla*$_{ADC-3}$	AY178995
89848	*A. baumannii*	*bla*$_{ADC-32}$	EU687477
26750	*A. baumannii*	*bla*$_{ADC-6}$	AY325306
181329	*A. baumannii*	New *bla*$_{ADC}$	NA
27623	*A. baumannii*	*bla*$_{ADC-39}$	EU652244
102450	*A. baumannii*	New *bla*$_{ADC}$	NA
29419	*A. baumannii*	*bla*$_{ADC-32}$	EU687477
14495	*A. baumannii*	*bla*$_{ADC-32}$	EU687477
13207	*A. baumannii*	*bla*$_{ADC-33}$	EU687478
100983	*A. baumannii*	*bla*$_{ADC-32}$	EU687477
14773	*A. baumannii*	New *bla*$_{ADC}$	NA
33047	*A. baumannii*	*bla*$_{ADC-39}$	EU652244
107228	*A. baumannii*	*bla*$_{ADC-39}$	EU652244
31364	*A. baumannii*	*bla*$_{ADC-29}$	EU604835
107532	*A. baumannii*	*bla*$_{ADC-39}$	EU652244
107245	*A. baumannii*	*bla*$_{ADC-39}$	EU652244
27021	*A. baumannii*	*bla*$_{ADC-6}$	AY325306
233651	*A. baumannii*	*bla*$_{ADC-29}$	EU604835
232982	*A. baumannii*	*bla*$_{ADC-39}$	EU652244
218722	*A. baumannii*	*bla*$_{ADC-39}$	EU652244
220165	*A. baumannii*	*bla*$_{ADC-32}$	EU687477
29540	*Acinetobacter* genosp. 3	*bla*$_{ADC-18}$	AM283523
12659	*Acinetobacter* genosp. 3	*bla*$_{ADC-18}$	AM283523
23269	*Acinetobacter* genosp. 3	*bla*$_{ADC-18}$	AM283523
14448	*Acinetobacter* genosp. 3	*bla*$_{ADC-18}$	AM283523
14748	*Acinetobacter* genosp. 3	*bla*$_{ADC-18}$	AM283523
28594	*Acinetobacter* genosp. 3	*bla*$_{ADC-18}$	AM283523
27616	*Acinetobacter* genosp. 3	*bla*$_{ADC-18}$	AM283523
32357	*Acinetobacter* genosp. 3	*bla*$_{ADC-18}$	AM283523
36277	*Acinetobacter* genosp. 3	*bla*$_{ADC-18}$	AM283523
103165	*Acinetobacter* genosp. 3	*bla*$_{ADC-18}$	AM283523
206479	*Acinetobacter* genosp. 3	*bla*$_{ADC-19}$	AM283522

Table 3-2 (Continued)

106770	*Acinetobacter* genosp. 3	bla_{ADC-18}	AM283523
27458	*Acinetobacter* genosp. 3	bla_{ADC-18}	AM283523
88276	*Acinetobacter* genosp. 3	bla_{ADC-19}	AM283522
105621	*Acinetobacter* genosp. 3	bla_{ADC-19}	AM283522
86096	*Acinetobacter* genosp. 3	bla_{ADC-16}	AM283525
107012	*Acinetobacter* genosp. 3	bla_{ADC-18}	AM283523
14027	*Acinetobacter* genosp. 3	bla_{ADC-22}	AM283519
30077	*Acinetobacter* genosp. 3	bla_{ADC-18}	AM283523
22344	*Acinetobacter* genosp. 3	bla_{ADC-18}	AM283523
12193	*Acinetobacter* genosp. 3	bla_{ADC-18}	AM283523
93743	*Acinetobacter* genosp. 3	bla_{ADC-16}	AM283525
35272	*Acinetobacter* genosp. 3	bla_{ADC-18}	AM283523
86661	*Acinetobacter* genosp. 3	bla_{ADC-18}	AM283523
12253	*Acinetobacter* genosp. 3	bla_{ADC-22}	AM283519
28933	*Acinetobacter* genosp. 3	bla_{ADC-18}	AM283523
20949	*Acinetobacter* genosp. 3	bla_{ADC-41}	FJ744160
31479	*Acinetobacter* genosp. 3	bla_{ADC-19}	AM283522
106498	*Acinetobacter* genosp. 3	bla_{ADC-18}	AM283523
106008	*Acinetobacter* genosp. 3	bla_{ADC-19}	AM283522
183436	*Acinetobacter* genosp. 3	bla_{ADC-41}	FJ744160
29420	*Acinetobacter* genosp. 3	bla_{ADC-18}	AM283523
14331	*Acinetobacter* genosp. 3	bla_{ADC-22}	AM283519
27035	*Acinetobacter* genosp. 3	bla_{ADC-18}	AM283523
25904	*Acinetobacter* genosp. 3	bla_{ADC-22}	AM283519
16198	*Acinetobacter* genosp. 3	bla_{ADC-22}	AM283519
82991	*Acinetobacter* genosp. 3	bla_{ADC-16}	AM283525
181882	*Acinetobacter* genosp. 3	bla_{ADC-18}	AM283523
13320	*Acinetobacter* genosp. 3	bla_{ADC-43}	FJ744162
101327	*Acinetobacter* genosp. 3	bla_{ADC-42}	FJ744161
91135	*Acinetobacter* genosp. 3	bla_{ADC-42}	FJ744161
106449	*Acinetobacter* genosp. 3	bla_{ADC-19}	AM283522
35168	*Acinetobacter* genosp. 3	bla_{ADC-16}	AM283525
232787	*Acinetobacter* genosp. 3	bla_{ADC-18}	AM283523
225991	*Acinetobacter* genosp. 3	bla_{ADC-18}	AM283523
33241	*A. calcoaceticus*	New bla_{ADC}	NA
92328	*A. calcoaceticus*	New bla_{ADC}	NA
183262	*A. calcoaceticus*	New bla_{ADC}	NA
20304	*A. calcoaceticus*	New bla_{ADC}	NA
28263	*A. calcoaceticus*	New bla_{ADC}	NA
100558	*A. calcoaceticus*	New bla_{ADC}	NA

NA, not applicable

3.3.4 Ambler class D OXA β-lactamase genes

None of the 117 isolates was positive for bla_{OXA-48}, or bla_{OXA} genes of OXA group I, group II, group III, group IV or OXA-24 group, respectively.

All 27 *A. baumannii* isolates, including the carbapenem-non-susceptible isolates 229437 and 11648, were found to be positive for $bla_{OXA-51-like}$ genes. No IS elements were detected upstream of the $bla_{OXA-51-like}$ genes. The $bla_{OXA-51-like}$ genes were not detected in any other species in this study.

A summary of antimicrobial resistance genes and associated genetic elements of the 13 carbapenem non-susceptible isolates is shown in Table 3-3. The 12 pan-carbapenem-resistant isolates were positive for $bla_{OXA-23-like}$ genes. Nucleotide sequencing confirmed the amplicons of all 12 isolates to be bla_{OXA-23} (100% sequence concordance with GenBank accession number EF127491). The IS*Aba1* element and the *ATPase* gene were also present in all 12 isolates upstream and downstream of the bla_{OXA-23}, respectively (see Figure 3-1). Plasmid studies revealed that the 12 pan-carbapenem-resistant isolates carried varying numbers of plasmids with sizes ranging from 2.5 MDa to 60 MDa. The bla_{OXA-23} genes in all 12 isolates were found to be plasmid-mediated. The bla_{OXA-23} genes of the *A. johnsonii* isolate 25868 and the ten *Acinetobacter* genospecies 3 isolates were all located on 40 MDa plasmids, while the bla_{OXA-23} of the *A. baumannii* isolate 229437 was located on a 35 MDa plasmid. IEF of the β-lactamase extracts of the 12 isolates revealed three pIs of 5.4, 6.7 and > 8.5 for each of the isolates. Together with the PCR and sequencing results, the above pI

Table 3-3. Summary of resistance genes and associated genetic elements in carbapenem non-susceptible *Acinetobacter* isolates (n = 13).

Isolate (Species)	Non-susceptibility to carbapenems	MBL Etest ratio	Resistance genes and associated genetic elements				
			bla$_{TEM}$	*bla*$_{ADC}$	*bla*$_{OXA}$	AME genes	integron
229437 (*A. baumannii*)	M, I, D	8	*bla*$_{TEM-1}$	IS*Aba1*/*bla*$_{ADC-25}$	IS*Aba1*/*bla*$_{OXA-23}$; *bla*$_{OXA-51-like}$	*aac(3)-Ia* *aph(3')-VIa*	Class 1
25868 (*A. johnsonii*)	M, I, D	12	*bla*$_{TEM-1}$	Unknown	IS*Aba1*/*bla*$_{OXA-23}$	*aac(3)-IIa*	None
29540 (AG3)	M, I, D	24	*bla*$_{TEM-1}$	*bla*$_{ADC-18}$	IS*Aba1*/*bla*$_{OXA-23}$	IS*Aba1*/ *aac(3)-IIa*	None
32357 (AG3)	M, I, D	24	*bla*$_{TEM-1}$	*bla*$_{ADC-18}$	IS*Aba1*/*bla*$_{OXA-23}$	IS*Aba1*/ *aac(3)-IIa*	None
36277 (AG3)	M, I, D	16	*bla*$_{TEM-1}$	*bla*$_{ADC-18}$	IS*Aba1*/*bla*$_{OXA-23}$	IS*Aba1*/ *aac(3)-IIa*	None
12659 (AG3)	M, I, D	12	*bla*$_{TEM-1}$	*bla*$_{ADC-18}$	IS*Aba1*/*bla*$_{OXA-23}$	ND	None
23629 (AG3)	M, I, D	12	*bla*$_{TEM-1}$	*bla*$_{ADC-18}$	IS*Aba1*/*bla*$_{OXA-23}$	ND	None
14448 (AG3)	M, I, D	16	*bla*$_{TEM-1}$	*bla*$_{ADC-18}$	IS*Aba1*/*bla*$_{OXA-23}$	ND	None
14748 (AG3)	M, I, D	16	*bla*$_{TEM-1}$	*bla*$_{ADC-18}$	IS*Aba1*/*bla*$_{OXA-23}$	ND	None
27616 (AG3)	M, I, D	12	*bla*$_{TEM-1}$	*bla*$_{ADC-18}$	IS*Aba1*/*bla*$_{OXA-23}$	ND	None
28594 (AG3)	M, I, D	12	*bla*$_{TEM-1}$	*bla*$_{ADC-18}$	IS*Aba1*/*bla*$_{OXA-23}$	ND	None
103165 (AG3)	M, I, D	12	*bla*$_{TEM-1}$	*bla*$_{ADC-18}$	IS*Aba1*/*bla*$_{OXA-23}$	ND	None
11648 (*A. baumannii*)	D	2	*bla*$_{TEM-1}$	IS*Aba1*/*bla*$_{ADC-29}$	*bla*$_{OXA-51-like}$	*aac(3)-Ia*	Class 1

AG3, *Acinetobacter* genospecies 3; M, meropenem; I, imipenem; D, doripenem; MBL, metallo-β-lactamase; AME, aminoglycoside-modifying enzyme; ND, not detected.

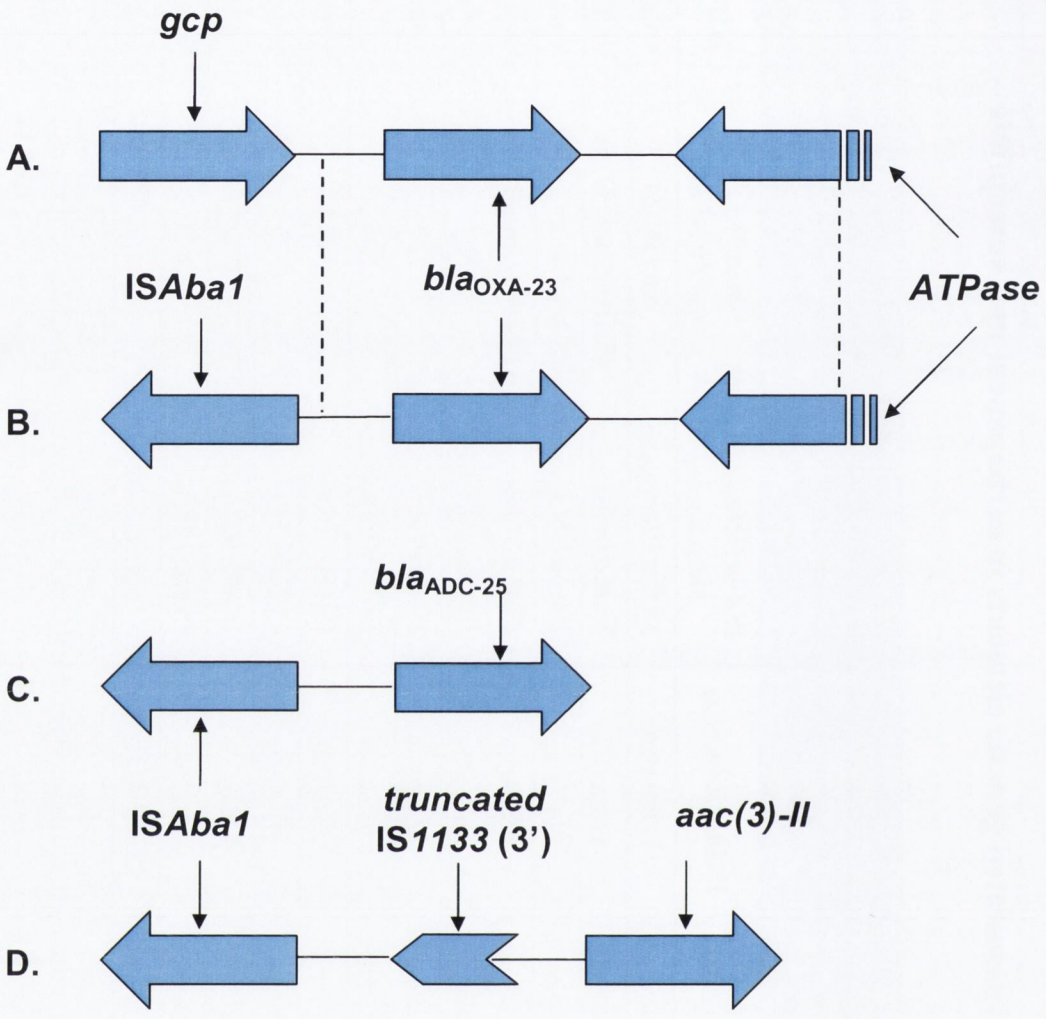


Figure 3-1. Genetic environment of resistance genes in *Acinetobacter radioresistens* and carbapenem-resistant *Acinetobacter* spp.

[A., *gcp*, bla_{OXA-23} and *ATPase* in carbapenem-susceptible *A. radioresistens* isolate 87374; B., IS*Aba1*, bla_{OXA-23} and *ATPase* of carbapenem-resistant *Acinetobacter* genospecies 3 isolate 36277 (the regions of A. and B. within the dashed lines were identical in sequence); C., IS*Aba1* and bla_{ADC-25} of *A. baumannii* isolate 229437; D., IS*Aba1*, IS*1133* and *aac(3)-II* of *Acinetobacter* genospecies 3 isolate 32357.]

values were consistent with the expression of TEM-1, OXA-23 and ADC enzymes, respectively.

The two carbapenem-susceptible *A. radioresistens* isolates (88427 and 87374) were also positive for $bla_{\text{OXA-23-like}}$ genes (see Figure 3-1). In both isolates, a putative O-sialoglycoprotein endopeptidase *gcp* gene was present upstream of $bla_{\text{OXA-23-like}}$ (both isolates with 98.4% sequence concordance with GenBank accession number EU571228), while downstream of $bla_{\text{OXA-23-like}}$ gene, *ATPase* gene was present in both (99% [88427] and 100% [87374] sequence concordance with GenBank accession number EU131372). Sequencing of the entire $bla_{\text{OXA-23-like}}$ genes revealed that 87374 carried $bla_{\text{OXA-23}}$ while 88427 carried a novel OXA β-lactamase gene $bla_{\text{OXA-134}}$. $bla_{\text{OXA-134}}$ has 99% sequence homology with $bla_{\text{OXA-23}}$, with eight nucleotide substitutions compared to the latter. Deduced amino acid sequence of $bla_{\text{OXA-134}}$ showed that OXA-134 differed from OXA-23 by 2 amino acid substitutions (Δ10: valine→phenylalanine and Δ33: valine→alanine). PCR for $bla_{\text{OXA-23-like}}$ was negative for 87374 and 88427, suggesting the genes were located chromosomally.

The $bla_{\text{OXA-58}}$ gene was detected by PCR and confirmed by nucleotide sequencing in two carbapenem-susceptible isolates, *Acinetobacter* genospecies 9 isolate 180361 and *Acinetobacter* genospecies 10 isolate 101055 (> 99% sequence concordance with GenBank accession number AY665723). In both isolates, an IS*Aba3*-like transposase was present upstream of $bla_{\text{OXA-58}}$ (see Figure 3-2). Sequencing of this transposase gene showed that the putative amino acid sequence of the C-terminus differed from the corresponding 23 amino acids of the IS*Aba3* transposase. Other insertion

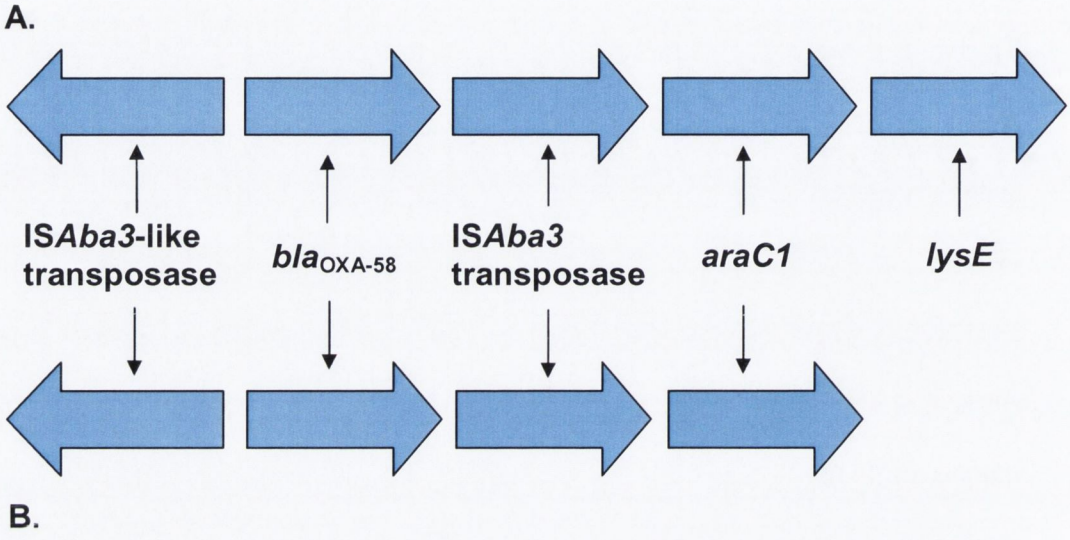


Figure 3-2. Genetic environment of *bla*$_{OXA-58}$ genes in *Acinetobacter* genospecies 10 and genospecies 9.

[A., *Acinetobacter* genosp. 10 isolate 101055; B., *Acinetobacter* genosp. 9 isolate 180361]

sequences IS*Aba2*, IS*Aba1* and IS*18* that were frequently found upstream of bla_{OXA-58} in carbapenem-resistant *Acinetobacter* isolates were absent in the two isolates. The insertion sequence IS*Aba3* as well as a transcriptional regulator gene *araC1* were found downstream of bla_{OXA-58} in both isolates. The gene for threonine efflux protein *lysE* was found downstream of the *araC1* gene in the *Acinetobacter* genospecies 10 isolate 101055 but not in the *Acinetobacter* genospecies 9 isolate 180361. Additional genes (putative esterase *est* and transcriptional regulator *araC2*) reported in some *A. baumannii* strains were not found in either of the isolates. PCR of the plasmid preparation from each isolate was positive for bla_{OXA-58}, implying that bla_{OXA-58} was plasmid-mediated in both isolates. Four plasmids were visualised for isolate 101055 (ranging from 5 MDa to 30 MDa) while six plasmids were visualised for 180361 (ranging from 1.5 MDa to 25 MDa). The bla_{OXA-58} genes were found on the 30 MDa plasmid and 22 MDa plasmid in isolate 101055 and 180361, respectively.

3.3.5 Integrons, aminoglycoside-modifying enzyme and *qnr* genes

The class 1 integrase gene *intI*1 was detected in two *A. baumannii* isolates: pan-carbapenem-resistant isolate 229437 and doripenem non-susceptible isolate 11648, thereby indicating the presence of class 1 integrons in these two isolates (see Table 3-3). No *Acinetobacter* isolate was positive for the class 2 integrase gene *intI*2.

Genes encoding aminoglycoside-modifying enzymes were found in all ten of the *Acinetobacter* isolates with aminoglycoside non-susceptibility or borderline susceptibility. The genes and enzymes are shown in Table 3-4. Aminoglycoside-modifying enzymes were found in various species and the aminoglycoside acetyltransferase gene AAC(3)-II was the most prevalent enzyme, being present in seven of the ten isolates tested. Notably, IS*Aba1* was found upstream of three *Acinetobacter* genospecies 3 isolates 29540, 32357 and 36277. However, the IS*Aba1* element and the *aac(3)-II* gene were separated by a truncated 3' end of another IS element IS*1133* (see Figure 3-2). The *A. baumannii* isolate 229437 carried genes encoding acetyltransferase AAC(3)-I and phosphotransferase APH(3')-VI, which accounted for gentamicin and amikacin resistance, respectively. *A. haemolyticus* isolate 89182, with borderline amikacin susceptibility, was positive for the acetyltransferase gene *aac(6')-Ib*.

None of the three transferable quinolone resistance genes (*qnrA*, *qnrB* and *qnrS*) was detected in the 117 *Acinetobacter* isolates.

3.3.6 GenBank® accession numbers

The following sequences were submitted to GenBank, NCBI, under the following accession numbers: EU827524 (*Acinetobacter* genospecies 3 insertion sequence IS*Aba1*/$bla_{\text{OXA-23}}$ gene/*ATPase* gene); EU827525 (*Acinetobacter johnsonii* insertion

Table 3-4. Aminoglycoside-modifying enzymes in *Acinetobacter* spp. in this study.

Isolate identifier	Species	Aminoglycoside -modifying enzyme	Gene (alternative nomenclature)	Reference GenBank accession no.
229437	*A. baumannii*	AAC(3)-I APH(3')-VI	*aac(3)-Ia* (*aacC1*) *aph(3')-VIa* (*aphA6*)	X15852 X07753
11648	*A. baumannii*	AAC(3)-I	*aac(3)-Ia* (*aacC1*)	X15852
29540	*Acinetobacter* genosp. 3	AAC(3)-II	*aac(3)-IIa* (*aacC2*)	X13543
32357	*Acinetobacter* genosp. 3	AAC(3)-II	*aac(3)-IIa* (*aacC2*)	X13543
36277	*Acinetobacter* genosp. 3	AAC(3)-II	*aac(3)-IIa* (*aacC2*)	X13543
14027	*Acinetobacter* genosp. 3	AAC(3)-II	*aac(3)-IIa* (*aacC2*)	X13543
25868	*A. johnsonii*	AAC(3)-II	*aac(3)-IIa* (*aacC2*)	X13543
101055	*Acinetobacter* genosp. 10	AAC(3)-II	*aac(3)-IIa* (*aacC2*)	X13543
183357	*A. ursingii*	AAC(3)-II	*aac(3)-IIa* (*aacC2*)	X13543
89182	*A. haemolyticus*	AAC(6')-I	*aac(6')-Ib* (*aacA4*)	M21682

AAC(3), aminoglycoside 3-*N*-acetyltransferase; APH(3'), aminoglycoside 3'-*O*-phosphotransferase; AAC(6'), aminoglycoside 6'-*N*-acteyltransferase.

sequence IS*Aba1*/bla_{OXA-23} gene/*ATPase* gene); EU827526 (*Acinetobacter baumannii* insertion sequence IS*Aba1*/bla_{OXA-23} gene/*ATPase* gene); EU835512 (*Acinetobacter baumannii* insertion sequence IS*Aba1*/bla_{ADC-25} gene); EU839488 (*Acinetobacter* genospecies 3 insertion sequence IS*Aba1*/aminoglycoside acetyltransferase gene *aac(3)-IIa*); EU872057 (*Acinetobacter johnsonii* aminoglycoside acetyltransferase gene *aac(3)-IIa*); FJ195388 (*A. radioresistens* putative O-sialoglycoprotein endopeptidase *gcp* gene/ bla_{OXA-23} gene/*ATPase* gene); FJ195387 (*A. radioresistens* putative O-sialoglycoprotein endopeptidase *gcp* gene/$bla_{OXA-134}$ gene/*ATPase* gene); FJ195389 (*Acinetobacter* genomospecies 10 IS*Aba3*-like transposase gene/bla_{OXA-58} gene/insertion sequence IS*Aba3*/*araC1* gene/*lysE* gene); FJ200197 (*Acinetobacter* genomospecies 9 IS*Aba3*-like transposase gene/bla_{OXA-58} gene/insertion sequence IS*Aba3*/*araC1* gene).

3.4 Discussion

MDR *Acinetobacter* spp. are becoming increasingly prevalent worldwide. However, many of the resistance mechanisms, particularly the Ambler class C *Acinetobacter*-derived cephalosporinases and class D OXA carbapenemases, had only been characterised within the last decade (Afzal-Shah *et al.*, 2001; Bou *et al.*, 2000; Brown & Amyes, 2005; Hujer *et al.*, 2005; Poirel *et al.*, 2004; Poirel & Nordmann, 2006a).

The present study aimed to characterise comprehensively the profiles of β-lactamase genes in isolates collected in an Irish tertiary-care hospital and to establish the local epidemiology of *Acinetobacter* β-lactamase genes and compare it with those of other centres worldwide.

The narrow-spectrum TEM-1 was found to be ubiquitous among the *Acinetobacter* isolates in this study. Several Ambler class A ESBLs have been reported in *Acinetobacter* spp. in recent years, including VEB-1, PER-1 and SHV-5 (Naas *et al.*, 2006a; Naas *et al.*, 2006b; Naas *et al.*, 2007). However, Ambler class A ESBLs were not detected in isolates collected in this study, including the 28 isolates with positive class A ESBL phenotypic screening results. At least two possible explanations can be offered for the above results. Firstly, the isolates carried rare and/or as yet uncharacterised class A ESBLs, and hence were not detected by the panel of primers for the range of ESBLs as stated in Table 3-1. Another possibility is that the ESBL screening results represented false-positive results, and that other mechanisms (such as other β-lactamases) were responsible for producing the positive ESBL screening results. This is plausible since some Ambler class D ESBLs such as OXA-18 from *P. aeruginosa* are susceptible to inhibition by clavulanic acid (Philippon *et al.*, 1997). However, OXA ESBLs were sought for and not detected in the isolates in this study. Furthermore, it has been reported that some *Acinetobacter* isolates can give false-positive class A ESBL screening results and are intrinsically susceptible to clavulanic acid, although it is still unclear what mechanism is responsible for the result (Beceiro *et al.*, 2008). Findings in this study supported the latter as a variety of species in this study gave positive class A ESBL screening results: seven of eight *A. johnsonii*, all

five *A. lwoffii*/*Acinetobacter* genospecies 9 and all four *Acinetobacter* genospecies 11 isolates subjected to ESBL screening gave positive results. The only *A. johnsonii* isolate (25868) that gave a negative ESBL screening result also expressed OXA-23, which would mask the effect of clavulanic acid. While it is still unclear why some isolates or species should be susceptible to clavulanic acid in the absence of Ambler class A ESBLs, caution should be exercised when interpreting positive ESBL screening results in *Acinetobacter* spp. The findings also suggest that the clavulanic acid-based ESBL screening tests may not be suitable for detecting Ambler class A ESBLs in *Acinetobacter* spp.

None of the genes encoding the five Ambler class B β-lactamases was found in the 117 *Acinetobacter* isolates in the study, including the 12 pan-carbapenem-resistant isolates that gave presumptive positive results with the MBL phenotypic screening test (see discussion below). The results were in contrast to the relatively high prevalence rates of such genes in some countries such as Korea and Japan (Lee *et al.*, 2004; Lee *et al.*, 2005; Yum *et al.*, 2002).

Ambler class C bla_{ADC} genes were characterised in *A. baumannii*, *Acinetobacter* genospecies 3 and *A. calcoaceticus* isolates, but those of other species have not been investigated. The primers used in this study were based on sequences of bla_{ADC} from *A. baumannii* and *Acinetobacter* genospecies 3. An Ambler class C bla_{ADC-8} from *Acinetobacter baylyi* was recently characterised and demonstrated only < 50% homology with the bla_{ADC} genes of *A. baumannii* and *Acinetobacter* genospecies 3 (Beceiro *et al.*, 2007). Hence, it is not entirely surprising that PCR using these primers

did not yield positive results for most of the other species as there is probably significant genetic heterogeneity amongst the bla_{ADC} genes of the various species. However, characterisation of bla_{ADC} (partial sequences) from all six *A. calcoaceticus* isolates was achieved, probably reflecting the genetic relatedness of the species within the *A. calcoaceticus-baumannii* complex. Collectively, the bla_{ADC} genes of *A. calcoaceticus* had < 90% sequence concordance with their counterparts in *A. baumannii* or *Acinetobacter* genospecies 3, and were not previously described in the literature or in GenBank database. Novel bla_{ADC} genes were also found in *A. baumannii* isolates 102450, 14773 and 181329. They had < 95% concordance with known sequences of *A. baumannii* bla_{ADC} genes in the GenBank database. Of the other known bla_{ADC} genes, $bla_{ADC\text{-}18}$ was the most prevalent bla_{ADC} in the *Acinetobacter* genospecies 3 isolates of this study, while there was greater heterogeneity of bla_{ADC} subtypes in *A. baumannii*. In spite of the heterogeneity of bla_{ADC} genes among the various species and isolates, the highest MICs for cefotaxime, ceftazidime and cefepime were recorded in the *A. baumannii* isolates 229437 and 11648, where IS*Aba1* was detected upstream of the bla_{ADC} genes, respectively. Therefore, the results support previous studies which demonstrated the importance of IS*Aba1* in promoting the overexpression of bla_{ADC} in *A. baumannii* (Héritier *et al.*, 2006; Segal *et al.*, 2004).

There was also a relatively high prevalence of genes encoding Ambler class D carbapenemases among isolates in this study (42 of 117 isolates; 35.9%). Ambler class D β-lactamases are also called oxacillinases or OXA β-lactamases because they hydrolyse oxacillin faster than the classical penicillins such as benzylpenicillin (Bush

et al., 1995). Over 50 of these oxacillinases have carbapenem-hydrolysing activities (Poirel & Nordmann, 2006b; Walther-Rasmussen & Høiby, 2006). The OXA carbapenemases have been discussed in sections 1.3.3.1.4 and 1.4.2.

There is strong circumstantial evidence to suggest that the OXA-51 group of genes are naturally occurring genes in *A. baumannii*. In this study, the OXA-51 group of bla_{OXA} genes were detected in all 27 *A. baumannii* isolates but not in any other species within the *Acinetobacter* genus. This group of bla_{OXA} genes were found in both carbapenem-susceptible and carbapenem-resistant *A. baumannii* isolates. Therefore, the results in this study support the findings of other studies that the OXA-51 group of bla_{OXA} genes are ubiquitous in *A. baumannii*, irrespective of the carbapenem susceptibility (Brown & Amyes, 2005; Héritier *et al.*, 2005a; Turton *et al.*, 2006).

The OXA-23 group of genes were found in isolates of several species in this study: *A. radioresistens*, *A. baumannii*, *Acinetobacter* genospecies 3 and *A. johnsonii*. This study also reported a novel $bla_{OXA-134}$ in the *A. radioresistens* isolate 88427 (GenBank accession number FJ195387), which differs from bla_{OXA-23} by two amino acid substitutions. Therefore, another new member has been added to this rapidly expanding cluster of bla_{OXA} genes.

In the study, the 12 pan-carbapenem-resistant *Acinetobacter* isolates (ten *Acinetobacter* genospecies 3 isolates, one *A. baumannii* isolate and one *A. johnsonii* isolate) were all found to carry plasmid-mediated bla_{OXA-23}. Although the *A. baumannii* isolate 229437 was also positive for $bla_{OXA-51-like}$ gene, the IEF results

would strongly suggest that bla_{OXA-23} was the most probable β-lactamase gene contributing to the carbapenem resistance in all 12 isolates. Interestingly, although the 12 isolates gave presumptive positive MBL phenotypic screening results, the PCR amplification of MBL genes indicated that the screening results were false-positive results. False-positive MBL screening results in *Acinetobacter* isolates carrying bla_{OXA-23} genes have been previously documented, and the above results in this study reiterated the importance of exercising caution when interpreting MBL screening results in *Acinetobacter* spp. with carbapenem resistance (Boo *et al.*, 2006; Segal & Elisha, 2005). The reason for the false-positive result with OXA-23-positive isolates is unclear. One possibility is that OXA-23 may exist as a highly active dimer, and EDTA may have converted the enzyme to a less active monomeric form by chelating and destabilising the cation dimeric bond (Segal & Elisha, 2005). In support of this, other OXA enzymes such as OXA-10 and OXA-14 exist in highly active dimers and less active monomers; and divalent metal cations such as Zn^{2+} and Cu^{2+} stabilise the dimerisation of such enzymes and prevent their conversion to less active monomeric forms (Danel *et al.*, 2001).

The present study also illustrated the unusual epidemiology of a predominance of *Acinetobacter* genospecies 3 within the OXA-23-positive *Acinetobacter* spp. in St. James's Hospital, which may partly be due to the relative distribution of the various *Acinetobacter* spp. locally (see section 2.3.1). This was in contrast to the predominance of *A. baumannii* in OXA-23-positive isolates in other reports worldwide (Coelho *et al.*, 2006; Corvec *et al.*, 2007; Lee *et al.*, 2009; Valenzuela *et al.*, 2007; Villegas *et al.*, 2007). This study was also the first report of a carbapenem-

resistant *A. johnsonii* clinical isolate mediated by $bla_{\text{OXA-23}}$, as there were no other reports of carbapenem resistance in this species in the literature. Elsewhere, carbapenem resistance in less commonly encountered *Acinetobacter* spp. was also increasingly reported (Marti *et al.*, 2008; Peleg *et al.*, 2006b). These findings indicated that potentially clinically important species other than *A. baumannii* were also developing carbapenem resistance. This is yet another worrisome escalation in antimicrobial resistance within the *Acinetobacter* genus.

In this study, all 12 pan-carbapenem-resistant *Acinetobacter* isolates were found to possess the insertion sequence IS*Aba1* immediately upstream of the $bla_{\text{OXA-23}}$ gene. This finding strongly supported the likelihood that there was overexpression of $bla_{\text{OXA-23}}$ in these isolates. The presence of IS*Aba1* associated with bla_{OXA} in carbapenem-resistant isolates and conversely, the lack of it in carbapenem-susceptible isolates, provided important evidence for its role in the regulation of the bla_{OXA} genes. This was corroborated by the findings in this study, where IS were absent upstream of $bla_{\text{OXA-23-like}}$ and $bla_{\text{OXA-51-like}}$ in carbapenem-susceptible *A. radioresistens* (see discussion below) and *A. baumannii* isolates, respectively, along with the presence of IS*Aba1* upstream of $bla_{\text{OXA-23}}$ in the carbapenem-resistant isolates. IS are transposable elements that carry genes encoding transposases and have short inverted terminal repeats at the ends of their DNA (see section 1.3.2). Several novel IS elements had been characterised in *Acinetobacter* spp.: IS*Aba1*, IS*Aba2*, IS*Aba3*, IS*Aba4* and IS*18*. They were found in different *Acinetobacter* strains and species in varying copy numbers but were not present in other genera such as *Enterobacteriaceae* and *P. aeruginosa* (Corvec *et al.*, 2007; Marti *et al.*, 2008; Poirel & Nordmann, 2006a; Segal

et al., 2005). The origins of these IS elements were unclear, but they encoded transposases with high levels of similarity to a putative transposase from *Deinococcus radiodurans*, suggesting possible acquisition from this organism (Segal *et al.*, 2003). *D. radiodurans* is a Gram-positive bacterium normally found in a variety of soil-based habitats, and has been intensely studied for its high level of resistance to radiation. While it is the subject of intense research, its clinical significance is unknown (Cox & Battista, 2005). The pivotal role that these IS elements played in the antimicrobial resistance of *Acinetobacter* spp. was exemplified in the regulation of carbapenem resistance by IS*Aba1*. The latter, which belongs to the IS*4* family, was found upstream of $bla_{\text{OXA-23}}$, $bla_{\text{OXA-27}}$, $bla_{\text{OXA-51-like}}$ and $bla_{\text{OXA-58}}$ genes in carbapenem-resistant *Acinetobacter* isolates (Corvec *et al.*, 2007; Naas *et al.*, 2007; Poirel & Nordmann, 2006a; Segal *et al.*, 2003; Turton *et al.*, 2006). In *A. baumannii* isolates carrying $bla_{\text{OXA-51-like}}$ genes but not other OXA groups, IS*Aba1* was found upstream of the bla_{OXA} genes only in the carbapenem-resistant isolates (Turton *et al.*, 2006; Naas *et al.*, 2007). Furthermore, IS*Aba1* was shown to possess promoter sequences involved in the up-regulation of $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-58}}$ expression respectively in *A. baumannii* isolates (Corvec *et al.*, 2007; Poirel & Nordmann, 2006a). Since the OXA carbapenemases had weak to moderate carbapenem hydrolytic activities, overexpression of these enzymes afforded by the presence of IS*Aba1* was probably necessary to confer the carbapenem-resistant phenotype (Héritier *et al.*, 2005a, Walther-Rasmussen & Høiby, 2006). Moreover, the central role of IS*Aba1* in the regulation of β-lactamase expression was further strengthened by studies, including this present study, demonstrating its importance in the overexpression of ADC enzymes in *A. baumannii* (Héritier *et al.*, 2006; Naas *et al.*, 2007; Segal *et al.*, 2004).

Ultimately, the paradigm of β-lactam resistance was probably best represented by the *A. baumannii* isolate 229437, where IS*Aba1*, by promoting the overexpression of both $bla_{\text{OXA-23}}$ and $bla_{\text{ADC-25}}$, had a profound influence on β-lactamase expression resulting in high-level resistance to all subclasses of β-lactam agents (see Table 3-2 and Figure 3-1).

It is interesting to postulate how carbapenem resistance might have arisen in *Acinetobacter* isolates in our institution. The unusual epidemiology of a predominance of *Acinetobacter* genospecies 3 among the carbapenem-resistant isolates, which has not been reported elsewhere, would argue against the importation of resistant strains into our hospital. Moreover, the demonstration through PFGE typing of clonality among some of the carbapenem-susceptible and carbapenem-resistant *Acinetobacter* genospecies 3 strains would further support the hypothesis that carbapenem resistance has arisen *de novo* among endemic strains due perhaps to selective antimicrobial pressure such as the increased use of carbapenems in the clinical setting. It is also possible that the carbapenem-resistant isolates have acquired the $bla_{\text{OXA-23}}$ genes from local *A. radioresistens* isolates via plasmids. Interestingly, $bla_{\text{OXA-23}}$ genes of the isolates of *Acinetobacter* genospecies 3 and *A. johnsonii* were all carried on plasmids of similar sizes (40 MDa), thereby raising the possibility of horizontal plasmidic transfer of $bla_{\text{OXA-23}}$ between different strains of the same species (ie. *Acinetobacter* genospecies 3) as well as between different species (ie. *Acinetobacter* genospecies 3 and *A. johnsonii*). This was further supported by the genetic similarities of the flanking sequences of $bla_{\text{OXA-23}}$ among the different isolates. There were significant

clinical implications with these findings. Apart from the potential therapeutic difficulties associated with carbapenem resistance in these *Acinetobacter* isolates, it will also be very challenging to control the dissemination of the resistance genes since the above findings imply that multiple mechanisms such as clonal expansion of particular strains (see section 2.3.3.1) and horizontal plasmidic transfer between strains/species were likely to be involved in promoting the spread of carbapenem resistance within the *Acinetobacter* isolates in this study.

In addition to the 12 carbapenem-resistant *Acinetobacter* isolates, the two carbapenem-susceptible *A. radioresistens* isolates also possessed OXA-23 group of genes, $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-134}}$, respectively. The deduced amino acid sequence of the novel $bla_{\text{OXA-134}}$ differs from OXA-23 by two amino acid substitutions. Whereas the $bla_{\text{OXA-23}}$ genes in carbapenem-resistant isolates were located on plasmids, the $bla_{\text{OXA-23-like}}$ genes in *A. radioresistens* isolates were located chromosomally. The findings in this study provided further evidence of *A. radioresistens* as the progenitor of the $bla_{\text{OXA-23-like}}$ genes. *A. radioresistens* isolates have recently been found to possess chromosomally located $bla_{\text{OXA-23}}$ or closely related $bla_{\text{OXA-102}}$, $bla_{\text{OXA-103}}$ and $bla_{\text{OXA-105}}$ genes respectively (Poirel *et al.*, 2008b). An *ATPase* gene was also found downstream of the $bla_{\text{OXA-23-like}}$ genes. These isolates were carbapenem-susceptible, implying that additional mechanisms were involved in the development of carbapenem resistance in other *Acinetobacter* isolates. Since the $bla_{\text{OXA-23-like}}$ genes in other *Acinetobacter* spp. were usually found in plasmids, it was most probable that these genes were initially mobilised from the *A. radioresistens* genome and subsequently transferred horizontally to other species via plasmids. In support of this,

ATPase gene with high sequence homology with that in *A. radioresistens* was found downstream of the plasmid-mediated bla_{OXA-23} in carbapenem-resistant *A. baumannii* isolates (Corvec *et al.*, 2007; Poirel *et al.*, 2008b). The results of this present study were compatible with such a hypothesis, with the bla_{OXA-23} in carbapenem-resistant *Acinetobacter* isolates being of plasmidic origins while the $bla_{OXA-23-like}$ genes in the carbapenem-susceptible *A. radioresistens* isolates were chromosomal in location. Moreover, *ATPase* gene homologous to that of *A. radioresistens* was also found downstream of bla_{OXA-23} in the carbapenem-resistant *Acinetobacter* isolates (see Figure 3-1).

An unexpected finding in this study was the detection of bla_{OXA-58} gene in two carbapenem-susceptible isolates, *Acinetobacter* genospecies 9 (isolate 180361) and genospecies 10 (isolate 101055), respectively. While bla_{OXA-58} was mostly reported in carbapenem-resistant *A. baumannii* isolates, it was also occasionally described in carbapenem-resistant *Acinetobacter* genospecies 3 and *Acinetobacter junii* (Marti *et al.*, 2008; Peleg *et al.*, 2006b). The findings in this study were unusual in that the two isolates (*Acinetobacter* genospecies 9 and 10, respectively) represented the first reported cases of bla_{OXA-58} carriage in these species and that they were phenotypically susceptible to carbapenems (see Appendix 1). The detection of silent carriage of bla_{OXA-58} genes in relatively uncommon *Acinetobacter* spp. suggested that the gene may be more widely disseminated than was previously believed, as there were no previous studies investigating the prevalence of bla_{OXA-58} in carbapenem-susceptible *Acinetobacter* spp.

The genetic structures surrounding bla_{OXA-58} in the two bla_{OXA-58}-positive isolates in the study suggested that these genes may be the precursor forms of bla_{OXA-58} elements found in carbapenem-resistant *Acinetobacter* isolates. In these two isolates, the bla_{OXA-58} was bracketed by transposases in a similar arrangement as that of a composite transposon. Both isolates had an intact IS*Aba3*-like transposase upstream of bla_{OXA-58} but without any other upstream IS (see figure 3-2). On the other hand, several IS elements were associated with the bla_{OXA-58} gene in carbapenem-resistant *A. baumannii*. IS*Aba1*, IS*Aba2* and IS*18* were found upstream of bla_{OXA-58}, while IS*Aba3* was consistently found downstream (Poirel & Nordmann, 2006a). Interestingly, the above three IS were flanked by direct repeats and a truncated form of IS*Aba3*-like element, suggesting that the IS*Aba3*-like element was disrupted by the later event of insertion by IS*Aba1*, IS*Aba2* and IS*18* respectively (Poirel & Nodmann, 2006a). The intact IS*Aba3*-like transposase upstream of bla_{OXA-58} in the two bla_{OXA-58}-positive isolates of the study provided supportive evidence that these genetic structures were precursor forms of the bla_{OXA-58} elements found in carbapenem-resistant isolates. Since both isolates in the study were fully susceptible to the three carbapenems, it is possible that in carbapenem-resistant isolates, subsequent genetic events involving the insertion of other IS such as IS*Aba2* were necessary in conferring the carbapenem-resistant phenotype associated with bla_{OXA-58}. Moreover, as promoter sequences were found in IS*Aba1*, IS*Aba2* and IS*18*, the findings in the study would imply that the IS*Aba3*-like element was a relatively weak promoter of bla_{OXA-58} expression. Although *A. baumannii* and *A. radioresistens* are the probable progenitors of $bla_{OXA-51-like}$ and $bla_{OXA-23-like}$ genes, respectively, the progenitor of bla_{OXA-58} has not been identified so far. The finding in this study that the bla_{OXA-58} genes were

located on plasmids in isolates of two different species makes it unlikely that either *Acinetobacter* genospecies 9 or genospecies 10 is the progenitor of bla_{OXA-58}.

In addition, other associated transferable elements in antimicrobial resistance such as aminoglycoside-modifying enzymes, Qnr resistance elements and integrons were also investigated. This study has demonstrated that genes encoding aminoglycoside-modifying enzymes were present in all isolates with non-susceptibility to gentamicin and/or amikacin. The predominant aminoglycoside-modifying enzyme among the *Acinetobacter* spp. was the acetyltransferase enzyme AAC(3)-II. While it is tempting to surmise that such enzymes were the main mechanisms mediating aminoglycoside resistance in these isolates, it should be borne in mind that other mechanisms of resistance might also have contributed to aminoglycoside resistance. In particular, overexpression of multidrug efflux pumps such as AdeABC can confer resistance to aminoglycosides and other classes of antimicrobial agents in *A. baumannii* (Magnet *et al.*, 2001). Interestingly, IS*Aba1* was also found upstream of *acc(3)-II* genes of some isolates. Nevertheless, the aminoglycoside MICs of such isolates were not higher than those of isolates possessing *aac(3)-II* but without an upstream IS*Aba1* (see Table 3-4 and Appendix 1). However, it should be noted that the IS*Aba1* was separated from *acc(3)-II* by a truncated form of IS*1133*, which could have interfered with the gene-regulatory function of IS*Aba1*. A recent report also supported the role of IS*Aba1* in the regulation of genes encoding aminoglycoside-modifying enzymes in *A. baumannii* (Naas *et al.*, 2007). Hence, it is still unclear as to the role that IS*Aba1* plays in the regulation of aminoglycoside resistance genes.

In the present study, the prevalence of integrons in *Acinetobacter* isolates was also low. Only two of 117 isolates (1.7%) carried class 1 integrons. As class 1 integrons were commonly associated with epidemic strains of MDR *Acinetobacter* spp., the low prevalence in our study was probably unsurprising since most isolates were obtained in the sporadic setting rather than in the setting of large outbreaks (Koeleman *et al.*, 2001; Nemec *et al.*, 2004; Turton *et al.*, 2005). Consistent with the observations of other reports, the two isolates carrying class 1 integrons were also resistant to multiple classes of antimicrobial agents (Koeleman *et al.*, 2001; Lévesque *et al.*, 1995; Nemec *et al.*, 2004; Turton *et al.*, 2005). None of the *qnr* genes was detected in any of the 117 isolates, which was not surprising since such genes had not been reported in *Acinetobacter* spp. so far.

The findings in this chapter of the study highlighted the complexity of genetic interactions involved in the dissemination, regulation and expression of antimicrobial resistance genes, particularly of bla_{OXA} genes. Such findings have significant therapeutic and infection control implications. The expression of class C and D β-lactamases as well as aminoglycoside-modifying enzymes in *Acinetobacter* spp. significantly limits the choice of antibiotics available for the treatment of infections caused by these MDR isolates. Formidable challenges in hospital infection control are also presented by such MDR strains. Measures such as isolation policies and stringent antimicrobial stewardship are all urgently required to contain the dissemination of resistance genes such as bla_{OXA-23}, especially since these genes were carried on plasmids and there was evidence supporting inter-strain and inter-species dissemination of such genes. Further evidence supporting the *de novo* development of

carbapenem resistance among endemic strains underscores the need for prudent use of antibiotics such as carbapenems in our hospital. The study also highlighted the under-recognised threat of silent reservoirs of bla_{OXA} carbapenemase genes. The $bla_{OXA\text{-}51\text{-}like}$, $bla_{OXA\text{-}23\text{-}like}$ and $bla_{OXA\text{-}58}$ genes were found in phenotypically susceptible isolates of *A. baumannii*, *A. radioresistens* and *Acinetobacter* genospecies 9 and 10, respectively. In most centres, resources and efforts in the detection of such genes as well as subsequent infection control measures would generally have been focused on phenotypically MDR organisms. Hence, the findings in this present study reiterated the importance of stringent adherence to standard infection control precautions such as hand hygiene and scrupulous healthcare environmental cleaning, since such measures target a wide range of micro-organisms (MDR phenotypes or otherwise) and hence reduce the risk of dissemination of resistance genes from unrecognised sources.

The transfer of yet another class of β-lactamase genes from relatively obscure organisms like *A. radioresistens* and *Shewanella* spp. to clinically important species like *A. baumannii*, *Acinetobacter* genospecies 3 and *K. pneumoniae* was yet another significant development in the acquisition and emergence of β-lactam resistance in Gram-negative bacteria (Poirel *et al.*, 2008b; Poirel *et al.*, 2004). Numerous β-lactamase genes had been transferred via mobile elements such as plasmids from various organisms to clinically important pathogens. While *Kluyvera* spp. such as *K. ascorbata* were the probable progenitors of the Ambler class A $bla_{CTX\text{-}M}$ genes found in *Enterobacteriaceae*, the plasmidic Ambler class C genes bla_{ACC} and bla_{CMY} found predominantly in *Klebsiella* spp. and *E. coli* most probably originated from *Hafnia alvei* and *Citrobacter freundii* respectively (Humeniuk *et al.*, 2002; Nadjar *et al.*,

2000; Wu *et al.*, 1999). The above examples underscore the extent and complexity of genetic interactions between different bacterial genera and species, including the exchange of resistance genes such as β-lactamase genes. The development of carbapenem resistance is particularly worrying since carbapenems constitute a very broad-spectrum and versatile class of β-lactams with stability against the majority of β-lactamases of Ambler class A and C. Hence the emergence and spread of OXA carbapenemases such as OXA-23 in *Acinetobacter* spp. portend an ominous trend in Gram-negative antimicrobial resistance.

Chapter 4

Investigation of non-β-lactamase mechanisms of carbapenem resistance in *Acinetobacter* species

4.1 Introduction

While β-lactamases play important roles in carbapenem resistance in *Acinetobacter* spp., other mechanisms also have contributory roles in the development of such resistance. It has been well characterised in *P. aeruginosa* that carbapenem resistance is often multifactorial, involving the interplay of efflux of antibiotics out of the cell, decreased permeability of outer membrane to antimicrobial penetration and β-lactamase activity in the periplasmic space (Cavallo *et al.*, 2002; Quale *et al.*, 2006). Increasingly, carbapenem resistance in *Acinetobacter* spp. has been attributed to other mechanisms in addition to β-lactamases (Bou *et al.*, 2000; Fernández-Cuenca *et al.*, 2003; Héritier *et al.*, 2005; Huang *et al.*, 2008; Mussi *et al.*, 2005; Quale *et al.*, 2003; Siroy *et al.*, 2005).

Efflux-mediated resistance to various antimicrobial agents including carbapenems has been increasingly appreciated as an important component contributing to bacterial multidrug resistance. The different families of efflux pumps associated with antimicrobial resistance have been explained in section 1.3.1. The effects of such active drug transport systems are wide-ranging, from the conferring of low intrinsic susceptibility in organisms to the development of cross-resistance in pathogens to chemically unrelated classes of antibiotics such as quinolones, tetracyclines, aminoglycosides and β-lactams (Ghisalberti *et al.*, 2006; Mahamoud *et al.*, 2007; Pradel & Pagès, 2002). Moreover, such mechanisms can reinforce the effects of other resistance mechanisms such as mutations or modifications of drug targets (Poole,

2005). Of particular concern is the finding that some efflux mechanisms can accommodate both antibiotics as well as biocides, the latter of which are a cornerstone in infection control measures and disinfection (Levy, 2002; Poole, 2005). This raises the prospect of further escalation of drug resistance through the selection of biocide-resistant, antibiotic-resistant pathogens.

Efflux pump inhibitors (EPIs) are currently employed in research laboratories to investigate the role of efflux mechanisms in antimicrobial resistance. The various types of EPIs have been described in section 1.3.10. As mentioned, EPIs have different spectra of activity against organisms and efflux pumps. For example, quinolines are active against AcrAB-TolC pumps in *Enterobacteriaceae* but have poor activity against MexAB-OprM efflux pump in *P. aeruginosa*. EPIs can also affect different antibiotic classes in the same organism, such as the effect of PAβN versus NMP in *A. baumannii*. NMP alters susceptibility to quinolones, β-lactams, tetracyclines and linezolid while PAβN mainly affects macrolides, β-lactams, rifampicin and linezolid (Pannek *et al.*, 2006). The mechanism(s) for the observed differences is still unclear. Several studies investigated the role of efflux pumps in carbapenem resistance in *Acinetobacter* spp. using EPIs, but did not obtain results that supported a significant role of efflux mechanism in carbapenem resistance (Bou *et al.*, 2000; Quale *et al.*, 2003; Sinha & Srinivasa, 2007). However, some of these studies investigated efflux mechanisms using reserpine, which is an inhibitor of Gram-positive efflux pumps (Bou *et al.*, 2000; Li & Nikaido, 2004; Sinha & Srinivasa, 2007). Some studies used CCCP as the EPI, and they yielded conflicting results of the role of efflux mechanisms in carbapenem resistance (Huang *et al.*, 2009; Quale *et al.*,

2003). The EPI PAβN had been used to assess the role of efflux mechanisms in tigecycline resistance in *Acinetobacter* spp., but has not been used to investigate efflux-mediated carbapenem resistance (Peleg *et al.*, 2007a). However, by studying the effects of inactivation (knockout) of efflux genes, there is now growing evidence that efflux mechanisms may also be involved in carbapenem resistance in *Acinetobacter* spp. (Chau *et al.*, 2004; Peleg *et al.*, 2007a; Ruzin *et al.*, 2007). It is unclear whether EPIs such as PAβN may be more appropriate in investigating efflux-mediated carbapenem resistance in *Acinetobacter* spp. than reserpine or CCCP.

In *A. baumannii*, efflux pumps of the various families have also been described (see section 1.3.1). QacE1 SMR efflux pumps confer resistance to ammonium antiseptic compounds, while Tet(A) and CmlA pumps are narrow-spectrum MFS efflux pumps targeting tetracyclines and chloramphenicol, respectively (Fournier *et al.*, 2006; Vila *et al.*, 2007). AbeM is a recently characterised multidrug efflux pump of the MATE family and recognises several compounds including quinolones, gentamicin, triclosan, doxorubicin, rhodamine and ethidium bromide (Su *et al.*, 2005; Vila *et al.*, 2007). Several RND efflux pumps were also characterised in *A. baumannii* and *Acinetobacter* genospecies 3, namely AdeABC, AdeDE, AdeIJK and AdeXYZ (Chau *et al.*, 2004; Chu *et al.*, 2006; Damier-Piolle *et al.*, 2008; Magnet *et al.*, 2001). AdeIJK has > 97% amino acid sequence identity to AdeXYZ, hence they are probably homologues in *A. baumannii* and *Acinetobacter* genospecies 3, respectively (Damier-Piolle *et al.*, 2008). Otherwise, AdeB, AdeE and AdeY have < 55% amino acid sequence identity when compared with one another (Chau *et al.*, 2004; Chu *et al.*, 2006). In conjunction with heterogeneity in amino acid sequences, the substrate

specificity profiles were different as well, although multiples classes of antibiotics were involved in each of the efflux pumps. Aminoglycosides, quinolones, tetracycline, tigecycline, erythromycin, cefotaxime and ethidium bromide are the main substrates of AdeABC, while imipenem, ceftazidime, amoxicillin, ticarcillin, rifampicin, clindamycin and sulphonamides are not significant substrates (Damier-Piolle *et al.*, 2008; Magnet *et al.*, 2001). Ticarcillin, clindamycin, tetracycline, chloramphenicol and cefotaxime are the main substrates for AdeIJK, while imipenem, rifampicin, quinolones and erythromycin and ethidium bromide are weak substrates (Damier-Piolle *et al.*, 2008). However, meropenem was not investigated as a substrate for AdeABC or AdeIJK by either group of researchers. Ceftazidime, rifampicin, tetracycline, erythromycin, quinolones, aminoglycosides, chloramphenicol and ethidium bromide are significant substrates of AdeDE while cefotaxime, cefepime, piperacillin are not significant substrates (Chau *et al.*, 2004). Following inactivation of AdeDE in *Acinetobacter* genospecies 3, meropenem susceptibility was increased as indicated by the decrease in MIC value, suggesting that meropenem was a substrate of the pump (Chau *et al.*, 2004). However, such studies were carried out on carbapenem-susceptible isolates, and the roles of such RND efflux pumps in carbapenem resistance were still unclear. Recently, however, the results of two studies carried out on carbapenem-resistant *A. baumannii* isolates supported the role of AdeABC efflux pump in meropenem resistance but not in imipenem resistance (Huang *et al.*, 2008; Wong *et al.*, 2009).

Decreased permeability of the outer membrane has been another important mechanism for the development of carbapenem resistance in Gram-negative bacteria

(see section 1.3.1). The decreased permeability to carbapenems is achieved through reduced expression of outer membrane porins (OMPs) through which the carbapenems traverse into the periplasmic space. The reduced expression of OprD in *P. aeruginosa* is a well-characterised mechanism of resistance for both imipenem and meropenem (Köhler *et al.*, 1999; Quale *et al.*, 2006). Decreased expression of both OmpF and Omp C porin analogues in *K. pneumoniae* (OmpK35 and OmpK36, respectively) and in *Enterobacter aerogenes* are associated with reduced carbapenem susceptibility (Crowley *et al.*, 2002; Doumith *et al.*, 2009; Yigit *et al.*, 2002). In *A. baumannii*, carbapenem resistance has been associated with decreased expression of several OMPs including 22 kDa-, 29 kDa-, 33 kDa-, 37 kDa- and 44 kDa-OMPs (Bou *et al.*, 2000; Fernández-Cuenca *et al.*, 2003; Mussi *et al.*, 2005; Quale *et al.*, 2006; Siroy *et al.*, 2005). Of the above OMPs, the 29 kDa OMP (also designated CarO porin) was characterised in great detail (Lu *et al.*, 2009; Mussi *et al.*, 2005; Poirel & Nordmann, 2006b; Siroy *et al.*, 2005). CarO has been shown to have channel-forming properties and belongs to a family of β-barrel OMPs found only in species of the *Moraxellaceae* family such as *Acinetobacter* spp., *Moraxella* spp. and *Psychrobacter* spp. (Mussi *et al.*, 2005; Siroy *et al.*, 2005). Loss of *carO* expression is associated with both imipenem and meropenem resistance (Mussi *et al.*, 2005; Poirel and Nordmann, 2006b). Moreover, in some isolates, the genetic basis for the loss of its expression has been found to be the insertional inactivation of insertion sequences such as IS*Aba1*, IS*Aba125* and IS*Aba825* (Lu *et al.*, 2009; Mussi *et al.*, 2005; Poirel and Nordmann, 2006b). Carbapenem resistance in other *Acinetobacter* spp. such as *Acinetobacter* genospecies 3 has been reported less commonly (Boo *et al.*, 2006;

Marti *et al.*, 2008). OMP profiles associated with carbapenem resistance in such species have also not been investigated to date.

This chapter aimed to investigate the role of efflux mechanisms in the development of carbapenem resistance in *Acinetobacter* spp. using efflux pump inhibitor as well as quantitative analysis of efflux gene expression of carbapenem-resistant as well as carbapenem-susceptible isolates. The study also aimed to investigate the role of permeability in carbapenem resistance through the evaluation of OMP profiles of the *Acinetobacter* isolates.

4.2 Materials and Methods

4.2.1 Efflux mechanisms of carbapenem resistance in *Acinetobacter* spp.

4.2.1.1 Characterisation of RND efflux genes in *Acinetobacter* spp.

Characterisation of RND-type multidrug efflux pumps in *Acinetobacter* spp. was performed on all 117 *Acinetobacter* isolates. It was carried out using PCR detection of the genes encoding the multidrug transporter components of the RND-type efflux pumps AdeABC, AdeDE, AdeIJK and AdeXYZ, ie. *adeB*, *adeE*, *adeJ* and *adeY*,

respectively. The primers for the above efflux transporter genes are shown in Table 4-1. PCR was performed with the Qiagen PCR core kit (Qiagen, GmbH, Hilden, Germany) using the methodology as described in section 2.2.1.2.1. The annealing temperatures for the PCR of the individual genes are also shown in Table 4-1.

4.2.1.2 Selection of strains for investigation of efflux mechanisms of resistance

Isolates giving positive results for section 4.2.1.1 were included for selection. As none of the *A. johnsonii* isolates gave positive PCR results for the efflux genes, the investigation of efflux contribution to carbapenem resistance in *A. johnsonii* was not performed. For *A. baumannii* and *Acinetobacter* genospecies 3, two carbapenem-non-susceptible isolates were selected for each species. For *A. baumannii*, the carbapenem-non-susceptible isolates were the pan-carbapenem-resistant *A. baumannii* isolate 229437 and the doripenem-non-susceptible *A. baumannii* isolate 11648. For *Acinetobacter* genospecies 3, the carbapenem-resistant isolates were two genetically distinct (by PFGE) isolates 36277 and 27616. Carbapenem-susceptible strains of the two species carrying the same complement of efflux transporter genes as the resistant strains were selected for the same experiments. For *A. baumannii*, as none of the carbapenem-susceptible isolates was clonally related to the non-susceptible strains, the ATCC 19606 reference strain and a randomly selected carbapenem-susceptible isolate 100983 were selected for the experiments. For *Acinetobacter* genospecies 3, isolate 27458 was selected as it was clonally indistinguishable from isolate 27616 by

Table 4-1. Primers used in PCR and qRT-PCR experiments for efflux genes in *Acinetobacter* isolates.

Primers used for PCR			
Gene	**Primers**	**Ann. Temp. (°C)**	**Reference**
adeB	F: 5'-TGAAATATTGGCCAACAGCA-3' R: 5'-CCCGATGACGTATCGAAGTT-3'	59.0	This study
adeJ	F: 5'-AATTGAAGCCGTGATTTTGG-3' R: 5'-AATCCGGCAGTTACACCAAG-3'	59.0	This study
adeE	F: 5'-GTAGTAGTTCGGCAGGACAA-3' R: 5'-GCGGTTCTAACATCTGATGG-3'	58.2	Chu *et al.*, 2006
adeY	F: 5'-CAATCTGCAACTGCGCTT-3' R: 5'-TCAACAGCTTCTGCGGTA-3'	58.5	Chu *et al.*, 2006
carO	F: 5'-ATGAAAGTATTACGTGTTTTAGTGACA-3' R: 5'-TTACCAGTAGAAGTTTACACCAACTTT-3'	58.2	This study
Primers used for qRT-PCR			
Gene	**Primers**	**Ann. Temp. (°C)**	**Reference**
16S rRNA	F: 5'-GCTGCCTTCGCCATCGGTATTC-3' R: 5'-CTGGGCGTAAAGCGTGCGTAGG-3'	57.4	This study
adeB	F: 5'-CAAACGGCGCTGTGTCG-3' R: 5'-GTGCTAACGCCGTGAAAACTG-3'	51.7	This study
adeJ	F: 5'-TTGCCCTTTTAGTCTTCTTGTTCC-3' R: 5'-CCACGGTGTCTTTTTCATTCATAA-3'	52.8	This study
adeE	F: 5'-TTCGCGGTACTAGTGGTGAGATGA-3' R: 5'-TGCCGCAGAGCCTTGTATTTG-3'	54.8	This study
adeY	F: 5'-CGCCCTAATGGTCAAGAAGATACA-3' R: 5'-GGCCACGGTCAACGAAGTC-3'	53.7	This study
bla OXA-23	F: 5'-AAAATGTTGAATGCCCTGAT-3' R: 5'-ATGGTCCTACCAACCAGAAA-3'	54.5	This study

F, Forward; R, Reverse; Ann. Temp., Annealing Temperature.

PFGE (see section 2.3.3.1); in addition, a genetically distinct isolate 106770 was also randomly selected for the experiments.

4.2.1.3 Carbapenem susceptibility testing and EPI testing with PAβN

Meropenem and imipenem susceptibility testing was performed for the above isolates using Etest gradient MIC method (AB Biodisk) as per manufacturer's instructions as well as CLSI breakpoints for susceptibility interpretation (CLSI, 2009). As the upper limit of the Etest meropenem MIC is 32 μg/mL, the exact meropenem MICs of the isolates were also determined by the microbroth dilution method as per CLSI guidelines (CLSI, 2009). To assess the efflux inhibitory effects of PAβN on *Acinetobacter* spp., particularly with respect to carbapenem susceptibility, meropenem and imipenem MICs were determined with the Etest method using Mueller-Hinton medium in the presence of PAβN at a concentration of 100 μg/mL, as used in other studies (Pannek *et al.*, 2006; Peleg *et al.*, 2007a).

4.2.1.4 RNA extraction from *Acinetobacter* culture in antibiotic-free medium and in medium containing meropenem

Before performing RNA extraction, growth of the isolates in liquid medium over 24 hours was performed and growth curves were plotted in order to ascertain the time and inoculum at which the mid-logarithmic phase of growth occurred for each of the isolates. Isolates were first subcultured onto sheep blood agar and incubated at 37 °C

overnight. A single colony of each isolate was then inoculated into 3 mL of Luria-Burtani (LB) broth and incubated overnight at 37 °C in the Forma Orbital Shaker (ThermoElectron Corporation, Basingstoke, Hampshire, UK). On the following day, the broth cultures were inoculated into 500ml of LB broth to give an optical density at 600nm (OD_{600}) of 0.05 as measured using the BioMate 5 UV-VIS spectrophotometer (ThermoSpectronic, Delaware, US). The broth cultures were in turn incubated in the Forma Orbital Shaker at 37 °C and OD_{600} measurements were taken at half-hourly intervals for the first 6 hours and at hourly intervals for the next 4 hours. Final OD_{600} measurements of the broth cultures were taken at 24 hours of incubation. Growth curves were plotted for the individual isolates.

For RNA extraction of isolates in antibiotic-free medium, the growth of the isolates in LB broth was performed as described above. An aliquot of 1.5 mL for each of the broth cultures was taken at the mid-logarithmic phase of growth. Each aliquot was centrifuged at > 10,000 x *g* for 2 minutes and the supernatant was removed thereafter. The precipitate was re-suspended in 1 mL of RNA stabilisation solution (RNA*later* solution, Ambion, Austin, Texas, US) and stored at -20 °C until further steps in the RNA extraction process were to be performed. RNA extraction was carried out using the RiboPure™-Yeast RNA extraction kit (Ambion, Austin, Texas, US) according to the manufacturer's instructions. Contaminating genomic DNA was eliminated by DNase I treatment according to the manufacturer's instructions and its absence was confirmed by the inclusion of a reverse transcriptase-minus control on each RNA sample. The concentration of the purified RNA for each isolate was estimated at an absorbance of 260 nm using the ND-1000 V3.3 nucleic acid spectrophotometer

(NanoDrop Technologies, Inc., Wilmington, Detroit, US). The purified RNA samples were stored at -20 °C until the performance of reverse transcriptase PCR. For each strain, extraction and purification of RNA were performed in triplicates.

For RNA extraction of cultures in the presence of sub-MIC concentrations of meropenem, isolates were initially subcultured onto blood agar and incubated overnight at 37 °C. On the following day, colonies were subcultured into LB broth as per the protocol above. On the following day, 500 mL of LB broth were prepared for each strain. Using the meropenem MIC data derived from the microbroth dilution method in section 4.2.1.3, meropenem was added to the broth to make up a final concentration which was two dilutions lower than the MIC of the individual isolate. Inoculation, growth measurements and RNA extraction of the respective strains in sub-MIC concentrations of meropenem were carried out in the same manner as for isolates cultured in the absence of meropenem. RNA extraction and purification were also performed in triplicates for each strain.

4.2.1.5 Validation of quantitative reverse transcriptase real-time PCR (qRT-PCR) assay for investigation of efflux gene expression

For validation of qRT-PCR experiments, standard curves were constructed to determine the amplification efficiencies of the housekeeping gene *16S rRNA* as well as the respective target efflux genes. The following strains were used for the preparation of the standard curves:

Gene	Strain	Species
adeB	100983	*A. baumannii*
adeJ	ATCC 19606	*A. baumannii*
adeE	106770	*Acinetobacter* genospecies 3
adeY	106770	*Acinetobacter* genospecies 3

A 1-in-5 dilution series of seven standards of RNA templates (6.25, 1.25, 0.25, 0.05, 0.01, 0.002 and 0.0004 ng, respectively) was prepared for qRT-PCR of the housekeeping and target genes. Each dilution series was amplified in triplicate using the Mastercycler® ep realplex^4 real-time PCR system (Eppendorf, Hamburg, Germany). The qRT-PCR methodology was described in the following section (4.2.1.6). The C_T values obtained from the quantitation report were used to construct standard curves for the two genes by plotting the mean C_T values against the logarithm of the template (RNA) concentrations. The optimal range of the slope was between -3.3 and -3.4 for the standard curve. Amplification efficiencies of the two genes were then compared by plotting the difference in C_T values of the two genes (ie. ΔC_T; C_T *ade* gene - C_T *16S rRNA*) against the logarithm of the RNA concentrations. The amplification efficiencies were considered comparable if the slope (M) of the resulting line was < 0.1.

4.2.1.6 qRT-PCR for the investigation of efflux gene expression in *Acinetobacter* strains

Aliquots of the purified RNA samples of the respective strains were diluted to a concentration of 0.25 ng/µl using the Eppendorf epMotion® 5070 automated pipetting system (Eppendorf, Hamburg, Germany). The primers and annealing temperatures for qRT-PCR of the respective efflux genes are shown in Table 4-1. qRT-PCR was performed using a QuantiTect SYBR Green RT-PCR kit (Qiagen, Hilden, Germany). The qRT-PCR was carried out in a 25 µl reaction mixture containing the following:

12.5 µl	2X QuantiTect SYBR Green RT-PCR Master Mix
1 µl (10 pmol)	Forward primer
1 µl (10 pmol)	Reverse primer
2.25 µl	Nuclease-free sterile water
0.25 µl	QuantiTect RT Mix Enzyme
8 µl (2 ng)	Purified RNA template

The qRT-PCR experiments were performed using the Mastercycler® ep realplex^4 real-time PCR system (Eppendorf). Initial reverse transcription was carried out at 50 °C for 30 min. It was followed by a denaturation step at 95 °C for 15 min. Amplification was then performed over 35 cycles with each cycle consisting of 15 sec at 94 °C, 30 sec at the respective annealing temperatures (see Table 4-1) and 30 sec at 72 °C. The final extension step was carried out at 72 °C for 10 min.

For the experiments, the *16S rRNA* gene of each isolate was assumed to be transcribed at a constant rate throughout the different growth conditions. Relative gene expression was calculated using the threshold cycle method (Livak & Schmittgen, 2001). Briefly, relative quantification of cDNA levels was calculated using the $\Delta\Delta C_T$ method. ΔC_T for each sample was determined by calculating the difference between C_T value of the target *ade* gene and the C_T value of the housekeeping gene *16S rRNA*. $\Delta\Delta C_T$ was then determined by subtracting the ΔC_T of the reference sample (eg. isolate grown in the absence of meropenem) from the ΔC_T of the new sample (eg. isolate grown in the presence of meropenem), depending on the nature of the comparison. If the amplification efficiencies of the target (*ade*) and housekeeping (*16S rRNA*) genes were comparable, the normalised level of target gene expression was calculated using the formula of $2^{\Delta\Delta CT}$.

4.2.2 Permeability mechanisms of carbapenem resistance in *Acinetobacter* spp.

4.2.2.1 Extraction of outer membrane proteins (OMP)

Suspension of the strain in LB broth was prepared to give a starting OD_{600} of 0.01 as per the method described in section 4.2.1.4. Growth in the liquid culture was monitored and at the mid-exponential phase of growth (between OD_{600} of 0.6 to 0.7),

50 mL of the liquid culture were harvested. The harvested liquid culture was centrifuged at 10,000 x *g* for ten minutes and the supernatant was then removed. The cells were re-suspended in 1 mL of ice-cold sonication buffer (10% sucrose; 50 mM Tris-HCl, pH 7.5; 100 mM NaCl; 1 mM EDTA, pH 8.0; and 5 mM dithiothreitol (DTT) and 1 table of Complete Mini, Protease Inhibitor Cocktail (Roche Diagnostics, GmbH, Mannheim, Germany)). 20 µL of lysozyme (concentration of 200 µg/mL) plus 4 µL of DNAse I (Ambion) were added to the cell suspension and incubated on ice for 30 minutes. Complete lysis was achieved by four cycles of sonication (at 5-8 microns; 30 sec/cycle) with cooling on ice between sonication cycles. The suspension was centrifuged at 10,000 x *g* for five minutes and the supernatant was transferred to a new tube. 0.3% N-laurylsarcosine was added to the supernatant and incubated for 30 minutes with continuous gentle shaking to solubilise the inner membranes. The sarcosine-insoluble outer membrane fraction was harvested by centrifugation at 14,000 x *g* for > 120 minutes and re-suspended in 50 µL of PCR-grade water. The concentration of the OMP extract was estimated using the ND-1000 V3.3 nucleic acid spectrophotometer (NanoDrop Technologies) at an absorbance of 280 nm.

4.2.2.2 Polyacrylamide gel electrophoresis with sodium dodecyl sulphate (SDS-PAGE)

Outer membrane proteins (OMPs) were separated by SDS-PAGE using 12% Precise™ 12-well pre-cast protein gels (ThermoScientific, Rockford, Illinois, US). 40 µg of the OMP extract was loaded into each well, and SDS-PAGE was carried out in Tris-HEPES-SDS running buffer at 30 mA for 60 minutes.

Bands on the gel were visualised by staining with Imperial™ Protein Stain (ThermoScientific). The gel was first washed in a clean tray for five minutes with 200 mL of ultra-pure water. 20 mL of Imperial™ Protein Stain was added for the gel, and stained for an hour with gentle shaking. The gel was then placed in ultra-pure water for over an hour. The gel was then visualised and digitally photographed using the Gel Logic 1500 Imaging System (Advanced Molecular Vision, Bacharach, Germany).

4.2.2.3 PCR and sequencing of *carO* gene in *A. baumannii* isolates

PCR and sequencing of *carO* gene in *A. baumannii* isolates were performed using the primers as shown in Table 4-1 to detect any mutations or the presence of insertion sequences (IS*Aba1*) within the *carO* gene as reported in some carbapenem-resistant isolates with reduced expression of CarO OMP (Lu *et al.*, 2009; Mussi *et al.*, 2005; Poirel & Nordmann, 2006b).

4.2.3 qRT-PCR investigation of *bla*$_{\text{OXA-23}}$ gene expression in carbapenem-resistant *Acinetobacter* isolates

qRT-PCR was also carried out for *bla*$_{\text{OXA-23}}$ using the same extracted RNA samples of the relevant isolates (ie. isolates 36277, 27616 and 229437). Validation of qRT-PCR experiments for *bla*$_{\text{OXA-23}}$ was performed using *16S rRNA* as the housekeeping gene and *Acinetobacter* genospecies 3 isolate 36277 as the reference strain. The validation

procedure was carried out in the same manner as in section 4.2.1.5. qRT-PCR assay was performed to assess the relative levels of bla_{OXA-23} expression both in the absence and presence of meropenem in the OXA-23-positive *A. baumannii* isolate 229437 and *Acinetobacter* genospecies 3 isolates 36277 and 27616. The quantitation procedure was carried out in triplicates for each of the three isolates using the protocol as mentioned in section 4.2.1.6. Relative quantification of bla_{OXA-23} expression under different growth conditions or between different isolates was calculated using the $\Delta\Delta C_T$ method as mentioned in section 4.2.1.6 as well.

4.3 Results

4.3.1 Investigation of efflux mechanisms of carbapenem resistance

4.3.1.1 Characterisation of RND efflux genes in *Acinetobacter* spp.

Genes for multidrug transporter component of known RND efflux pumps in *Acinetobacter* spp. were characterised for 118 isolates (inclusive of the reference *A. baumannii* strain ATCC 19606). The results are shown in Table 4-2. The majority of *A. baumannii* isolates (23/28) had both *adeB* and *adeJ* genes. One isolate had *adeB* only (isolate 233651), three isolates had *adeJ* only (isolates 26756, 89848 and 14495) and one isolate had neither *adeB* nor *adeJ* (isolate 102450). The majority of

Table 4-2. Distribution of RND efflux genes in *Acinetobacter* spp. (n = 117 + ATCC 19606).

***Acinetobacter* species (No. of isolates)**	**No. of isolates with RND-type multidrug transporter efflux genes**						
	adeB* + *adeJ	***adeB* only**	***adeJ* only**	***adeY* + *adeE***	***adeY* only**	***adeE* only**	**None of the four *ade* genes**
A. baumannii (28)	23	1	3	0	0	0	1
Acinetobacter genospecies 3 (45)	0	0	0	35	6	0	4
A. calcoaceticus (6)	0	0	0	0	2	4	0
A. haemolyticus (1)	0	0	0	0	1	0	0
Other *Acinetobacter* spp. (38)	0	0	0	0	0	0	38

Acinetobacter genospecies 3 isolates (35/45) had both *adeE* and *adeY* genes. Six isolates had *adeY* only (isolates 14331, 12253, 14027, 25904, 16198 and 13320) and four isolates had neither *adeY* nor *adeE* (isolates 86096, 82991, 35168 and 93743). *A. calcoaceticus* isolates had either *adeE* or *adeY* gene but not both. Apart from isolates of the *A. calcoaceticus-baumannii* complex, the only isolate which was positive for one of the four *ade* genes was the *A. haemolyticus* isolate 89182 (*adeY*).

4.3.1.2 Carbapenem susceptibility testing and EPI testing with PAβN

Meropenem and imipenem MIC values of the eight isolates were measured in the absence and presence of PAβN, respectively. The MIC results are shown in Table 4-3. In the presence of 100 μg/mL of PAβN, there were four-fold to eight-fold reductions in meropenem MICs for all the isolates except *A. baumannii* isolate 100983, which had a two-fold reduction in meropenem MIC in the presence of PAβN. In contrast, there were only modest changes in imipenem MICs when exposed to PAβN. The isolates demonstrated no changes in imipenem susceptibility or only produced two-fold reductions in MICs in the presence of 100 μg/mL of PAβN. Meropenem MICs obtained with the microbroth dilution method also correlated closely with those obtained with the Etest method. The meropenem MICs using broth microdilution method were as follows:

A. baumannii	MIC (μg/mL)
ATCC 19606	2
100983	0.5
11648	4
229437	128

Table 4-3. Meropenem and imipenem MICs of *Acinetobacter* isolates in the absence and presence of the efflux pump inhibitor PAβN using Etest method.

Acinetobacter species	Isolate	Meropenem MIC (μg/mL)		Imipenem MIC (μg/mL)	
		No PAβN	PAβN 100 μg/mL	No PAβN	PAβN 100 μg/mL
Acinetobacter baumannii	ATCC 19606	2	0.25	1	0.5
	100983	0.5	0.25	0.25	0.25
	11648	4	0.5	4	2
	229437	≥ 32 (128)[a]	16	≥ 32	≥ 32
Acinetobacter genospecies 3	106770	0.5	0.125	0.5	0.25
	27458	1	0.125	0.5	0.25
	27616	≥ 32 (32)[a]	4	≥ 32	≥ 32
	36277	≥ 32 (64)[a]	8	≥ 32	≥ 32

[a]MICs obtained using CLSI microbroth dilution method (CLSI, 2009).

Acinetobacter genosp. 3	MIC (μg/mL)
106770	0.5
27458	1
27616	32
36277	64

4.3.1.3 qRT-PCR results for the efflux gene expression in *Acinetobacter* strains

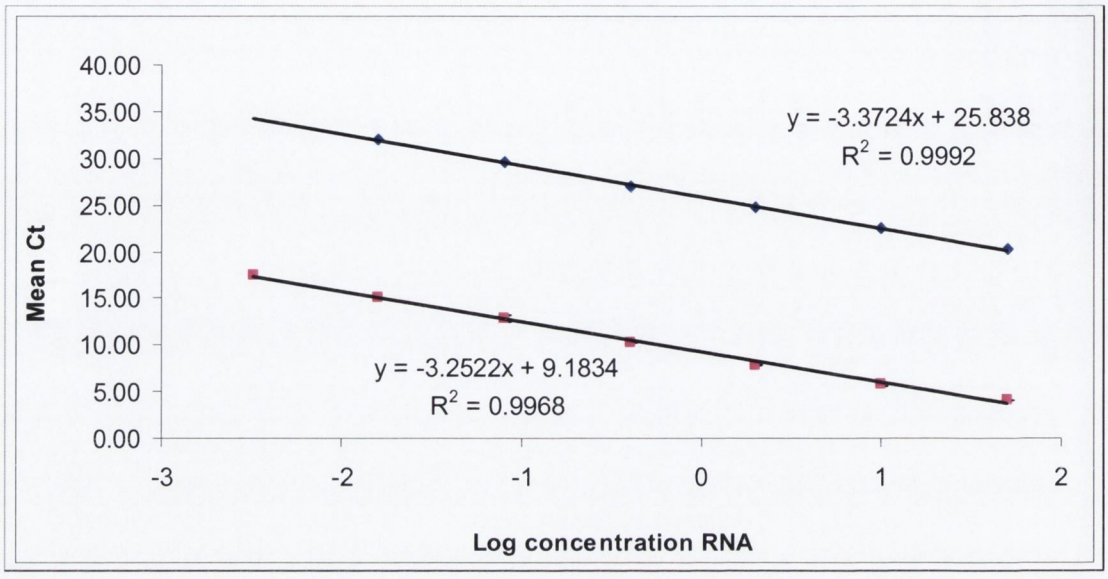
The results of the isolates' growth kinetics in liquid broth culture were shown in Appendix 2. The inocula at which the isolates were in the mid-logarithmic phase of growth were all between OD_{600} 0.6 and 0.7. The *A. baumannii* isolates reached mid-logarithmic phase of growth earlier than the *Acinetobacter* genospecies 3 isolates. Among the *A. baumannii* isolates, 229437 was the first to reach the mid-logarithmic phase. Among the *Acinetobacter* genospecies 3 isolates, 36277 was the last to reach the mid-logarithmic phase. At 24 hours, *Acinetobacter* genospecies 3 isolates reached higher inocula (OD_{600} of 4.52 to 5.78) in liquid culture compared to the *A. baumannii* isolates (OD_{600} of 2.68 to 4.72).

In the qRT-PCR validation experiments, the housekeeping gene *16S rRNA*, the four *ade* genes and bla_{OXA-23} all achieved satisfactory slope values of between -3.25 and -3.45 when the mean C_T values were plotted against the log RNA concentrations. The amplification efficiencies between the respective *ade* genes and the housekeeping

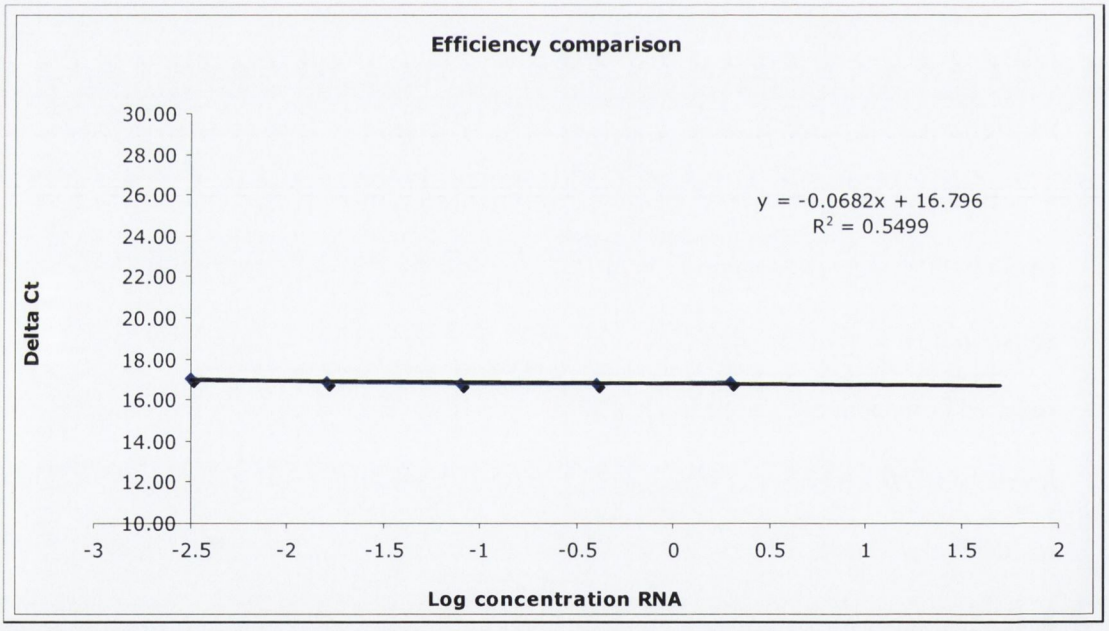
gene *16S rRNA* were also comparable, achieving slope values of between 0.06 and 0.08 when the ΔC_T values were plotted against the log RNA concentrations. As an example, the validation results for *adeY* gene are shown in Figure 4-1.

The qRT-PCR results of the four *ade* genes are shown in Appendix 3 and Tables 4-4 to 4-7. Triplicate C_T values of qRT-PCR results of the *16S rRNA* gene and the *ade* genes as well as the respective ΔC_T values are shown in Appendix 3. The average ΔC_T values for the respective *ade* genes are shown in Table 4-4. Relative quantitation of gene expression was calculated using the method as stated in section 4.2.1.6.

Using the carbapenem-susceptible *A. baumannii* ATCC 19606 as the reference strain, relative baseline expression levels of *adeB* and *adeJ* (ie. in antibiotic-free liquid medium) for the other three *A. baumannii* isolates were calculated and shown in Table 4-5. Baseline expression level of *adeB* was highest in the *A. baumannii* ATCC 19606 strain, while *adeB* expression in another carbapenem-susceptible strain 100983 was substantially lower (1%) than that of the ATCC 19606 strain. The isolates with reduced carbapenem susceptibility (229437 and 11648, respectively) had intermediate levels of *adeB* expression (37% and 33% of the expression level by ATCC 19606, respectively). The results suggested that there was no direct correlation between baseline *adeB* expression level and carbapenem susceptibility. There was less variation in the expression of *adeJ* among the *A. baumannii* isolates (expression levels ranging from 44% to 185% of *adeJ* expression by ATCC 19606, respectively). As with *adeB* expression, the baseline expression of *adeJ* did not correlate directly to the carbapenem susceptibility of the isolates.



A. Standard curves for qRT-PCR of *adeY* (upper) and *16SrRNA* (lower).



B. Comparison (ΔC_T) of amplification efficiencies for *adeY* and *16S rRNA*.

Figure 4-1. Validation experiments for qRT-PCR of *adeY* gene of *Acinetobacter* genospecies 3 isolate 106770.

Table 4-4. Average ΔC_T values of qRT-PCR results of *Acinetobacter* isolates for expression of *ade* and bla_{OXA-23} genes.

Gene	Species	Isolate	Average ΔC_T	
			Absence of meropenem	Presence of meropenem
bla_{OXA-23}	*Acinetobacter* genosp. 3	27616	13.27	13.28
		36277	12.61	12.57
	A. baumannii	229437	12.41	12.4
adeB	*A. baumannii*	ATCC 19606	22.94	22.15
		100983	29.89	29.34
		11648	24.52	23.95
		229437	24.39	23.78
adeJ	*A. baumannii*	ATCC 19606	14.34	13.7
		100983	13.45	13.33
		11648	14.61	14.48
		229437	15.53	15.23
adeE	*Acinetobacter* genosp. 3	106770	23.03	22.61
		27458	22.63	22.39
		27616	22.62	22.39
		36277	21.49	21.17
adeY	*Acinetobacter* genosp. 3	106770	16.97	16.98
		27458	16.96	16.85
		27616	17.04	16.83
		36277	16.62	16.44

Table 4-5. Baseline *adeB* and *adeJ* expression (in antibiotic-free medium) of *A. baumannii* isolates relative to ATCC 19606 strain.

***A. baumannii* isolate**	**Carbapenem susceptibility pattern**	***adeB* expression**	***adeJ* expression**
ATCC 19606	Mpm-S; Ipm-S; Dpm-S	1.00	1.00
100983	Mpm-S; Ipm-S; Dpm-S	0.01	1.85
11648	Mpm-S; Ipm-S; Dpm-I	0.33	0.83
229437	Mpm-R; Ipm-R; Dpm-R	0.37	0.44

Mpm, meropenem; Ipm, imipenem; Dpm, doripenem; S, susceptible; I, intermediate; R, resistant.

In *Acinetobacter* genospecies 3, relative baseline expression levels of *adeE* and *adeY* (ie. in antibiotic-free liquid medium) for the four isolates were calculated using the carbapenem-susceptible isolate 106770 as the reference strain and are shown in Table 4-6. Compared to isolate 106770, the baseline expression level of *adeE* was significantly higher in the carbapenem-resistant isolate 36277 (almost three times that of 106770). Isolates 27458 (carbapenem-susceptible) and 27616 (carbapenem-resistant), which were clonally indistinguishable, had nearly identical *adeE* expression levels, which were 32% and 33% higher than that of isolate 106770, respectively. No clear correlation between *adeE* baseline expression level and carbapenem susceptibility could be deduced from the results, although this may also be due to the small number of isolates analysed. Although *adeE* expression in isolate 36277 was significantly higher than isolate 106770, the expression levels in isolates 27458 and 27616 were almost identical, suggesting that there were intrinsic differences in *adeE* expression among the strains irrespective of carbapenem susceptibility. The range of variation in *adeY* expression was less among the *Acinetobacter* genospecies 3 isolates, with baseline *adeY* expression levels ranging from 95% (isolate 27616) to 127% (isolate 36277) of that in isolate 106770. This suggested that the *adeY* expression among different isolates was relatively constant.

Gene expression in the presence of sub-inhibitory concentrations of meropenem was calculated in relation to the isolates' baseline expression and the results are shown in Table 4-7. In the four *A. baumannii* isolates, expression levels of *adeB* increased by 46% to 73% following exposure to meropenem. On the other hand, with the exception of ATCC 19606, expression levels of *adeJ* only increased by 9% to 23%. In ATCC

Table 4-6. Baseline *adeE* and *adeY* expression (in antibiotic-free medium) of *Acinetobacter* genospecies 3 isolates relative to isolate 106770.

Acinetobacter genosp. 3 isolate	Carbapenem susceptibility pattern	*adeE* expression	*adeY* expression
106770	Mpm-S; Ipm-S; Dpm-S	1.00	1.00
27458	Mpm-S; Ipm-S; Dpm-S	1.32	1.01
27616	Mpm-R; Ipm-R; Dpm-R	1.33	0.95
36277	Mpm-R; Ipm-R; Dpm-R	2.91	1.27

Mpm, meropenem; Ipm, imipenem; Dpm, doripenem; S, susceptible; I, intermediate; R, resistant.

Table 4-7. Relative *ade* gene expression of isolates in the presence of meropenem compared to baseline expression.

***Acinetobacter* species**	**Isolate**	**Gene expression relative to baseline expression**	
		adeB	***adeJ***
A. baumannii	ATCC 19606	1.73	1.56
	100983	1.46	1.09
	11648	1.48	1.09
	229437	1.53	1.23
***Acinetobacter* species**	**Isolate**	**Gene expression relative to baseline expression**	
		adeE	***adeY***
Acinetobacter genospecies 3	106770	1.34	0.99
	27458	1.18	1.08
	27616	1.17	1.15
	36277	1.25	1.13

19606, *adeJ* expression increased by 56% the presence of meropenem. The results suggested that upregulation of *adeB* expression occurred in the presence of meropenem for the four *A. baumannii* isolates, which in turn suggested that meropenem was a substrate of the efflux pump AdeABC. It was unclear if meropenem was a significant substrate of the AdeIJK efflux pump, as there was greater variability in the increase in *adeJ* expression (9% to 56%) following exposure to meropenem.

In the *Acinetobacter* genospecies 3 isolates, there were increases of 17% to 34% in *adeE* expression level in the presence of meropenem. On the other hand, *adeY* expression levels demonstrated less variability in the presence of meropenem, with expression levels ranging from a decrease of 1% (isolate 106770) to an increase of 15% (isolate 27616) (see Table 4-7). Based on *adeE* expression results, meropenem may be a substrate of the efflux pump AdeDE in *Acinetobacter* genospecies 3. Based on *adeY* expression results, it was unclear whether meropenem was a substrate of the AdeXYZ efflux pump.

In *A. baumannii*, in comparing the baseline expression of *adeJ* and *adeB*, expression levels of *adeJ* (ΔC_T values of 13.45 to 15.53) were substantially higher than those of *adeB* (ΔC_T values of 22.94 to 29.89) (see Table 4-4). The results raised the possibility that *adeJ* expression in *A. baumannii* was constitutive rather than inducible. Similarly in *Acinetobacter* genospecies 3, in comparing the baseline expression of *adeY* and *adeE*, expression levels of *adeY* (ΔC_T values of 16.62 to 17.04) were also substantially

higher than those of *adeE* (ΔC_T values of 21.49 to 23.03) (see Table 4-4). Therefore, the results also raised the possibility that *adeY* expression in *Acinetobacter* genospecies 3 was constitutive rather than inducible. The implication was that expression levels of such efflux genes following exposure to meropenem might not change substantially even if meropenem was indeed a substrate of such efflux pumps.

4.3.2 OMP profiles and *carO* gene characterisation

OMP profiles following 12% SDS-PAGE for the *Acinetobacter* isolates were shown in Figure 4-2.

In *A. baumannii*, expression of a 29 kDa OMP consistent with CarO was absent in isolate 229437, while isolate 11648 showed reduced expression of the same OMP relative to ATCC 19606. No other differences in the *A. baumannii* OMP profiles were observed. PCR and sequencing of *carO* gene were performed on the four *A. baumannii* isolates. All four isolates produced amplicons of 750 bp with PCR consistent with the size of the entire *carO* gene. Following sequencing, no mutations or insertion sequences such as IS*Aba1* and IS*Aba825* were found within the *carO* gene of the four isolates (reference GenBank accession number EF646275).

No significant differences in OMP profiles of the four *Acinetobacter* genospecies 3 isolates were identified (see Figure 4-2).

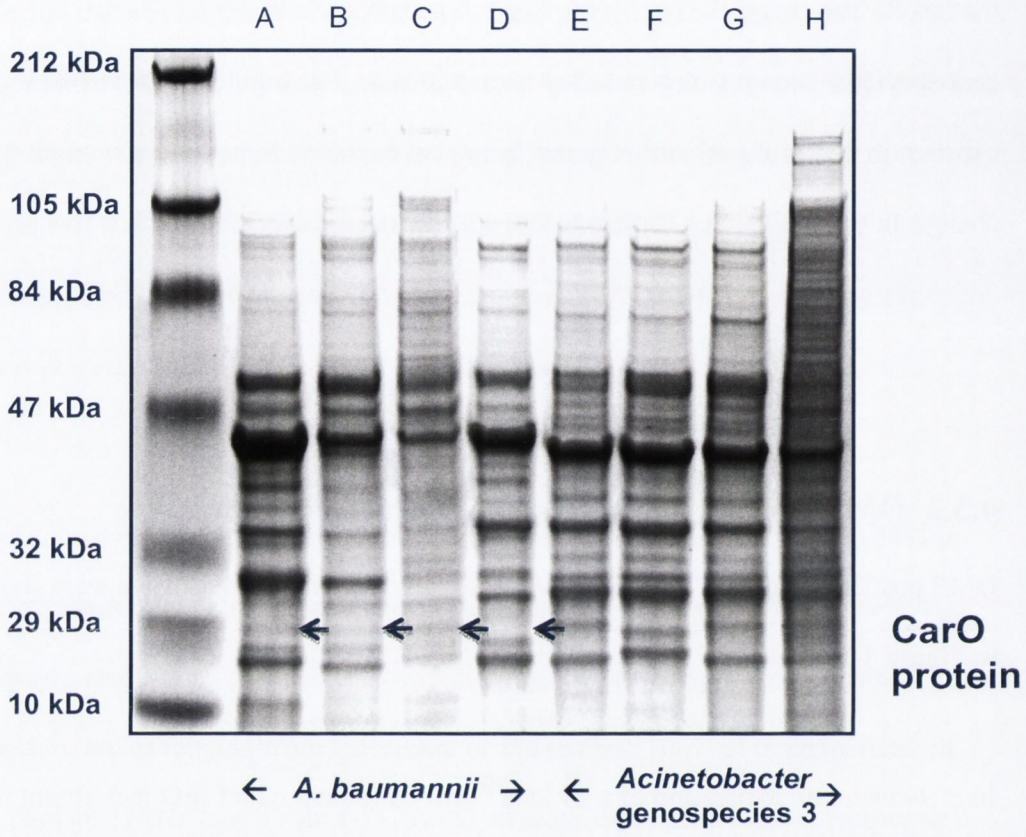


Figure 4-2. Outer membrane protein profiles of *Acinetobacter* isolates following SDS-PAGE.

(A, ATCC 19606; B, 11648; C, 100983; D, 299437; E, 27458; F, 106770; G, 27616; H, 36277) The arrows indicate the presence or absence in *A. baumannii* isolates (A-D) of the 29 kDa protein corresponding to CarO protein.

4.3.3 qRT-PCR results for *bla*$_{OXA-23}$ expression in carbapenem-resistant *Acinetobacter* isolates

The contribution of *bla*$_{OXA-23}$ expression to carbapenem resistance was investigated. Triplicate C_T and ΔC_T values of qRT-PCR assays of *bla*$_{OXA-23}$ expression in the three carbapenem-resistant isolates (229437, 27616 and 36277) are shown in Appendix 3. Average ΔC_T values are also shown in Table 4-4. Using *Acinetobacter* genospecies 3 isolate 27616 as the reference strain, relative baseline expression levels of *bla*$_{OXA-23}$ in the three isolates are shown in Table 4-8. Baseline *bla*$_{OXA-23}$ expression levels of *A. baumannii* isolate 229437 and *Acinetobacter* genospecies 3 isolate 36277 were 82% and 58% higher than that of 27616, respectively. The differences in *bla*$_{OXA-23}$ expression may have a significant impact on the level of carbapenem resistance in the isolates, as indicated by the MIC values. Changes in *bla*$_{OXA-23}$ expression in the three isolates following exposure to meropenem are shown in Table 4-9. There were no significant changes in the expression of *bla*$_{OXA-23}$ in the three isolates following exposure to meropenem. Post-exposure expression levels ranged from 1% lower to 3% higher than the baseline levels. The results suggested that the *bla*$_{OXA-23}$ was constitutively expressed in the three isolates.

Table 4-8. Baseline *bla*$_{OXA-23}$ expression (in antibiotic-free medium) of carbapenem-resistant *Acinetobacter* isolates relative to *Acinetobacter* genospecies 3 isolate 27616.

Isolate	Species	Relative *bla*$_{OXA-23}$ expression
27616	*Acinetobacter* genosp. 3	1.00
36277	*Acinetobacter* genosp. 3	1.58
229437	*A. baumannii*	1.82

Table 4-9. Relative *bla*$_{OXA-23}$ expression of carbapenem-resistant *Acinetobacter* isolates in the presence of meropenem compared to baseline expression.

Isolate	Species	Relative *bla*$_{OXA-23}$ expression
27616	*Acinetobacter* genosp. 3	0.99
36277	*Acinetobacter* genosp. 3	1.03
229437	*A. baumannii*	1.01

4.4 Discussion

Four RND-type efflux pumps have been characterised in *Acinetobacter* spp. over the last decade (Chau *et al.*, 2004; Chu *et al.*, 2006; Magnet *et al.*, 2001; Damier-Piolle *et al.*, 2008). The present study showed that these efflux pumps were found mainly in species within the *Acinetobacter calcoaceticus-baumannii* complex (Acbc). Both *adeB* and *adeJ* were found exclusively in *A. baumannii* isolates, implying that the corresponding efflux pumps, AdeABC and AdeIJK, respectively, were found in this species only. Consistent with the findings of others, the majority of *A. baumannii* isolates in this study possessed *adeB* (86%) and *adeJ* (93%), respectively, with 82% of the isolates possessing both genes (Chu *et al.*, 2006; Damier-Piolle *et al.*, 2008). The *adeE* and *adeY* genes were found predominantly in *Acinetobacter* genospecies 3 and *A. calcoaceticus* isolates. The majority (91%) of *Acinetobacter* genospecies 3 isolates were positive for *adeY* while 78% of isolates possessed *adeE*. All isolates possessing *adeE* were also positive for *adeY* (see Table 4-2). *A. calcoaceticus* isolates possessed either *adeE* or *adeY*, while *adeY* was also found in the *A. haemolyticus* isolate 89182. A previous study also found *adeE* and *adeY* in the majority of *Acinetobacter* genospecies 3 isolates and in a minority of *Acinetobacter* genospecies 13TU and genospecies 17 (Chu *et al.*, 2006). Hence, the results in the study were consistent with the observation that AdeDE and AdeXYZ efflux pumps are found predominantly in Acbc isolates other than *A. baumannii*, and occasionally in non-Acbc isolates as well.

In the current study, PAβN was investigated as an agent for demonstrating efflux-related mechanisms in carbapenem resistance. PAβN belongs to a large family of peptidomimetics that exhibit EPI properties. Analysis of the inhibitory mechanism has demonstrated that PAβN is a competitive inhibitor of substrate of efflux pumps (Lomovskaya & Bostian, 2006). In this study, PAβN resulted in eight-fold reductions in meropenem MICs for most isolates, but did not result in significant reductions in imipenem MIC values. The PAβN results demonstrated the summative effect of efflux mechanisms on carbapenem resistance, thereby providing strong evidence that they contributed significantly to meropenem resistance in *Acinetobacter* spp. Furthermore, the findings in this study also demonstrated the suitability of PAβN as an EPI for the investigation of meropenem resistance. The most likely targets of PAβN in the *Acinetobacter* isolates were the RND-type efflux pumps, since studies suggested that AdeABC efflux pump had a role in meropenem resistance (Huang *et al.*, 2008; Wong *et al.*, 2009). Other studies also provided evidence supporting PAβN as an inhibitor of RND-type efflux pumps in various GNBs. PAβN restored susceptibility in *P. aeruginosa* and *E. coli* to various classes of antibiotics including quinolones, macrolides and chloramphenicol (Lomovskaya & Bostian, 2006; Lomovskaya *et al.*, 2001; Renau *et al.*, 1999). In addition, the co-crystallization of the RND multidrug transporter AcrB with various substrates provided further supportive evidence that PAβN bind to the same affinity pocket inside the AcrB cavity of *E. coli* and MexB cavity of *P. aeruginosa* used by the antibiotics (Mahamoud *et al.*, 2007; Yu *et al.*, 2003; Yu *et al.*, 2005). PAβN has also been shown to be active in *A. baumannii*, increasing susceptibility of the organism to antibiotics such as rifampicin, clarithromycin, cefepime, moxifloxacin tetracycline and linezolid (Pannek et al.,

2006). Furthermore, PAβN restored the susceptibility of tigecycline in *A. baumannii* with AdeABC overexpression, supporting the view that PAβN is an inhibitor of RND-type efflux pumps in *Acinetobacter* spp. (Peleg *et al.*, 2007a). The lack of effect by PAβN on imipenem susceptibility in this study, along with results of other studies demonstrating no significant change in imipenem susceptibility following inactivation of AdeABC and AdeIJK efflux pumps, respectively, suggest that imipenem is not a significant substrate of efflux pumps in *Acinetobacter* spp. (Damier-Piolle *et al.*, 2008; Wong *et al.*, 2009). This is probably not too surprising considering that imipenem susceptibility in *P. aeruginosa* is also not significantly affected by efflux mechanisms (Köhler *et al.*, 1999; Quale *et al.*, 2006).

While the PAβN results in this study, along with data from other studies mentioned above, supported a significant role of efflux mechanisms (in particular, RND-type efflux pumps) in meropenem resistance in *Acinetobacter* spp., it was more challenging to determine which RND-type efflux pump was mainly responsible for contributing to meropenem resistance. The interpretation of qRT-PCR results for efflux gene expression was confounded by the presence of additional mechanisms (such as OXA-23 carbapenemase and loss of OMP) in some isolates, while the potentially constitutive expression of some efflux genes (see section 4.3.1.3) confounded the interpretation of changes (or the lack of them) in expression of these genes following meropenem exposure. Nevertheless, the results from the qRT-PCR assays provided supportive evidence for some conclusions derived from other studies.

In this study, the results of qRT-PCR assays of *adeB* gene expression were suggestive of a significant role of the efflux pump AdeABC in meropenem resistance in *A. baumannii*. Based on the *adeJ* qRT-PCR results, the role of the efflux pump AdeIJK was less certain. The significant role of AdeABC in meropenem resistance was most strongly supported by the results showing changes in *adeB* expression in the presence of meropenem, where all four isolates showed substantial increases in gene expression of 46% to 73% compared to baseline levels (see Table 4-7). Although baseline *adeB* expression did not appear to have a direct correlation with the overall meropenem phenotype for the four *A. baumannii* isolates, the interpretation was confounded by the presence of additional mechanisms of resistance in the two carbapenem-non-susceptible isolates, 229437 and 11648. In isolate 229437, there was evidence showing the overexpression of $bla_{\text{OXA-23}}$ and $bla_{\text{ADC-25}}$ (see sections 3.3.4 and 3.3.3, respectively), while in isolate 11648, there was overexpression of $bla_{\text{ADC-29}}$ (see section 3.3.3). While OXA carbapenemases have well-established roles in carbapenem resistance, increased expression of ADC was also associated with carbapenem resistance in some *Acinetobacter* isolates (Quale *et al.*, 2003). Both isolates in this study also had reduced expression of OMP CarO (see discussion below). In comparing the remaining isolates of ATCC 19606 and 100983, there was a substantial difference in the baseline expression of *adeB* (see Table 4-5). The baseline expression of *adeB* in ATCC 19606 was 100 times higher than in 100983. The difference in *adeB* expression between the two isolates was even greater following exposure to meropenem (see Table 4-7). As the meropenem MICs of ATCC 19606 and 100983 were 2 μg/mL and 0.5 μg/mL, respectively, the four-fold difference in MIC values may be the result of differential *adeB* expression, especially since both

isolates lacked carbapenemases and had no significant differences in OMP profiles. This view is consistent with the observations in a study showing higher baseline *adeB* expression in carbapenem-resistant *A. baumannii* isolates compared to carbapenem-susceptible isolates in the absence of additional resistance mechanisms (Huang *et al.*, 2008). Furthermore, a recent study demonstrated four-fold reductions in meropenem MICs in carbapenem-resistant *A. baumannii* following inactivation of *adeB* (Wong *et al.*, 2009).

The correlation of *adeJ* expression to carbapenem susceptibility in *A. baumannii* was unclear. Three of the four *A. baumannii* isolates showed only small changes (increases of 9% to 23%) in *adeJ* expression following exposure to meropenem (see Table 4-7). However, the above results did not necessarily imply that the AdeIJK efflux pump was not involved in carbapenem resistance. The expression of *adeJ* may be constitutive since the baseline expression levels of *adeJ* were at least 170 times higher than those of *adeB* (see Table 4-4 and section 4.3.1.3). Therefore, the role of AdeIJK in meropenem susceptibility may be clarified definitively following inactivation of *adeJ* using methodologies as described by Damier-Piolle *et al.* (Damier-Piolle *et al.*, 2008).

In *Acinetobacter* genospecies 3, the results of qRT-PCR for *adeE* expression suggested a potentially significant role for the efflux pump AdeDE in meropenem resistance. All four isolates showed increases of 17% to 34% in *adeE* expression following the exposure to meropenem, suggesting possible induction of expression by this antibiotic. The interpretation of *adeE* expression results was confounded by the

presence of bla_{OXA-23} in the carbapenem-resistant isolates of 36277 and 27616. However, in comparing the carbapenem-susceptible isolates of 106770 and 27458, the baseline *adeE* expression in 27458 was 32% higher than in 106770, although the *adeE* expression in 27458 was only 16% higher than in 106770 in the presence of meropenem. The relatively small differences in *adeE* expression could have accounted for the small difference in meropenem MIC values of the two isolates (1 μg/mL in 27458 and 0.5 μg/mL in 106770). Thus, the above results provided further evidence in support of the potential role of the AdeDE efflux pump in meropenem resistance in *Acinetobacter* genospecies 3. In further support of AdeDE's role in carbapenem susceptibility, a study demonstrated that the inactivation of *adeE* led to further reductions in meropenem MIC values in carbapenem-susceptible *Acinetobacter* genospecies 3 isolates (Chau *et al.*, 2004). In comparing the carbapenem-resistant isolates of 27616 and 36277, both of which expressed OXA-23, the expression of *adeE* in 36277 was more than 100% higher than that of 27616 (see Tables 4-6 and 4-7). While it was tempting to surmise that increased *adeE* expression was responsible for the higher meropenem MIC in 36277 (64 μg/mL) compared to that in 27616 (32 μg/mL), such a conclusion was confounded by the finding the level of bla_{OXA-23} expression in 36277 was 58% higher than in 27616 as well (see Table 4-8). Therefore, the impact of increased *adeE* expression can only be accurately determined following inactivation of bla_{OXA-23} in both isolates. Nevertheless, the above results suggested a potential role of AdeDE in determining meropenem susceptibility in *Acinetobacter* genospecies 3.

Based on qRT-PCR results of *adeY* expression, the role of AdeXYZ in meropenem resistance in *Acinetobacter* genospecies 3 was unproven. The baseline expression levels of *adeY* in the four *Acinetobacter* genospecies 3 isolates were very similar (see Table 4-6). Following exposure to meropenem, there were only small changes in *adeY* expression in the four isolates (99% to 115% of baseline expression; see Table 4-7). The baseline *adeY* expression was significantly higher (at least 20 times higher) than that of *adeE* (see Tables 4-4 and 4-6) in the isolates, raising the possibility of constitutive expression of *adeY* in *Acinetobacter* genospecies 3. Hence, as with other efflux genes, the inactivation of *adeY* can probably elucidate the role of efflux pump AdeXYZ in influencing meropenem susceptibility in *Acinetobacter* genospecies 3.

In this study, the role of porin loss in carbapenem resistance in *A. baumannii* was also difficult to prove. The interpretation of OMP results was confounded by the concomitant presence of β-lactamases in isolates with reduced porin expression (isolates 229437 and 11648). Moreover, there were variations in expression levels of efflux pump genes among the four isolates. The complexity of the relative contributions by the different potential mechanisms of resistance was illustrated by the comparison of *A. baumannii* isolates 11648 and ATCC 19606. While isolate 11648 had reduced CarO expression and overexpression of $bla_{\text{ADC-29}}$, ATCC 19606 had a higher level of *adeB* expression. The assessment of the role of CarO in carbapenem susceptibility may only be achieved following the inactivation of other mechanisms such as efflux pumps and β-lactamases. Nonetheless, the association of reduced CarO expression with reduced carbapenem susceptibility in isolates suggested the possibility that this porin may have a role in influencing carbapenem

susceptibility. However, others have suggested that reduced porin expression in *A. baumannii* may only play a minor role in carbapenem resistance since, in the presence of carbapenemases, the loss of CarO was not associated with significant differences in carbapenem MICs (Lu *et al.*, 2009). No mutations or insertion sequences were found on sequence analysis of the *carO* genes of isolates 229437 and 11648, respectively. The results suggested that regulatory mechanisms other than insertional inactivation by insertion sequences were also involved in the regulation of *carO* expression (Mussi *et al.*, 2005; Poirel & Nordmann, 2006b). Such a view was supported by the finding by Lu *et al.* that only 5.4% of the *A. baumannii* isolates with reduced *carO* expression had insertional inactivation by IS*Aba1* of the gene (Lu *et al.*, 2009). In *Acinetobacter* genospecies 3, altered permeability through reduced OMP expression did not appear to play a significant role in carbapenem resistance as no significant differences in OMP profiles were observed for the four isolates.

The study also emphasised the importance of the OXA carbapenemases in conferring the carbapenem-resistant phenotype. The impact of OXA carbapenemases was clearly illustrated when the comparison between carbapenem-resistant isolates (carrying $bla_{\text{OXA-23}}$) and carbapenem-susceptible isolates was made. In comparing the two *Acinetobacter* genospecies 3 isolates 27458 (carbapenem-susceptible) and 27616 (carbapenem-resistant), which had indistinguishable PFGE typing patterns, *adeE* and *adeY* expression as well as OMP profiles were all virtually identical in the two isolates. Therefore, thirty two-fold difference in meropenem MIC values (1 μg/mL and 32 μg/mL in 27458 and 27616, respectively) was most probably attributable to OXA-23 expression by isolate 27616. When comparing *A. baumannii* isolates 229437

and 11648, both isolates had bla_{ADC} overexpression and similar levels of *adeB* expression. While isolate 11648 had reduced CarO expression, isolate 229437 had no detectable expression of CarO. Although there were probably small differences in the levels of CarO expression, the thirty two-fold difference in meropenem MIC values (128 μg/mL and 4 μg/mL in 229437 and 11648, respectively) was most likely due mainly to OXA-23 expression in isolate 229437. Although not performed in the study, the exact role of OXA-23 can be more definitively ascertained by the inactivation of bla_{OXA-23} in carbapenem-resistant isolates. There were potentially significant differences in the level of bla_{OXA-23} expression on comparing the three OXA-23-positive isolates. Expression levels in *A. baumannii* isolate 229437 and *Acinetobacter* genospecies 3 isolate 36277 were 82% and 58% higher than that in isolate 27616, respectively (see Table 4-7). As mentioned above, the higher meropenem MIC of *Acinetobacter* genospecies 3 isolate 36277 (64 μg/mL) compared with that of isolate 27616 (32 μg/mL) may be due to a higher (58%) level of bla_{OXA-23} expression (see Table 4-8) and/or a higher (> 100%) level of *adeE* expression (see Table 4-6) in the former isolate. As all three isolates had IS*Aba1* as a promoter upstream of bla_{OXA-23}, the differences in expression level may be the result of different copy numbers of the plasmids or of the bla_{OXA-23} gene, since the level of carbapenem resistance was shown to be influenced by the copy number of bla_{OXA-58} gene in *A. baumannii* isolates (Bertini *et al.*, 2007). Consistent with the findings of other researchers that IS*Aba1* resulted in constitutive upregulation of bla_{OXA} genes, the three OXA-23-positive isolates did not demonstrate any significant changes in the levels of bla_{OXA-23} expression following exposure to meropenem as compared to the baseline (antibiotic-

free) levels of expression (see Table 4-9) (Corvec *et al.*, 2007; Poirel & Nordmann, 2006a; Segal *et al.*, 2003).

In both species, multiple mechanisms were most likely involved in carbapenem resistance. For example, OXA-23 expression, efflux mechanism(s), loss of CarO expression and overexpression of ADC were all probably involved in carbapenem resistance in *A. baumannii* isolate 229437. It is difficult to quantify the relative contribution of each mechanism to the development of meropenem resistance, as such mechanisms may also interact synergistically to produce a highly resistant phenotype (Bou *et al.*, 2000; Fernández-Cuenca *et al.*, 2003; Quale *et al.*, 2003). Nevertheless, the qRT-PCR and PAβN results of *ade* and bla_{OXA-23} expression in the study would suggest that in *A. baumannii* and *Acinetobacter* genospecies 3, carbapenemases played a significant role in carbapenem resistance, while efflux mechanisms such as AdeABC and AdeDE also contributed substantially to the resistant phenotype. The role of reduced expression of CarO in influencing the carbapenem susceptibility in *A. baumannii* remained unproven.

In addition to carbapenems, the results from efflux pump studies may also explain the observed differences in cefotaxime and ceftazidime MICs of *Acinetobacter* spp. Cefotaxime MICs were generally higher than ceftazidime MICs (see section 2.3.2.2 and Tables 2-7 and 2-8). Cefotaxime was shown to be a substrate of AdeABC and AdeIJK pumps while ceftazidime was not to be a substrate of AdeABC (Damier-Piolle *et al.*, 2008; Magnet *et al.*, 2001). Therefore, efflux mechanisms may have a significant contribution for the higher cefotaxime MIC values compared to

ceftazidime MIC values. The quantitative results in the study may also provide some supportive evidence for the role of efflux pumps in influencing the cephalosporin susceptibility profiles. Since AdeXYZ of *Acinetobacter* genospecies 3 has high amino acid identity (>97%) to AdeIJK of *A. baumannii* (Damier-Piolle *et al.*, 2008), it is reasonable to assume that their substrate specificity profiles are similar. Therefore, it is likely that cefotaxime is also a substrate of AdeXYZ. Ceftazidime, but not cefotaxime, is a substrate of the AdeDE efflux pump of *Acinetobacter* genospecies 3 (Chau *et al.*, 2004). Since the quantitative results showed that *adeJ* was more highly expressed than *adeE* in *Acinetobacter* genospecies 3 isolates, it is plausible that differential activities of the two efflux pumps within the same isolate may have resulted in differences in susceptibility to the respective cephalosporins.

The growth kinetics results also revealed interesting growth characteristics of *A. baumannii* and *Acinetobacter* genospecies 3, which may represent differences in virulence and pathogenicity in clinical infections for both species. *A. baumannii* isolates reached the mid-logarithmic phase of growth earlier than *Acinetobacter* genospecies 3 isolates; while on the other hand, *Acinetobacter* genospecies 3 achieved higher inocula than *A. baumannii* at 24 hours. The growth characteristic of *A. baumannii* isolate 229437 was also indicative of its fitness. In spite of the burden of exhibiting the MDR phenotype, it demonstrated a faster rate of growth than the other three *A. baumannii* isolates. The fitness of MDR strains like 229437 and 11648 is probably an important factor explaining why MDR *A. baumannii* strains are so prevalent in hospitals and outbreaks worldwide. In comparison, the MDR phenotype in *Acinetobacter* genospecies 3 (such as in strain 36277) may be at the expense of its

fitness of growth. However, it may also reflect inter-strain differences in growth characteristics, as another MDR strain 27616 did not demonstrate any significant differences in growth rate compared to the more susceptible strains of 106770 and 27458. Comparison of isolates 27616 and 27458, which were indistinguishable by PFGE, the results suggested that carbapenem resistance *per se* did not affect the fitness of growth of the strain.

There are limitations in the study. Consequently, some issues in the chapter remain unresolved. The most pertinent unresolved issue is the relative quantitation of the contributions of the various mechanisms to meropenem resistance in *A. baumannii* and *Acinetobacter* genospecies 3. While the study has produced data supporting the roles of bla_{OXA-23} and *adeB* in meropenem resistance, the roles of efflux pumps such as AdeIJK and AdeXYZ as well as OMP CarO remained uncertain as a result of the presence of multiple mechanisms in the same isolates. As discussed already, the role of the individual mechanism as well as the synergy between different mechanisms can best be assessed following inactivation (knockout) of one or more of the respective resistance genes. In addition, to show definitively that PAβN binds to RND-type efflux pumps in *Acinetobacter* spp., co-crystallisation experiments of RND efflux pumps and PAβN can also be performed in the same manner as those conducted on AcrB efflux pump (Yu *et al.*, 2003; Yu *et al.*, 2005).

The results of the study strongly suggests that both OXA-23 carbapenemase and efflux mechanisms play important roles in the development of meropenem resistance in *A. baumannii* and *Acinetobacter* genospecies 3 isolates. AdeABC and Ade DE

efflux pumps have potentially significant roles in meropenem resistance in *A. baumannii* and *Acinetobacter* genospecies 3, respectively. The roles of AdeIJK and AdeXYZ efflux pumps as well as CarO porin remain unproven.

Chapter 5

General discussion

5.1 General discussion

Acinetobacter spp. are remarkable Gram-negative pathogens. They originate as biochemically inert commensals and saprophytes found on organic matter and inanimate surfaces (see section 1.2.1). However, some species, particularly those of the *Acinetobacter calcoaceticus-baumannii* complex, have established a niche in healthcare-associated settings such as intensive care units (Dijkshoorn *et al.*, 2007; Giamarellou *et al.*, 2008; Peleg *et al.*, 2008). Consequently, they have evolved to become one of the most formidable and dreaded bacterial pathogens in the clinical setting.

In addition to a wide array of serious clinical infections that can be caused by *Acinetobacter* spp. (see section 1.2.3), they are also particularly difficult to eradicate in the healthcare settings. The immense challenge faced by infection control specialists in the prevention or control of MDR *Acinetobacter* dissemination is evident from the recent reports of outbreaks caused by these organisms worldwide (Coelho *et al.*, 2006; Jones *et al.*, 2004a; Lee *et al.*, 2009; Lolans *et al.*, 2006; Naas *et al.*, 2006a; Peleg *et al.*, 2006a; Poirel & Nordmann, 2006b; Quale *et al.*, 2003; Schulte *et al.*, 2005; Turton *et al.*, 2005; Valenzuela *et al.*, 2007; Vila *et al.*, 1999).

5.1.1 Identification and epidemiology of *Acinetobacter* spp.

A crucial factor in the timely recognition and investigation of *Acinetobacter* outbreaks is the accurate identification of *Acinetobacter* isolates from clinical samples. Phenotypic methods of identification have been shown to be highly unreliable while molecular methods are much more accurate in the identification of *Acinetobacter* spp. (see section 1.1.3). Results of the present study also reiterated this finding. The study demonstrated the very high error rate (74.4%) in the speciation of *Acinetobacter* isolates by VITEK 2 phenotypic identification system when compared to the validated molecular method of identification by *rpoB* sequencing. However, the VITEK 2 system showed high levels of sensitivity and specificity in identifying isolates to the level of *Acinetobacter calcoaceticus-baumannii* complex (Acbc). The implication for laboratories utilising phenotypic methods for the identification of *Acinetobacter* isolates is the need for modification of the reported results. Isolates should be reported as an Acbc isolate or as a non-Acbc isolate, pending further identification by a validated molecular method. Although molecular methods such as *rpoB* sequencing and ARDRA are more ideal for *Acinetobacter* speciation, they are nonetheless relatively time-consuming methods requiring specialised equipment and skills that are not routinely available in many clinical microbiology diagnostic laboratories. Therefore, the future challenge in the identification of *Acinetobacter* spp. is the development of accurate, simple and rapid molecular methods that can be routinely applied to the clinical laboratory setting. Methods currently in the process of development and validation include DNA and RNA microarrays, which facilitates the simultaneous detection of multiple genes and their levels of expression through the

use of numerous genetic probes incorporated into small microchips. With the help of specialised analytical software, genetic characterisation of isolates can be performed rapidly and accurately (Weile & Knabbe, 2009).

The present study has also established the epidemiological pattern of clinical *Acinetobacter* isolates in a tertiary-care hospital in the Republic of Ireland, and the data obtained may provide a useful foundation for a larger epidemiological study of *Acinetobacter* isolates on a national level. The results of this study showed a predominance of *Acinetobacter* genospecies 3 among the clinical isolates in this hospital, an epidemiological pattern that was relatively uncommon worldwide. With the overwhelming number of studies reporting *A. baumannii* as the predominant species, the unusual epidemiology was only occasionally reported in centres in Sweden and Germany (Bouvet & Grimont, 1986; Traub & Bauer, 2000). The reason(s) for the predominance of *Acinetobacter* genospecies 3 rather than *A. baumannii* is not clear. Notably, this study also provided important antimicrobial susceptibility data on this clinically important species, since such data were reported infrequently in the literature (Ribera *et al.*, 2004; Seifert *et al.*, 1993a; Traub & Spohr, 1989).

5.1.2 Susceptibility testing in *Acinetobacter* spp.

Several problems in susceptibility testing of *Acinetobacter* spp. were also highlighted in this study. An unacceptably high rate of very major errors in piperacillin-

tazobactam susceptibility testing was found with the VITEK 2 susceptibility test system. The results of the study also highlighted problematic areas in susceptibility testing pertaining to carbapenem breakpoints as well as the effects of different test media on tigecycline susceptibility interpretation. There is a need for greater consensus among the major microbiological organisations in areas of antimicrobial susceptibility interpretation as well as test methodologies. Greater uniformity in these areas would not only generate more accurate antimicrobial susceptibility data but would also facilitate more meaningful and reproducible comparison of such data between centres, countries and/or regions.

5.1.3 Antimicrobial resistance in *Acinetobacter* spp.

One significant reason for the remarkable success of *Acinetobacter* spp. as one of the emerging pathogens in hospital infections and outbreaks is the ability for rapid development of resistance to biocides and antibiotics. Resistance to biocides such as quarternary ammonium compounds confers *Acinetobacter* spp. enhanced survival potential in an otherwise hostile hospital environment for microbes (Fournier *et al.*, 2006). Similarly, the ability of *Acinetobacter* spp. to develop resistance to a wide range of antibiotics underlies their persistence in the healthcare settings in the face of mounting antimicrobial pressure. The resistance potential of *Acinetobacter* spp. was demonstrated by a recent study revealing the presence of a large 86-kb resistance island containing resistance genes of various classes of antibiotics, heavy metals and biocides in an MDR *A. baumannii* strain (Fournier *et al.*, 2006). Over the years,

resistance to important classes of antibiotics such as broad-spectrum cephalosporins, aminoglycosides and fluoroquinolones has risen steadily. Unfortunately, carbapenem resistance has also emerged, particularly over the last decade (see section 1.4). As it is a very versatile and useful group of β-lactams, resistance to carbapenems is of particular clinical concern. In this study, over 10% of *Acinetobacter* isolates, involving different species (*A. baumannii*, *Acinetobacter* genospecies 3 and *A. johnsonii*), were resistant to carbapenems. Such isolates were also resistant to other antibiotics such as quinolones and other β-lactams. Moreoever, these isolates had reduced susceptibility to tigecycline and doripenem, which are two of a limited number of new antimicrobial agents available against highly-resistant Gram-negative bacteria. In this study, the resistance potential of *Acinetobacter* spp. was exemplified by the *A. baumannii* isolate 229437, which was fully resistant to all β-lactams, ciprofloxacin, gentamicin, amikacin, trimethoprim-sulphamethoxazole and tigecycline. Only colistin had consistent *in vitro* activity against the carbapenem-resistant isolates. The results highlighted the daunting therapeutic challenge facing clinicians and microbiologists when tackling infections caused by these highly resistant pathogens. The need for clinicians to resort to treatment with colistin, which had previously fallen out of favour due to toxicity problems, underpinned the dire clinical situation posed by these organisms. The study highlighted the urgent need for the development of new therapeutic agents and also emphasised the importance of stringent antimicrobial stewardship in tackling these MDR *Acinetobacter* isolates.

β-lactamase mechanisms of resistance were also investigated in this study. Clavulanic acid-based and EDTA-based phenotypic screening tests for class A ESBL and class B

MBL β-lactamase genes, respectively, were shown to have poor positive predictive value in *Acinetobacter* spp. None of the isolates with screen-positive results was found by PCR to carry known class A ESBL or class B MBL genes, respectively. The results suggest that these phenotypic screening methods are not reliable for the detection of the above genes in the *Acinetobacter* genus.

Along with class C β-lactamase genes in Acbc isolates, the study also found a relatively high prevalence (35.9%) of class D β-lactamase genes in the *Acinetobacter* isolates. Most importantly, plasmid-mediated $bla_{\text{OXA-23}}$ gene was found in all 12 of the *Acinetobacter* isolates with high-level carbapenem resistance, and each had the insertion sequence IS*Aba1* upstream of $bla_{\text{OXA-23}}$. Subsequent qRT-PCR assays of $bla_{\text{OXA-23}}$ expression also demonstrated the constitutive nature of its expression even in the absence of meropenem. Moreover, IS*Aba1* was also found upstream of class C cephalosporinase genes of the two *A. baumannii* isolates with high-level resistance to third- and fourth-generation cephalosporins. Hence, the results of this study provide strong evidence that this element played a pivotal role in the constitutive overexpression of β-lactamase genes in *Acinetobacter* spp., further supporting results from previous studies (Corvec *et al.*, 2007; Poirel & Nordmann, 2006a). Chromosomally-located $bla_{\text{OXA-23-like}}$ genes were also found in carbapenem-susceptible *A. radioresistens* isolates, hence supporting the view that this species is the progenitor of $bla_{\text{OXA-23}}$ found in carbapenem-resistant *Acinetobacter* isolates (Poirel *et al.*, 2008b). The study also showed that several mechanisms are probably involved in the dissemination of $bla_{\text{OXA-23}}$ among the carbapenem-resistant isolates. While PFGE results in this study showed clonal expansion of *Acinetobacter*

genospecies 3 strains carrying bla_{OXA-23}, plasmid studies also suggested that intra-species plasmidic transfer of the gene between different *Acinetobacter* genospecies 3 strains as well as inter-species transfer between *Acinetobacter* genospecies 3 and *A. johnsonii* might have occurred. The multiple mechanisms involved in bla_{OXA-23} dissemination emphasise the need for stringent infection control measures in order to control the spread of such MDR *Acinetobacter* isolates. Furthermore, the discovery of $bla_{OXA-23-like}$ and bla_{OXA-58} genes in carbapenem-susceptible *Acinetobacter* isolates highlights the challenge posed by unrecognised reservoirs of such OXA carbapenemase genes and reiterates the need for concomitant implementation of standard infection control measures along with those measures to deal with the MDR isolates. While the discovery of OXA carbapenemases in *Acinetobacter* spp. represents yet another ominous development in antimicrobial resistance in this genus, it also presents to scientists and clinicians another potential target for therapeutic intervention. Further characterisation of OXA carbapenemases using methods such as X-ray crystallography may reveal potential target sites for the development of novel agents inhibiting these β-lactamases and can therefore augment the arsenal of agents available against MDR *Acinetobacter* isolates.

5.1.4 Efflux and permeability mechanisms in carbapenem resistance in *Acinetobacter* spp.

Apart from OXA carbapenemases, other mechanisms were also found to contribute significantly to carbapenem resistance in *Acinetobacter* spp. The PAβN results in this

study strongly support a significant role of efflux mechanisms in meropenem resistance. The qRT-PCR assays provided further evidence that certain RND-type efflux pumps such as AdeABC and AdeDE may have contributory roles in meropenem resistance in *A. baumannii* and *Acinetobacter* genospecies 3, respectively. OMP profiling also suggests that CarO porin had a possible role in carbapenem resistance in *A. baumannii*. In the face of ever-increasing prevalence of MDR pathogens, coupled with the paucity of development of new anti-infective drugs, the inhibition of efflux mechanisms provides an attractive measure that could potentially increase intrinsic susceptibility of organisms to antibiotics, restore susceptibility of MDR pathogens to current agents or reduce the capacity for acquiring additional resistance (Mahamoud *et al.*, 2007). In particular, the inhibition of RND-type efflux pumps can have very significant therapeutic potential since restoration of susceptibility to multiple classes of antibiotics may be possible. However, none of the EPIs currently utilised in laboratory research is suitable for clinical usage. The concentrations of general inhibitors such as reserpine and verapamil required for inhibition of bacterial efflux pumps would be too neurotoxic (Li & Nikaido, 2004). Peptidomimetics such as PAβN have numerous toxic properties which precluded clinical use, while arylpiperazines, due to their serotonin agonist properties, are probably too toxic therapeutically as well (Levy, 2002; Mahamoud *et al.*, 2007; Poole, 2005). With mounting antimicrobial resistance in pathogens, the envisaged therapeutic potential of EPIs as well as the toxicity of current agents in laboratory usage, the research and development of clinically effective and yet safe efflux pump inhibitors (EPIs) take on greater urgency and impetus.

5.1.5 Interplay of mechanisms in carbapenem resistance

Another confounding factor in tackling antimicrobial resistance in pathogens is the presence of multiple mechanisms interacting with one another to produce the MDR phenotype. The complexity of the problem is exemplified by the PAβN experiments on the *Acinetobacter* isolates. In the meropenem-resistant *Acinetobacter* isolates, as a result of the presence of OXA-23, meropenem MICs (4 to16 μg/mL) were still significantly higher than fully susceptible isolates even in the presence of high concentrations of PAβN. Restoration of full susceptibility can probably only be achieved with inhibition of the various resistance mechanisms; in the case of the meropenem-resistant *Acinetobacter* isolates, the concomitant inhibition of OXA-23 and efflux mechanisms would probably be required for restoration of meropenem susceptibility. This would represent an alternative approach in tackling the escalating antimicrobial resistance in organisms such as *Acinetobacter* spp. This approach would aim at restoring utility of existing antibiotics through the inhibition of resistance mechanisms to these agents. In tandem with the development of novel antibiotics with new mechanisms of action, this approach can increase the therapeutic potential in tackling these MDR organisms.

5.2 Concluding remarks

In summary, the study has yielded several interesting and important findings. Using accurate molecular methods, an unusual epidemiological pattern in *Acinetobacter* isolates was found in St. James's Hospital. This finding reiterates the clinical importance of *Acinetobacter* genospecies 3. In addition, there is a worrying level of multidrug resistance, especially carbapenem resistance, among clinical isolates of *Acinetobacter* spp. in the hospital. Relatively few therapeutic options are available to tackle these carbapenem-resistant isolates. Moreover, carbapenem resistance is mediated by multiple mechanisms, including a carbapenemase (OXA-23) that is carried on highly mobile plasmids. The multiple modalities of dissemination of resistance present an immense challenge to infection control efforts to limit their spread. Tackling the emerging problem of carbapenem-resistance in *Acinetobacter* will require an integrated approach of resistance surveillance, infection control, antimicrobial stewardship and translational research.

Further research should be performed based on the results on the study. Potential therapeutic options should be investigated, such as efflux pump inhibitors and OXA carbapenemase-inhibitors. The roles of efflux pumps, porins and carbapenemases in carbapenem resistance should be more accurately elucidated using gene knockouts to isolate the effects of individual mechanisms. Investigation into factors that influence

the predominance of one *Acinetobacter* species over another in the clinical setting may be of therapeutic benefit and infection control significance.

Acinetobacter spp. represent the paradigm of antimicrobial resistance in Gram-negative pathogens. They have the capacity to acquire and utilise an array of resistance genes against a wide variety of antibiotics and biocides, and are also able to disseminate such genes efficiently through multiple mechanisms. These attributes were clearly demonstrated in the case of carbapenem resistance. In tackling such versatile and adaptable pathogens, further translational research and development in the areas of diagnostic methodologies and therapeutic interventions need to take place urgently and in tandem with each other. In the future, the use of new technologies such as gene and expression microarrays may allow accurate and rapid identification of *Acinetobacter* isolates as well as early characterisation of resistance mechanisms. However, such diagnostic developments would only be of academic importance without the concomitant development of clinically useful agents against such versatile pathogens. In an era of escalating antimicrobial resistance and diminishing options to counter such developments, there is an ever greater urgency to utilise all available measures in infection control, antimicrobial stewardship, diagnostic methodology, therapeutic intervention and scientific research in the fight against formidable pathogens such as *Acinetobacter* spp.

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Appendices

Appendix 1. Antimicrobial susceptibility results of the *Acinetobacter* isolates using three AST methods.

A. baumannii	Disc diffusion method (mm)								
	Ctx	Caz	Cfp	Tzp	Mpm	Cip	Gen	Amk	Cot
229437	≤ 6	≤ 6	≤ 6	≤ 6	≤ 6	≤ 6	≤ 6	≤ 6	≤ 6
11648	≤ 6	≤ 6	12	15	16	≤ 6	≤ 6	19	≤ 6
26756	16	19	18	27	20	8	18	19	20
101049	11	14	13	20	18	8	20	19	20
20802	17	18	21	19	21	9	23	21	21
217068	12	16	18	20	20	17	21	20	21
12937	11	17	17	21	19	22	20	19	18
89848	23	20	22	26	23	24	18	22	20
26750	16	18	20	18	24	22	19	20	19
181329	17	16	19	21	22	25	22	21	22
27623	15	18	18	18	20	21	20	22	21
102450	16	17	17	18	21	23	20	20	21
29419	20	23	23	28	23	24	18	22	21
14495	25	28	26	27	22	32	20	19	23
13207	17	22	21	21	21	25	18	21	18
100983	23	26	24	26	22	29	22	23	22
14773	17	21	23	26	25	30	19	22	20
33047	16	22	23	26	21	30	20	21	20
107228	22	23	24	27	24	31	23	24	22
107532	21	22	24	26	25	31	22	24	23
107245	22	21	24	28	24	30	23	24	22
31364	17	19	18	19	20	25	21	23	21
27021	16	21	23	26	21	24	19	22	19
233651	17	21	20	24	27	26	20	20	23
232982	20	23	21	26	26	23	20	21	19
218722	15	18	20	23	25	24	21	22	21
220165	22	21	22	24	26	27	22	23	21
A. calcoaceticus									
33241	16	21	22	24	23	28	21	23	23
92328	15	16	19	25	24	26	22	20	20
183262	13	14	18	18	21	28	20	21	24
20304	13	15	19	24	25	26	20	20	19
28263	15	16	18	23	27	25	21	22	20
100558	16	15	19	19	23	25	21	23	22

Ctx, cefotaxime; Caz, ceftazidime; Cfp, cefepime; Tzp, piperacillin-tazobactam; Mpm, meropenem; Cip, ciprofloxacin; Gen, gentamicin; Amk, amikacin; Cot, trimethoprim-sulphamethoxazolel; AG3, *Acinetobacter* genospecies 3.

AG3	Disc diffusion method (mm)								
	Ctx	Caz	Cfp	Tzp	Mpm	Cip	Gen	Amk	Cot
29540	10	12	10	10	≤ 6	8	13	26	≤ 6
32357	12	14	12	8	≤ 6	9	13	24	≤ 6
36277	12	14	12	10	≤ 6	8	14	27	≤ 6
12659	14	18	15	13	≤ 6	10	19	24	22
23269	13	17	14	15	≤ 6	≤ 6	20	21	23
14448	15	19	14	16	≤ 6	11	21	23	22
14748	14	18	15	16	≤ 6	11	22	22	24
27616	15	18	16	14	≤ 6	13	20	20	23
103165	13	15	12	15	≤ 6	8	21	21	21
28594	16	20	17	15	≤ 6	14	19	19	22
206479	22	25	23	23	22	24	18	24	25
106770	13	19	19	22	20	26	19	24	24
27458	17	17	16	20	19	11	18	20	22
88276	20	22	24	27	22	27	19	21	22
105621	16	21	20	28	24	25	23	23	24
86096	22	22	19	21	22	23	19	22	23
107012	16	16	17	22	22	24	18	24	24
14027	17	17	16	28	20	≤ 6	11	19	20
30077	17	15	18	20	19	14	20	22	22
22344	16	16	20	21	19	12	22	24	23
12193	14	17	17	21	18	22	20	22	22
93743	13	17	17	19	20	21	19	22	24
35272	17	16	16	20	18	20	18	23	23
86661	14	17	17	23	18	22	18	23	25
12253	16	18	15	24	18	23	18	22	21
28933	17	15	17	22	19	21	20	23	23
20949	16	16	19	26	21	23	19	21	22
31479	21	21	21	28	22	24	19	22	22
106498	16	19	19	19	20	27	20	24	25
106008	16	20	20	26	22	26	20	24	25
183436	17	16	21	26	20	27	19	20	25

Ctx, cefotaxime; Caz, ceftazidime; Cfp, cefepime; Tzp, piperacillin-tazobactam; Mpm, meropenem; Cip, ciprofloxacin; Gen, gentamicin; Amk, amikacin; Cot, trimethoprim-sulphamethoxazole.

AG3	Disc diffusion method (mm)								
	Ctx	Caz	Cfp	Tzp	Mpm	Cip	Gen	Amk	Cot
29420	14	17	18	27	21	25	18	21	22
14331	14	16	16	21	19	24	18	24	21
27035	17	16	19	26	21	25	19	22	22
25904	16	18	16	22	21	24	18	22	22
16198	16	16	18	28	22	24	20	23	23
82991	18	16	21	28	23	25	20	23	25
181882	16	19	22	23	21	26	21	22	25
13320	15	20	22	24	22	27	20	22	21
101327	20	17	20	23	21	26	20	24	24
91135	22	21	21	27	24	27	19	24	23
106449	24	22	24	28	24	28	21	24	24
35168	22	21	24	26	23	26	22	22	21
232787	21	20	23	25	21	25	21	22	22
225991	21	22	19	24	20	26	19	22	20
A. johnsonii									
25868	20	18	13	10	≤ 6	8	≤ 6	22	≤ 6
100441	24	21	12	28	24	8	19	21	24
182952	23	20	12	27	25	29	21	19	23
26612	24	25	21	26	23	30	23	22	23
181441	19	22	19	24	26	25	20	23	22
28001	22	23	15	26	28	26	19	20	24
226419	25	21	17	28	28	28	22	24	21
214737	24	25	18	26	27	29	21	21	23
215759	25	22	17	25	27	32	24	21	23
215194	27	21	21	25	28	27	21	23	21
215687	27	26	24	27	28	27	20	23	22
221358	26	25	25	27	27	29	21	21	24
A. ursingii									
88389	10	12	21	21	23	21	21	19	22
183357	20	17	23	25	26	26	10	22	24
89302	13	13	22	23	24	25	19	21	23
90772	15	13	22	29	22	25	20	20	21
217818	27	23	24	28	28	29	25	23	21
91411	16	15	21	22	24	23	21	19	20
91352	17	17	22	22	23	24	20	19	22
30431	19	19	21	26	23	26	22	22	22

AG3, *Acinetobacter* genospecies 3; Ctx, cefotaxime; Caz, ceftazidime; Cfp, cefepime; Tzp, piperacillin-tazobactam; Mpm, meropenem; Cip, ciprofloxacin; Gen, gentamicin; Amk, amikacin; Cot, trimethoprim-sulphamethoxazole.

A. lwoffii/ AG9	Disc diffusion method (mm)								
	Ctx	Caz	Cfp	Tzp	Mpm	Cip	Gen	Amk	Cot
203910	21	18	21	28	25	26	23	21	21
22104	24	23	27	27	26	32	22	23	20
190152	18	16	25	19	20	22	20	22	≤ 6
107690	28	25	27	30	24	25	25	24	24
184576	20	20	24	29	23	28	22	21	24
180361	27	27	28	27	26	26	24	21	23
23063	23	22	25	27	23	24	23	22	22
AG11									
101559	16	19	21	20	27	28	22	19	22
219537	20	21	24	25	26	27	26	20	22
27611	21	21	23	25	23	23	21	19	23
105947	21	22	24	21	24	25	23	20	24
Other spp.									
20715 (AG13)	17	15	21	18	19	≤ 6	21	21	23
30293 (AG13)	22	18	24	24	25	28	24	20	22
89182 (AH)	25	25	27	28	23	27	18	16	21
88427 (AR)	20	20	20	27	21	25	20	20	22
87374 (AR)	25	23	22	29	24	27	22	20	20
227313 (AS)	27	21	28	24	29	26	24	22	23
103727 (AT)	22	19	25	28	22	23	17	19	23
101055 (AG10)	24	23	24	27	18	≤ 6	8	22	24

Ctx, cefotaxime; Caz, ceftazidime; Cfp, cefepime; Tzp, piperacillin-tazobactam; Mpm, meropenem; Cip, ciprofloxacin; Gen, gentamicin; Amk, amikacin; Cot, trimethoprim-sulphamethoxazole; AG, *Acinetobacter* genospecies; AH, *A. haemolyticus*; AR, *A. radioresistens*; AS, *A. schindleri*; AT, *A. tjernbergiae*.

A. baumannii	VITEK 2 MIC method (μg/mL)								
	Ctx	**Caz**	**Cfp**	**Tzp[a]**	**Mpm**	**Cip**	**Gen**	**Amk**	**Cot[b]**
229437	≥ 64	≥ 64	≥ 64	≥ 128	≥ 16	≥ 4	≥ 16	≥ 32	≥ 320
11648	≥ 64	≥ 64	16	16	1	≥ 4	≥ 16	≤ 2	≥ 320
26756	32	8	8	≤ 4	0.5	≥ 4	≤ 1	≤ 2	≤ 20
101049	≥ 64	16	16	8	2	≥ 4	≤ 1	≤ 2	≤ 20
20802	32	8	4	≤ 4	0.5	≥ 4	≤ 1	≤ 2	≤ 20
217068	≥ 64	16	8	8	0.5	1	≤ 1	≤ 2	≤ 20
12937	≥ 64	16	8	16	1	1	≤ 1	≤ 2	≤ 20
89848	8	4	2	≤ 4	≤ 0.25	1	≤ 1	≤ 2	≤ 20
26750	16	8	4	≤ 4	≤ 0.25	0.5	≤ 1	≤ 2	≤ 20
181329	32	16	8	8	0.5	0.5	≤ 1	≤ 2	≤ 20
27623	32	8	8	16	0.5	1	≤ 1	≤ 2	≤ 20
102450	32	16	8	8	0.5	0.5	≤ 1	≤ 2	≤ 20
29419	16	4	2	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
14495	4	≤ 1	≤ 1	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
13207	16	4	2	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
100983	8	2	2	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
14773	16	4	2	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
33047	16	4	2	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
107228	16	4	2	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
107532	16	4	2	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
107245	16	4	2	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
31364	32	8	8	8	0.5	≤ 0.25	≤ 1	≤ 2	≤ 20
27021	16	4	2	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
233651	16	4	4	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
232982	16	4	4	≤ 4	≤ 0.25	0.5	≤ 1	≤ 2	≤ 20
218722	32	8	4	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
220165	16	8	4	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
A. calco-aceticus									
33241	32	4	2	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
92328	32	16	4	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
183262	≥ 64	16	8	8	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
20304	32	16	8	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
28263	32	16	8	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
100558	32	16	4	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20

Ctx, cefotaxime; Caz, ceftazidime; Cfp, cefepime; Tzp[a], piperacillin-tazobactam (piperacillin component); Mpm, meropenem; Cip, ciprofloxacin; Gen, gentamicin; Amk, amikacin; Cot[b], trimethoprim-sulphamethoxazole (sulphamethoxazole component).

AG3	VITEK 2 MIC method (µg/mL)								
	Ctx	Caz	Cfp	Tzp[a]	Mpm	Cip	Gen	Amk	Cot[b]
29540	≥ 64	16	32	≥ 128	≥ 16	≥ 4	≥ 16	≤ 2	≥ 320
32357	≥ 64	16	32	≥ 128	≥ 16	≥ 4	≥ 16	≤ 2	≥ 320
36277	≥ 64	16	≥ 64	≥ 128	≥ 16	≥ 4	≥ 16	≤ 2	≥ 320
12659	≥ 64	8	32	≥ 128	≥ 16	≥ 4	≤ 1	≤ 2	≤ 20
23269	≥ 64	16	32	≥ 128	≥ 16	≥ 4	≤ 1	≤ 2	≤ 20
14448	32	8	16	≥ 128	≥ 16	≥ 4	≤ 1	≤ 2	≤ 20
14748	≥ 64	16	32	64	≥ 16	≥ 4	≤ 1	≤ 2	≤ 20
27616	≥ 64	8	32	≥ 128	≥ 16	≥ 4	≤ 1	≤ 2	≤ 20
103165	≥ 64	16	32	≥ 128	≥ 16	≥ 4	≤ 1	≤ 2	≤ 20
28594	32	4	16	≥ 128	≥ 16	≥ 4	≤ 1	4	40
206479	8	2	2	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
106770	32	16	8	8	0.5	≤ 0.25	≤ 1	≤ 2	≤ 20
27458	32	16	16	16	0.5	≥ 4	≤ 1	≤ 2	≤ 20
88276	8	4	2	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
105621	16	4	4	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
86096	16	8	4	≤ 4	≤ 0.25	0.5	≤ 1	≤ 2	≤ 20
107012	32	16	16	8	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
14027	32	16	16	≤ 4	0.5	≥ 4	4	4	≤ 20
30077	32	16	16	16	0.5	≥ 4	≤ 1	≤ 2	≤ 20
22344	32	16	8	16	0.5	≥ 4	≤ 1	≤ 2	≤ 20
12193	≥ 64	16	16	8	1	1	≤ 1	≤ 2	≤ 20
93743	≥ 64	16	16	8	0.5	1	≤ 1	≤ 2	≤ 20
35272	32	16	16	8	0.5	1	≤ 1	≤ 2	≤ 20
86661	≥ 64	16	16	8	0.5	1	≤ 1	≤ 2	≤ 20
12253	32	16	16	8	0.5	0.5	≤ 1	≤ 2	≤ 20
28933	32	16	16	8	0.5	1	≤ 1	≤ 2	≤ 20
20949	32	16	8	≤ 4	≤ 0.25	0.5	≤ 1	≤ 2	≤ 20
31479	8	4	4	≤ 4	≤ 0.25	0.5	≤ 1	≤ 2	≤ 20
106498	32	8	8	8	0.5	≤ 0.25	≤ 1	≤ 2	≤ 20
106008	16	8	8	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
183436	32	16	8	≤ 4	0.5	≤ 0.25	≤ 1	≤ 2	≤ 20

AG3, *Acinetobacter* genospecies 3; Ctx, cefotaxime; Caz, ceftazidime; Cfp, cefepime; Tzp[a], piperacillin-tazobactam (piperacillin component); Mpm, meropenem; Cip, ciprofloxacin; Gen, gentamicin; Amk, amikacin; Cot[b], trimethoprim-sulphamethoxazole (sulphamethoxazole component).

AG3	VITEK 2 MIC method (µg/mL)								
	Ctx	Caz	Cfp	Tzp[a]	Mpm	Cip	Gen	Amk	Cot[b]
29420	32	16	8	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
14331	≥ 64	16	16	8	0.5	≤ 0.25	≤ 1	≤ 2	≤ 20
27035	32	16	16	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
25904	32	16	16	8	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
16198	32	16	8	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
82991	32	16	4	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
181882	32	4	4	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
13320	16	4	4	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
101327	16	8	4	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
91135	8	4	2	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
106449	8	4	2	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
35168	16	4	2	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
232787	16	4	4	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
225991	16	4	8	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
A. johnsonii									
25868	16	8	32	≥ 128	≥ 16	≥ 4	≥ 16	≤ 2	≥ 320
100441	8	4	32	≤ 4	≤ 0.25	≥ 4	≤ 1	≤ 2	≤ 20
182952	8	4	32	≤ 4	≤ 0.25	≤ 0.25	≤ 1	4	≤ 20
26612	8	2	4	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
181441	8	4	8	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
28001	8	4	16	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
226419	4	4	8	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
214737	4	2	8	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
215759	4	4	8	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
215194	4	4	4	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
215687	2	2	2	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
221358	2	2	2	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
A. ursingii									
88389	≥ 64	≥ 64	4	8	≤ 0.25	1	≤ 1	≤ 2	≤ 20
183357	16	16	2	≤ 4	≤ 0.25	≤ 0.25	≥ 16	≤ 2	≤ 20
89302	≥ 64	32	4	8	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
90772	32	16	4	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
217818	2	2	2	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
91411	32	16	4	≤ 4	≤ 0.25	0.5	≤ 1	≤ 2	≤ 20
91352	16	16	2	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20
30431	16	8	2	≤ 4	≤ 0.25	≤ 0.25	≤ 1	≤ 2	≤ 20

AG3, *Acinetobacter* genospecies 3; Ctx, cefotaxime; Caz, ceftazidime; Cfp, cefepime; Tzp[a], piperacillin-tazobactam (piperacillin component); Mpm, meropenem; Cip, ciprofloxacin; Gen, gentamicin; Amk, amikacin; Cot[b], trimethoprim-sulphamethoxazole (sulphamethoxazole component).

A. lwoffii/ AG9	VITEK 2 MIC method (µg/mL)								
	Ctx	Caz	Cfp	Tzp[a]	Mpm	Cip	Gen	Amk	Cot[b]
203910	8	8	4	≤4	≤0.25	≤0.25	≤1	≤2	≤20
22104	4	2	≤1	≤4	≤0.25	≤0.25	≤1	≤2	≤20
190152	32	16	4	8	0.5	0.5	≤1	≤2	≥320
107690	≤1	≤1	≤1	≤4	≤0.25	≤0.25	≤1	≤2	≤20
184576	16	8	2	≤4	≤0.25	≤0.25	≤1	≤2	≤20
180361	2	≤1	≤1	≤4	≤0.25	≤0.25	≤1	≤2	≤20
23063	8	4	2	≤4	≤0.25	≤0.25	≤1	≤2	≤20
AG11									
101559	16	8	4	8	≤0.25	≤0.25	≤1	≤2	≤20
219537	16	4	2	≤4	≤0.25	≤0.25	≤1	≤2	≤20
27611	8	4	2	≤4	2	0.5	≤1	4	≤20
105947	16	4	2	8	≤0.25	≤0.25	≤1	≤2	≤20
Other spp.									
20715 (AG13)	16	16	4	32	1	≥4	≤1	≤2	≤20
30293 (AG13)	8	8	2	≤4	≤0.25	≤0.25	≤1	≤2	≤20
89182 (AH)	4	≤1	≤1	≤4	≤0.25	≤0.25	2	16	≤20
88427 (AR)	16	4	2	≤4	0.5	≤0.25	≤1	≤2	≤20
87374 (AR)	4	4	2	≤4	≤0.25	≤0.25	≤1	≤2	≤20
227313 (AS)	2	4	≤1	≤4	≤0.25	≤0.25	≤1	≤2	≤20
103727 (AT)	8	8	2	≤4	2	0.5	8	4	≤20
101055 (AG10)	4	2	4	≤4	1	≥4	≥16	≤2	≤20

Ctx, cefotaxime; Caz, ceftazidime; Cfp, cefepime; Tzp[a], piperacillin-tazobactam (piperacillin component); Mpm, meropenem; Cip, ciprofloxacin; Gen, gentamicin; Amk, amikacin; Cot[b], trimethoprim-sulphamethoxazole (sulphamethoxazole component); AG, *Acinetobacter* genospecies; AH, *A. haemolyticus*; AR, *A. radioresistens*; AS, *A. schindleri*; AT, *A. tjernbergiae*.

A. baumannii	Etest MIC method (µg/mL)								
	Ctx	**Caz**	**Cfp**	**Tzp[a]**	**Mpm**	**Cip**	**Gen**	**Amk**	**Cot**
229437	≥ 256	≥ 256	≥ 256	≥ 256	≥ 32	≥ 32	≥ 256	≥ 256	≥ 32/608
11648	≥ 256	≥ 256	32	16	4	≥ 32	≥ 256	1	≥ 32/608
26756	32	8	8	≤ 1	0.5	≥ 32	0.5	1	≤ 1/19
101049	128	32	32	64	2	≥ 32	0.5	1	≤ 1/19
20802	32	8	4	32	0.5	≥ 32	0.25	0.5	≤ 1/19
217068	128	16	8	32	1	2	0.5	1	≤ 1/19
12937	128	16	16	32	1	1	1	2	≤ 1/19
89848	8	4	2	≤ 1	0.25	0.5	1	0.5	≤ 1/19
26750	32	8	4	32	0.25	1	1	1	≤ 1/19
181329	32	16	8	16	0.5	0.5	0.5	1	≤ 1/19
27623	32	8	8	64	1	1	1	1	≤ 1/19
102450	32	16	16	128	0.5	0.5	0.5	1	≤ 1/19
29419	16	4	2	≤ 1	0.25	0.5	0.25	0.5	≤ 1/19
14495	4	≤ 1	1	≤ 1	0.25	≤ 0.125	1	2	≤ 1/19
13207	32	4	4	16	0.25	0.25	1	1	≤ 1/19
100983	8	2	2	≤ 1	0.5	≤ 0.125	0.5	0.5	≤ 1/19
14773	32	4	2	≤ 1	≤ 0.125	≤ 0.125	1	0.5	≤ 1/19
33047	16	4	2	≤ 1	0.25	≤ 0.125	0.5	1	≤ 1/19
107228	16	4	2	≤ 1	≤ 0.125	≤ 0.125	0.25	0.25	≤ 1/19
107532	16	4	2	≤ 1	≤ 0.125	≤ 0.125	0.25	0.25	≤ 1/19
107245	16	4	2	≤ 1	≤ 0.125	≤ 0.125	0.25	0.25	≤ 1/19
31364	32	8	8	32	0.5	0.25	0.5	0.5	≤ 1/19
27021	32	4	2	≤ 1	0.25	0.25	1	1	≤ 1/19
233651	32	4	4	4	≤ 0.125	0.25	1	1	≤ 1/19
232982	16	4	4	≤ 1	≤ 0.125	0.5	1	1	≤ 1/19
218722	32	8	4	≤ 1	0.25	0.25	0.5	0.5	≤ 1/19
220165	16	4	2	4	0.25	≤ 0.125	0.5	0.5	≤ 1/19
A. calcoaceticus									
33241	16	4	2	8	0.25	≤ 0.125	1	0.5	≤ 1/19
92328	32	16	4	2	0.25	0.25	0.5	1	≤ 1/19
183262	64	16	8	64	0.25	≤ 0.125	1	1	≤ 1/19
20304	64	16	8	4	≤ 0.125	0.25	1	1	≤ 1/19
28263	32	16	8	8	≤ 0.125	0.25	0.5	0.5	≤ 1/19
100558	32	16	4	32	0.25	0.25	1	0.5	≤ 1/19

Ctx, cefotaxime; Caz, ceftazidime; Cfp, cefepime; Tzp[a], piperacillin-tazobactam (piperacillin component); Mpm, meropenem; Cip, ciprofloxacin; Gen, gentamicin; Amk, amikacin; Cot, trimethoprim-sulphamethoxazole.

AG3	Etest MIC method (μg/mL)								
	Ctx	Caz	Cfp	Tzp[a]	Mpm	Cip	Gen	Amk	Cot
29540	≥ 256	32	128	≥ 256	≥ 32	≥ 32	32	1	≥ 32/608
32357	128	32	64	≥ 256	≥ 32	≥ 32	32	2	≥ 32/608
36277	128	16	64	≥ 256	≥ 32	≥ 32	32	0.5	≥ 32/608
12659	64	8	32	128	≥ 32	16	1	0.5	≤ 1/19
23269	64	16	32	128	≥ 32	≥ 32	1	2	≤ 1/19
14448	64	8	32	128	≥ 32	16	1	2	≤ 1/19
14748	64	8	32	128	≥ 32	16	1	1	≤ 1/19
27616	32	8	16	128	≥ 32	8	0.5	2	≤ 1/19
103165	64	16	64	≥ 256	≥ 32	≥ 32	1	2	≤ 1/19
28594	32	4	16	128	≥ 32	8	0.5	4	2/38
206479	8	2	2	4	0.25	0.25	1	0.5	≤ 1/19
106770	64	8	8	4	0.5	≤ 0.125	1	0.5	≤ 1/19
27458	32	16	16	32	1	16	1	2	≤ 1/19
88276	8	4	2	≤ 1	0.25	≤ 0.125	1	2	≤ 1/19
105621	32	4	4	≤ 1	≤ 0.125	0.25	0.25	0.5	≤ 1/19
86096	16	4	4	8	0.25	0.5	1	1	≤ 1/19
107012	32	16	8	8	0.25	0.25	1	0.5	≤ 1/19
14027	32	16	16	≤ 1	0.5	≥ 32	8	8	≤ 1/19
30077	32	16	8	16	0.5	8	0.5	0.5	≤ 1/19
22344	32	16	8	16	1	16	0.5	0.25	≤ 1/19
12193	64	8	16	16	1	1	0.5	0.5	≤ 1/19
93743	64	16	16	32	0.5	1	1	1	≤ 1/19
35272	32	16	16	16	1	1	1	0.5	≤ 1/19
86661	64	16	8	8	1	1	1	0.5	≤ 1/19
12253	32	8	16	8	1	0.5	1	0.5	≤ 1/19
28933	32	16	16	8	0.5	1	0.5	0.5	≤ 1/19
20949	32	16	8	≤ 1	0.25	0.5	1	1	≤ 1/19
31479	8	4	4	≤ 1	0.25	0.5	1	1	≤ 1/19
106498	32	8	8	16	0.5	≤ 0.125	1	0.25	≤ 1/19
106008	32	8	8	≤ 1	0.25	≤ 0.125	0.5	0.25	≤ 1/19
183436	32	16	4	≤ 1	0.5	≤ 0.125	1	1	≤ 1/19

AG3, *Acinetobacter* genospecies 3; Ctx, cefotaxime; Caz, ceftazidime; Cfp, cefepime; Tzp[a], piperacillin-tazobactam (piperacillin component); Mpm, meropenem; Cip, ciprofloxacin; Gen, gentamicin; Amk, amikacin; Cot, trimethoprim-sulphamethoxazole.

AG3	Etest MIC method (μg/mL)								
	Ctx	Caz	Cfp	Tzp[a]	Mpm	Cip	Gen	Amk	Cot
29420	64	16	8	≤ 1	0.25	0.25	1	1	≤ 1/19
14331	64	16	16	16	0.5	0.25	1	0.5	≤ 1/19
27035	32	16	8	≤ 1	0.25	0.25	1	0.5	≤ 1/19
25904	32	16	16	16	0.25	0.25	1	0.5	≤ 1/19
16198	32	16	8	≤ 1	0.25	0.25	0.5	0.5	≤ 1/19
82991	32	8	4	≤ 1	0.25	0.25	1	0.5	≤ 1/19
181882	32	4	4	8	0.25	≤ 0.125	0.5	1	≤ 1/19
13320	32	4	4	8	0.25	≤ 0.125	1	0.5	≤ 1/19
101327	16	8	4	8	0.25	≤ 0.125	0.5	0.25	≤ 1/19
91135	16	4	4	≤ 1	≤ 0.125	≤ 0.125	1	0.25	≤ 1/19
106449	8	4	2	≤ 1	≤ 0.125	≤ 0.125	0.5	0.5	≤ 1/19
35168	16	4	2	≤ 1	0.25	0.25	0.25	0.5	≤ 1/19
232787	16	4	2	≤ 1	0.25	0.25	0.5	1	≤ 1/19
225991	16	4	8	4	0.25	0.25	1	0.5	≤ 1/19
A. johnsonii									
25868	16	8	32	128	≥ 32	≥ 32	128	1	≥ 32/608
100441	8	4	32	≤ 1	0.25	≥ 32	1	1	≤ 1/19
182952	8	4	32	≤ 1	0.25	≤ 0.125	0.5	2	≤ 1/19
26612	4	1	4	≤ 1	0.25	≤ 0.125	0.25	0.5	≤ 1/19
181441	16	4	16	≤ 1	≤ 0.125	0.25	1	0.5	≤ 1/19
28001	8	4	16	≤ 1	≤ 0.125	0.25	1	1	≤ 1/19
226419	4	4	8	≤ 1	≤ 0.125	≤ 0.125	0.5	0.5	≤ 1/19
214737	4	2	8	≤ 1	≤ 0.125	≤ 0.125	0.5	1	≤ 1/19
215759	4	4	8	≤ 1	≤ 0.125	≤ 0.125	0.25	1	≤ 1/19
215194	2	2	4	≤ 1	≤ 0.125	0.25	1	0.5	≤ 1/19
215687	2	2	2	≤ 1	≤ 0.125	0.25	1	0.5	≤ 1/19
221358	2	1	4	≤ 1	≤ 0.125	≤ 0.125	0.5	1	≤ 1/19
A. ursingii									
88389	128	64	4	16	0.25	1	1	2	≤ 1/19
183357	16	16	2	≤ 1	≤ 0.125	0.25	32	1	≤ 1/19
89302	64	32	4	8	0.25	0.25	1	1	≤ 1/19
90772	32	32	4	8	0.25	0.25	1	2	≤ 1/19
217818	2	2	1	≤ 1	≤ 0.125	≤ 0.125	0.25	0.5	≤ 1/19
91411	32	16	4	8	0.25	0.5	1	2	≤ 1/19
91352	32	16	2	8	0.25	0.25	1	2	≤ 1/19
30431	16	8	4	≤ 1	0.25	0.25	1	1	≤ 1/19

AG3, *Acinetobacter* genospecies 3; Ctx, cefotaxime; Caz, ceftazidime; Cfp, cefepime; Tzp[a], piperacillin-tazobactam (piperacillin component); Mpm, meropenem; Cip, ciprofloxacin; Gen, gentamicin; Amk, amikacin; Cot, trimethoprim-sulphamethoxazole.

A. lwoffii /AG9	Etest MIC method (μg/mL)								
	Ctx	Caz	Cfp	Tzp[a]	Mpm	Cip	Gen	Amk	Cot
203910	8	8	4	≤ 1	≤ 0.125	0.25	0.5	1	≤ 1/19
22104	4	2	1	2	≤ 0.125	≤ 0.125	0.5	0.5	≤ 1/19
190152	32	16	4	64	0.5	0.5	1	1	≥ 32/608
107690	1	1	0.5	≤ 1	≤ 0.125	0.25	0.25	0.5	≤ 1/19
184576	16	8	2	≤ 1	0.25	≤ 0.125	0.5	1	≤ 1/19
180361	2	1	1	≤ 1	≤ 0.125	≤ 0.125	0.25	1	≤ 1/19
23063	8	4	2	≤ 1	0.25	0.25	0.5	1	≤ 1/19
AG11									
101559	32	8	4	32	≤ 0.125	≤ 0.125	0.5	1	≤ 1/19
219537	16	4	2	4	≤ 0.125	0.25	0.25	1	≤ 1/19
27611	16	4	2	2	0.25	0.5	1	4	≤ 1/19
105947	16	4	2	8	0.25	0.25	0.5	1	≤ 1/19
Other spp.									
20715 (AG13)	32	16	4	64	1	≥ 32	1	1	≤ 1/19
30293 (AG13)	8	8	2	8	≤ 0.125	≤ 0.125	0.5	2	≤ 1/19
89182 (AH)	4	1	0.5	≤ 1	0.25	≤ 0.125	2	16	≤ 1/19
88427 (AR)	16	4	4	≤ 1	0.5	0.25	1	1	≤ 1/19
87374 (AR)	4	2	2	≤ 1	0.25	≤ 0.125	0.5	1	≤ 1/19
227313 (AS)	2	4	0.25	8	≤ 0.125	0.25	0.5	0.5	≤ 1/19
103727 (AT)	16	8	2	≤ 1	0.5	0.5	2	4	≤ 1/19
101055 (AG10)	4	2	2	4	1	≥ 32	16	1	≤ 1/19

Ctx, cefotaxime; Caz, ceftazidime; Cfp, cefepime; Tzp[a], piperacillin-tazobactam (piperacillin component); Mpm, meropenem; Cip, ciprofloxacin; Gen, gentamicin; Amk, amikacin; Cot, trimethoprim-sulphamethoxazole; AG, *Acinetobacter* genospecies; AH, *A. haemolyticus*; AR, *A. radioresistens*; AS, *A. schindleri*; AT, *A. tjernbergiae*.

A. baumannii	Etest MIC method (μg/mL)						
	Amc[a]	Tic[b]	Ipm	Dpm	Col	Tig-MHA	Tig-ISA
229437	≥ 256	≥ 256	≥ 32	≥ 32	0.5	2	8
11648	16	64	4	2	1	2	8
26756	8	8	1	0.5	0.5	1	2
101049	8	8	2	1	1	1	2
20802	8	4	0.5	0.25	0.25	0.25	0.5
217068	8	8	0.5	0.5	0.5	1	2
12937	8	32	2	0.5	0.5	0.5	1
89848	4	4	0.25	≤ 0.125	≤ 0.125	0.0625	0.125
26750	8	8	0.5	0.25	0.25	0.5	1
181329	8	4	1	0.5	0.5	0.25	1
27623	8	4	2	1	1	0.5	2
102450	4	2	0.5	0.25	0.25	0.25	0.5
29419	4	4	0.25	≤ 0.125	≤ 0.125	0.5	2
14495	4	2	0.5	0.25	≤ 0.125	0.125	0.25
13207	8	4	0.5	≤ 0.125	0.25	0.25	0.5
100983	2	1	0.25	≤ 0.125	0.25	0.125	1
14773	4	4	0.25	≤ 0.125	≤ 0.125	0.25	0.5
33047	8	4	0.25	≤ 0.125	0.25	0.25	0.5
107228	4	4	≤ 0.125	≤ 0.125	≤ 0.125	0.25	0.5
107532	4	4	0.25	≤ 0.125	≤ 0.125	0.25	0.5
107245	4	4	0.25	≤ 0.125	0.25	0.25	0.5
31364	8	8	1	0.25	0.5	0.5	0.5
27021	8	8	0.5	0.25	0.25	0.25	0.5
233651	16	8	≤ 0.125	≤ 0.125	0.25	0.25	0.5
232982	8	4	≤ 0.125	≤ 0.125	0.25	0.25	0.5
218722	16	8	0.5	0.25	0.5	0.5	0.5
220165	2	1	0.25	≤ 0.125	0.25	0.25	0.5
A. calcoaceticus							
33241	8	8	0.5	≤ 0.125	0.25	0.125	0.25
92328	8	8	0.25	≤ 0.125	≤ 0.125	0.25	0.5
183262	16	16	0.25	0.25	0.5	0.125	0.125
20304	16	8	0.25	≤ 0.125	0.25	0.125	0.25
28263	8	4	≤ 0.125	≤ 0.125	0.25	0.125	0.25
100558	4	2	0.25	0.25	≤ 0.125	0.125	0.5

Amc[a], amoxicillin-clavulanic acid (amoxicillin component); Tic[b], ticarcillin-clavulanic acid (ticarcillin component); Ipm, imipenem; Dpm, doripenem; Col, colistin; Tig-MHA, tigecycline (Mueller-Hinton agar); Tig-ISA, tigecycline (IsoSensitest agar).

AG3	Etest MIC method (μg/mL)						
	Amc[a]	Tic[b]	Ipm	Dpm	Col	Tig-MHA	Tig-ISA
29540	≥ 256	≥ 256	≥ 32	≥ 32	0.5	0.5	2
32357	≥ 256	≥ 256	≥ 32	≥ 32	0.5	0.5	2
36277	≥ 256	≥ 256	≥ 32	≥ 32	0.5	0.5	2
12659	≥ 256	≥ 256	≥ 32	16	0.5	0.5	2
23269	≥ 256	≥ 256	≥ 32	16	0.5	0.5	2
14448	≥ 256	≥ 256	≥ 32	16	0.5	0.5	2
14748	≥ 256	≥ 256	≥ 32	16	0.5	0.5	2
27616	≥ 256	≥ 256	≥ 32	16	0.5	0.5	2
103165	≥ 256	≥ 256	≥ 32	16	0.5	0.5	2
28594	≥ 256	≥ 256	≥ 32	16	0.5	0.5	2
206479	8	4	0.5	0.25	0.25	0.125	0.25
106770	8	4	0.5	0.25	0.5	0.5	0.5
27458	8	8	0.5	0.25	0.5	1	2
88276	4	4	0.5	≤ 0.125	0.25	0.125	0.25
105621	4	2	0.25	≤ 0.125	≤ 0.125	0.125	0.25
86096	8	4	0.5	≤ 0.125	≤ 0.125	0.125	0.25
107012	8	4	0.5	0.25	0.25	0.25	0.5
14027	8	4	1	0.5	0.5	2	2
30077	8	8	0.5	0.25	0.5	0.25	0.5
22344	16	8	1	0.5	0.5	0.25	0.5
12193	16	16	2	0.5	0.5	0.25	0.5
93743	8	4	1	0.25	0.25	0.5	1
35272	8	8	2	1	0.5	0.25	0.5
86661	4	4	1	0.5	0.5	1	4
12253	8	16	2	1	0.5	0.25	0.5
28933	8	8	1	0.25	0.25	0.25	0.5
20949	16	4	0.25	0.25	0.25	0.25	0.25
31479	4	2	0.5	≤ 0.125	0.25	0.125	0.25
106498	8	8	0.5	≤ 0.125	0.25	0.25	0.25
106008	4	2	0.25	≤ 0.125	≤ 0.125	0.5	0.25
183436	8	4	1	0.25	0.5	0.25	0.25

AG3, *Acinetobacter* genospecies 3; Amc[a], amoxicillin-clavulanic acid (amoxicillin component); Tic[b], ticarcillin-clavulanic acid (ticarcillin component); Ipm, imipenem; Dpm, doripenem; Col, colistin; Tig-MHA, tigecycline (Mueller-Hinton agar); Tig-ISA, tigecycline (IsoSensitest agar).

AG3	Etest MIC method (µg/mL)						
	Amc[a]	Tic[b]	Ipm	Dpm	Col	Tig-MHA	Tig-ISA
29420	16	8	0.5	≤ 0.125	0.25	0.25	0.25
14331	8	8	1	0.25	0.5	0.5	0.5
27035	4	≤ 1	0.5	0.25	0.5	0.5	0.5
25904	8	16	0.25	≤ 0.125	0.25	0.25	0.5
16198	8	8	0.5	≤ 0.125	0.5	0.25	0.25
82991	4	4	0.25	≤ 0.125	≤ 0.125	0.125	0.125
181882	8	4	0.5	0.25	≤ 0.125	0.125	0.5
13320	8	8	0.5	0.25	0.25	0.25	0.25
101327	16	8	0.25	0.25	0.25	0.125	0.25
91135	4	2	≤ 0.125	≤ 0.125	≤ 0.125	0.125	0.25
106449	4	≤ 1	≤ 0.125	≤ 0.125	≤ 0.125	0.5	0.25
35168	8	4	0.5	≤ 0.125	0.25	0.25	0.5
232787	4	4	0.5	≤ 0.125	0.25	0.125	0.25
225991	8	8	0.5	0.25	0.25	0.25	0.5
A. johnsonii							
25868	≥ 256	≥ 256	≥ 32	≥ 32	0.5	0.5	0.25
100441	2	≤ 1	0.5	≤ 0.125	0.25	0.25	0.5
182952	2	≤ 1	0.25	≤ 0.125	0.5	0.125	0.25
26612	4	2	0.25	≤ 0.125	0.25	0.125	0.125
181441	2	≤ 1	0.25	≤ 0.125	0.25	0.0625	0.0625
28001	≤ 1	≤ 1	≤ 0.125	≤ 0.125	0.25	0.0625	0.125
226419	2	2	≤ 0.125	≤ 0.125	0.5	0.0625	0.125
214737	≤ 1	≤ 1	0.25	≤ 0.125	0.25	0.0625	0.125
215759	≤ 1	≤ 1	0.25	≤ 0.125	≤ 0.125	0.0625	0.0625
215194	2	≤ 1	0.25	≤ 0.125	0.25	0.0625	0.0625
215687	≤ 1	≤ 1	0.25	≤ 0.125	≤ 0.125	0.0625	0.0625
221358	≤ 1	≤ 1	≤ 0.125	≤ 0.125	0.25	0.0625	0.125
A. ursingii							
88389	2	≤ 1	0.25	≤ 0.125	≤ 0.125	0.5	0.5
183357	2	≤ 1	0.25	≤ 0.125	0.5	0.25	0.5
89302	2	≤ 1	0.5	0.25	≤ 0.125	1	1
90772	2	2	0.5	0.25	≤ 0.125	0.125	0.25
217818	≤ 1	≤ 1	≤ 0.125	≤ 0.125	≤ 0.125	0.5	1
91411	2	2	0.25	≤ 0.125	≤ 0.125	0.25	0.5
91352	2	2	0.25	≤ 0.125	≤ 0.125	0.25	0.5
30431	≤ 1	≤ 1	0.25	≤ 0.125	0.25	0.25	0.5

AG3, *Acinetobacter* genospecies 3; Amc[a], amoxicillin-clavulanic acid (amoxicillin component); Tic[b], ticarcillin-clavulanic acid (ticarcillin component); Ipm, imipenem; Dpm, doripenem; Col, colistin; Tig-MHA, tigecycline (Mueller-Hinton agar); Tig-ISA, tigecycline (IsoSensitest agar).

A. lwoffii /AG9	Etest MIC method (µg/mL)						
	Amc[a]	Tic[b]	Ipm	Dpm	Col	Tig-MHA	Tig-ISA
203910	8	4	≤0.125	≤0.125	≤0.125	0.125	0.125
22104	2	1	≤0.125	≤0.125	≤0.125	0.0625	0.125
190152	16	8	1	0.25	0.5	0.125	0.25
107690	1	≤0.125	≤0.125	≤0.125	0.25	0.0625	0.125
184576	2	≤0.125	0.25	0.25	0.25	0.125	0.125
180361	2	0.5	0.5	≤0.125	0.25	0.125	0.25
23063	2	1	0.25	≤0.125	0.25	0.125	0.25
AG11							
101559	8	8	0.5	≤0.125	≤0.125	0.25	0.5
219537	2	≤0.125	0.25	≤0.125	0.25	0.25	0.25
27611	8	4	0.25	0.25	0.25	0.25	0.5
105947	4	4	0.25	≤0.125	0.25	0.25	0.5
Other spp.							
20715 (AG13)	8	8	1	0.25	0.5	0.5	1
30293 (AG13)	8	32	0.25	≤0.125	0.25	0.125	0.125
89182 (AH)	1	0.5	0.5	0.25	0.5	0.25	0.5
88427 (AR)	2	1	0.5	0.25	0.25	0.25	0.5
87374 (AR)	2	0.5	0.5	≤0.125	0.25	0.25	0.5
227313 (AS)	2	1	0.25	≤0.125	≤0.125	0.125	0.25
103727 (AT)	4	2	1	0.25	0.25	0.125	0.25
101055 (AG10)	8	4	2	0.5	0.5	1	1

Amc[a], amoxicillin-clavulanic acid (amoxicillin component); Tic[b], ticarcillin-clavulanic acid (ticarcillin component); Ipm, imipenem; Dpm, doripenem; Col, colistin; Tig-MHA, tigecycline (Mueller-Hinton agar); Tig-ISA, tigecycline (IsoSensitest agar); AG, *Acinetobacter* genospecies; AH, *A. haemolyticus*; AR, *A. radioresistens*; AS, *A. schindleri*; AT, *A. tjernbergiae*.

Appendix 2. Growth kinetics of *A. baumannii* and *Acinetobacter* genospecies 3 isolates in LB broth.

Time (h)	OD_{600} measurements of growth of isolates							
	A. baumannii				*Acinetobacter* genosp. 3			
	ATCC 19606	100983	11648	229437	106770	27458	27616	36277
0	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005
0.5	0.008	0.009	0.008	0.009	0.006	0.007	0.007	0.005
1.0	0.018	0.018	0.015	0.023	0.010	0.009	0.012	0.010
1.5	0.048	0.047	0.040	0.070	0.020	0.021	0.024	0.014
2.0	0.140	0.154	0.128	0.238	0.061	0.055	0.062	0.030
2.5	0.312	0.360	0.296	0.558	0.132	0.126	0.135	0.057
3.0	0.744	0.796	0.660	1.145	0.343	0.318	0.345	0.114
3.5	1.27	1.30	1.18	1.63	0.724	0.658	0.690	0.270
4.0	1.64	1.42	1.59	1.82	1.47	1.18	1.29	0.466
4.5	1.97	1.66	1.91	2.11	1.79	1.59	1.60	0.825
5.0	2.00	1.79	2.33	2.42	2.23	2.13	2.11	1.30
6.0	2.18	2.18	2.83	2.98	2.98	2.59	2.58	2.09
7.0	2.28	2.25	3.25	3.09	3.29	3.23	3.25	2.69
8.0	2.34	2.37	3.58	3.35	3.62	3.58	3.36	3.01
9.0	2.47	2.42	3.99	3.38	3.83	3.69	3.80	3.13
10.0	2.54	2.48	4.42	3.74	4.22	4.10	4.00	3.34
24.0	2.68	3.46	4.72	4.00	4.52	5.56	5.78	4.52

Appendix 3. Results of qRT-PCR assays for the relative expression of *16S rRNA*, *ade* genes and bla_{OXA-23} in *Acinetobacter* isolates.

A. qRT-PCR results of *16S rRNA* and *adeB* expression in *A. baumannii* isolates.

Isolate	Absence of meropenem				Presence of meropenem			
	Tripli-cate no.	C_T *16S rRNA*	C_T *adeB*	ΔC_T	Tripli-cate no.	C_T *16S rRNA*	C_T *adeB*	ΔC_T
ATCC 19606	1	5.84	28.64	22.8	1	5.92	27.93	22.01
	2	5.87	28.79	22.92	2	5.72	27.9	22.18
	3	5.52	28.61	23.09	3	5.89	28.14	22.25
	Average ΔC_T			22.94	Average ΔC_T			22.15
100983	1	5.91	35.76	29.85	1	5.32	35.02	29.7
	2	6.4	36.25	29.85	2	5.78	34.93	29.15
	3	5.57	35.55	29.98	3	5.84	35.0	29.16
	Average ΔC_T			29.89	Average ΔC_T			29.34
11648	1	5.8	30.3	24.5	1	5.53	29.41	23.88
	2	5.55	30.38	24.83	2	5.55	29.6	24.05
	3	5.52	29.76	24.24	3	5.83	29.76	23.93
	Average ΔC_T			24.52	Average ΔC_T			23.95
229437	1	6.64	30.87	24.23	1	6.11	29.8	23.69
	2	6.2	30.74	24.54	2	5.85	29.29	23.44
	3	6.55	30.95	24.4	3	6.26	30.46	24.2
	Average ΔC_T			24.39	Average ΔC_T			23.78

B. qRT-PCR results of *16S rRNA* and *adeJ* expression in *A. baumannii* isolates.

Isolate	Absence of meropenem				Presence of meropenem			
	Tripli-cate no.	C_T *16S rRNA*	C_T *adeJ*	ΔC_T	Tripli-cate no.	C_T *16S rRNA*	C_T *adeJ*	ΔC_T
ATCC 19606	1	5.77	20.15	14.38	1	5.95	19.27	13.32
	2	5.99	20.34	14.35	2	5.89	20.09	14.2
	3	5.83	20.12	14.29	3	6.26	19.83	13.57
	Average ΔC_T			14.34	Average ΔC_T			13.7
100983	1	5.19	18.56	13.37	1	5.93	19.26	13.33
	2	5.87	19.16	13.29	2	6.2	19.44	13.24
	3	5.81	19.5	13.69	3	5.82	19.24	13.42
	Average ΔC_T			13.45	Average ΔC_T			13.33
11648	1	5.88	20.37	14.49	1	5.84	20.16	14.32
	2	5.6	20.26	14.66	2	5.66	20.17	14.51
	3	5.9	20.57	14.67	3	6.06	20.67	14.61
	Average ΔC_T			14.61	Average ΔC_T			14.48
229437	1	6.31	21.84	15.53	1	6.03	21.32	15.29
	2	6.74	22.24	15.5	2	6.19	21.44	15.25
	3	6.19	21.76	15.57	3	5.94	21.09	15.15
	Average ΔC_T			15.53	Average ΔC_T			15.23

C. qRT-PCR results of *16S rRNA* and *adeE* expression in *Acinetobacter* genospecies 3 isolates.

Isolate	Absence of meropenem				Presence of meropenem			
	Tripli-cate no.	C_T *16S rRNA*	C_T *adeE*	ΔC_T	Tripli-cate no.	C_T *16S rRNA*	C_T *adeE*	ΔC_T
106770	1	6.89	29.74	22.85	1	6.37	28.77	22.4
	2	5.84	28.55	22.71	2	5.9	28.74	22.84
	3	5.54	29.07	23.53	3	6.11	28.71	22.6
	Average ΔC_T			23.03	Average ΔC_T			22.61
27458	1	6.81	29.21	22.4	1	6.07	28.95	22.88
	2	6.7	29.63	22.93	2	6.08	28.4	22.32
	3	7.56	30.11	22.55	3	5.93	27.91	21.98
	Average ΔC_T			22.63	Average ΔC_T			22.39
27616	1	6.96	29.56	22.6	1	6.32	28.82	22.5
	2	6.8	29.18	22.38	2	5.61	28.25	22.64
	3	5.75	28.64	22.89	3	5.77	27.79	22.02
	Average ΔC_T			22.62	Average ΔC_T			22.39
36277	1	6.13	27.61	21.48	1	5.94	27.36	21.42
	2	6.06	27.63	21.57	2	7.49	28.49	21
	3	6.42	27.85	21.43	3	7.11	28.2	21.09
	Average ΔC_T			21.49	Average ΔC_T			21.17

D. qRT-PCR results of *16S rRNA* and *adeY* expression in *Acinetobacter* genospecies 3 isolates.

Isolate	Absence of meropenem				Presence of meropenem			
	Tripli-cate no.	C_T *16S rRNA*	C_T *adeY*	ΔC_T	Tripli-cate no.	C_T *16S rRNA*	C_T *adeY*	ΔC_T
106770	1	7.13	24.08	16.95	1	7.15	23.64	16.49
	2	7.38	24.31	16.93	2	7.29	23.84	16.55
	3	7.55	24.58	17.03	3	6.98	24.9	17.92
	Average ΔC_T			16.97	Average ΔC_T			16.98
27458	1	7.73	24.65	16.92	1	6.89	24.03	17.14
	2	7.5	24.59	17.09	2	6.76	23.49	16.73
	3	7.0	23.87	16.87	3	6.5	23.18	16.68
	Average ΔC_T			16.96	Average ΔC_T			16.85
27616	1	7.36	24.43	17.07	1	6.43	23.24	16.81
	2	7.01	23.97	16.96	2	7.06	24.04	17.02
	3	7.0	24.1	17.1	3	7.22	23.89	16.67
	Average ΔC_T			17.04	Average ΔC_T			16.83
36277	1	7.76	24.28	16.52	1	7.13	23.86	16.73
	2	7.77	24.77	17.0	2	7.18	23.62	16.44
	3	7.52	23.87	16.35	3	6.87	23.02	16.15
	Average ΔC_T			16.62	Average ΔC_T			16.44

E. qRT-PCR results of *16S rRNA* and *bla*$_{OXA-23}$ expression in *A. baumannii* and *Acinetobacter* genospecies 3 isolates.

Isolate	Absence of meropenem				Presence of meropenem			
	Triplicate no.	C_T *16S rRNA*	C_T *bla*$_{OXA-23}$	ΔC_T	Triplicate no.	C_T *16S rRNA*	C_T *bla*$_{OXA-23}$	ΔC_T
27616	1	7.50	20.44	12.94	1	5.67	18.96	13.29
	2	6.59	20.38	13.79	2	5.79	18.85	13.06
	3	6.33	19.28	12.95	3	7.06	20.56	13.5
	Average ΔC_T			13.27	Average ΔC_T			13.28
36277	1	7.82	20.38	12.56	1	7.18	19.49	12.31
	2	7.64	20.29	12.65	2	7.51	20.36	12.85
	3	8.03	20.66	12.63	3	6.14	18.69	12.55
	Average ΔC_T			12.61	Average ΔC_T			12.57
229437	1	8.04	20.32	12.28	1	7.31	20.0	12.69
	2	6.69	18.9	12.21	2	7.09	19.97	12.88
	3	6.57	19.31	12.74	3	7.25	18.88	11.63
	Average ΔC_T			12.41	Average ΔC_T			12.4

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First report of OXA-23 carbapenemase in clinical isolates of *Acinetobacter* species in the Irish Republic

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Sir,

Meropenem and imipenem are carbapenems that remain active against organisms carrying most Ambler classes A and C β-lactamases which include many Gram-negative bacilli, including *Acinetobacter* spp. However, carbapenem resistance is increasingly encountered in *Acinetobacter* isolates worldwide.[1] Mechanisms of carbapenem resistance include the loss of porins, increase in efflux activity and the production of Ambler class B metallo-β-lactamases such as VIM and IMP enzymes. Another mechanism is the production of Ambler class D oxacillinases (OXA enzymes) with carbapenem-hydrolysing activity, such as OXA-23 and OXA-51 carbapenemases. A multidrug-resistant OXA-23 carbapenemase-producing *Acinetobacter baumannii* clone has spread rapidly among UK hospitals in recent years.[2] We report two *Acinetobacter* isolates producing OXA-23 enzyme in the Irish Republic.

Two meropenem-resistant *Acinetobacter* isolates were encountered in our hospital in 2005. The first isolate (05/29540) was isolated from a biliary drain fluid specimen of a 62-year-old patient who underwent a laparotomy for gall bladder perforation and associated intra-abdominal sepsis. She had underlying pancreatic carcinoma and diabetes mellitus. Recent antimicrobial therapy included piperacillin/tazobactam, ciprofloxacin and vancomycin. The patient did not receive a carbapenem prior to the isolation of the organism. The second isolate (05/12659) was cultured from a sputum specimen of a 49-year-old patient with a history of complicated inflammatory bowel disease associated with recent intensive care (ICU) admission for intra-abdominal sepsis and methicillin-resistant *Staphylococcus aureus* vascular catheter-related bloodstream infection. Recent antimicrobial treatment included meropenem, ciprofloxacin, vancomycin and caspofungin. He was also on immunosuppressants, methotrexate and infliximab, and was neutropenic (neutrophil count of $<0.1 \times 10^9$/L) at the time of the organism's isolation. The two cases were epidemiologically unrelated in time or space.

Both isolates were presumptively identified as *A. baumannii* using the Vitek-2 automated identification and susceptibility test system (bioMerieux, Basingstoke, UK), but amplified ribosomal DNA restriction analysis (ARDRA) subsequently confirmed both isolates to be *Acinetobacter* genomic species 3. Both isolates demonstrated resistance to β-lactams including meropenem (MIC $\geq$ 16 mg/L) and to quinolones. 05/29540 was also resistant to gentamicin and co-trimoxazole but susceptible to tobramycin and amikacin. 05/12659 was susceptible to gentamicin, tobramycin, amikacin and co-trimoxazole. Antimicrobial susceptibility results with agar dilution and Etest (AB Biodisk, Solna, Sweden) methods using CLSI guidelines are shown in Table 1.

Using Etest metallo-β-lactamase (MBL) strips (imipenem MIC: imipenem + EDTA MIC) (AB Biodisk), ratios of $\geq$24 and $\geq$12 were obtained for 05/29540 and 05/12659, respectively, suggesting the presence of MBL activity. However, PCR using VIM and IMP primers did not reveal the presence of relevant amplicons on agarose gel electrophoresis. Isoelectric focusing revealed a β-lactamase with a pI value of 6.7 for both isolates as well as the OXA-23 positive control, suggesting the presence of OXA-23 carbapenemase activity. PCR using OXA-23-like primers revealed a single amplicon in the region between 800 and 900 bp in size for both isolates and the OXA-23 positive control.[3] Nucleotide sequencing of the amplicons demonstrated >99.5% and 100% homology with the $bla_{\text{OXA-23}}$ carbapenemase gene (GenBank database accession number AJ132105), from bp 24 to bp 822 for 05/29540 and from bp 31 to bp 822 for 05/12659.[3] Nucleotide sequences in the regions of variation between $bla_{\text{OXA-23}}$, $bla_{\text{OXA-27}}$ and $bla_{\text{OXA-49}}$ are all consistent with $bla_{\text{OXA-23}}$ for both isolates. PCR with OXA-51-like primers did not reveal the presence of a $bla_{\text{OXA-51-like}}$ carbapenemase gene in either isolate,[4] while amplicons of the expected size were obtained with the positive control as well as the *A. baumannii* ATCC 19606 strain. PCR also did not detect the presence of class 1 integrons in both isolates.[2] DNA macrorestriction followed by PFGE revealed the two isolates' profiles to be distinct from one another, suggesting that they are not clonally related.

OXA carbapenemases are increasingly encountered worldwide, especially in the nosocomial setting. To our knowledge, these are the first reported isolates of OXA-23 carbapenemases in *Acinetobacter* spp. in the Irish Republic. They were associated with risk factors for antimicrobial resistance such as prior antibiotic treatment, ICU admission, immunosuppression and severe underlying diseases. *Acinetobacter* genomic species 3, like *A. baumannii*, has been associated with nosocomial cross-infection and is the predominant *Acinetobacter* sp. in some institutions.[5] In view of their roles in nosocomial outbreaks, accurate speciation of *Acinetobacter* spp. is essential. Thus we would like to highlight the problem of misidentification of *Acinetobacter* spp. by commercial systems utilizing phenotypic tests (e.g.Vitek-2) and the need for further molecular typing for accurate speciation.

Interestingly, both isolates met the screening criterion (imipenem to imipenem/EDTA ratio of $\geq$8) for MBL activity using the Etest MBL strips, even though subsequent PCR assays did not indicate the presence of bla_{VIM} or bla_{IMP} genes. Such a phenomenon has also been observed by Segal *et al.*[6] However, the imipenem to imipenem/EDTA ratios of 05/29540 and 05/12659 were lower (8 and 4, respectively) with the agar dilution method, thus suggesting that the latter method may be more specific for the detection of MBL activity. Therefore, Etest for the detection of MBL in *Acinetobacter* spp. must be used with caution and requires further validation before a positive result is conclusive.

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Table 1. Antimicrobial susceptibility patterns of the *Acinetobacter* isolates

Antibiotic	05/29540	05/12659
MICs (mg/L) using the agar dilution method		
Ampicillin	>128	>128
Piperacillin	>128	>128
Amoxicillin/clavulanate	>128	>128
Piperacillin/tazobactam	128	128
Cefoxitin	128	>128
Cefotaxime	64	32
Ceftazidime	32	16
Meropenem	64	128
Meropenem + EDTA (320 mg/L)	8	32
Imipenem	128	128
Imipenem + EDTA (320 mg/L)	16	32
Nalidixic acid	>128	>128
Ciprofloxacin	8	4
Gentamicin	32	1
Amikacin	0.5	2
MICs (mg/L) using the Etest method		
Meropenem	≥32	≥32
Imipenem (MBL test)	24	12
Imipenem + EDTA (MBL test)	≤1	≤1
Tigecycline	0.25	0.25
Colistin	0.25	0.5

The presence of OXA-23 carbapenemase-producing *Acinetobacter* spp. in the Irish Republic has not yet been associated with outbreak problems as seen in the UK. Nevertheless, the emergence of such a resistance mechanism in *Acinetobacter* isolates represents a worrying trend, although it is probably unsurprising given the recent trends in carbapenem resistance elsewhere in the world. We would like to reiterate the importance of prudence in antimicrobial prescription and adherence to infection control measures in the efforts to control the rise of such resistance mechanisms, as well as the urgent need for new antimicrobial agents in the face of pan-β-lactam resistance.

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Transparency declarations

We have no affiliations with the pharmaceutical industry or any related commercial concerns.

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Antibiotic susceptibility of 50 clinical isolates of *Burkholderia pseudomallei* from Singapore

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Sir,

Burkholderia pseudomallei is the aetiological agent of melioidosis, a potentially fatal disease in humans and animals. In Singapore, a high incidence of melioidosis cases was observed in early 2004 with a high mortality rate (40%).[1] Prevention of exposure is difficult as this organism is common in the soil of many parts of south-east Asia. In the absence of a vaccine, antibiotic prophylaxis for those predisposed to melioidosis could be explored. Others have shown that oral doxycycline/ciprofloxacin could prevent melioidosis in experimentally infected mice.[2] We thus examined the *in vitro* susceptibility of 50 clinical isolates obtained from five local hospitals in Singapore, between the years 1996 and 2004, of which 31 were from the outbreak in 2004,[1] to four oral antibiotics, namely amoxicillin/clavulanic acid, doxycycline, ciprofloxacin and co-trimoxazole.

MICs were determined by the Etest (AB Biodisk, Solna, Sweden) method using Mueller–Hinton (MHII) agar (Oxoid, Basingstoke, UK) and the plates were read after incubation at 37°C for 24 h. The MIC of each antibiotic (in mg/L) for each *B. pseudomallei* isolate was reported as susceptible, intermediate or resistant as per CLSI[3] guidelines: ≤8/4, 16/8 and ≥32/16 for amoxicillin/clavulanic acid, ≤4, 8 and ≥16 for doxycycline, ≤2/38, – and ≥4/76 for co-trimoxazole and ≤1, 2 and ≥4 for ciprofloxacin. *E. coli* ATCC 25922 and *Pseudomonas aeruginosa*

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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 β-lactamase OXA-23 in all 12 isolates, with insertion sequence IS*Aba1* found upstream of bla_{OXA-23}. IS*Aba1* was also found upstream of bla_{ADC-25}, 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_{OXA-23} 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 β-lactam/β-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 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 β-lactams with the broadest spectra of activity. They are clinically effective against Gram-negative organisms producing Ambler classes A and C extended-spectrum β-lactamases. However, carbapenem resistance in *Acinetobacter* has been increasingly reported worldwide, attributable mainly to Ambler class D carbapenemases and class B metallo-β-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.

Abbreviations: AG3, *Acinetobacter* genomic species 3; CLSI, Clinical and Laboratory Standards Institute; ICU, intensive care unit; MBL, metallo β-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_{OXA-23}/ATPase), EU827525 (*Acinetobacter johnsonii* IS*Aba1*/bla_{OXA-23}/ATPase), EU827526 (*Acinetobacter baumannii* IS*Aba1*/bla_{OXA-23}/ATPase), EU835512 (*Acinetobacter baumannii* IS*Aba1*/bla_{ADC-25}), EU839488 (*Acinetobacter* genomic species 3 IS*Aba1*/*aacC2*), EU872057 (*Acinetobacter johnsonnii aacC2*).

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

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 β-lactamase-encoding genes bla_{VIM}, bla_{IMP}, bla_{SPM}, bla_{GIM} and bla_{SIM} (Castanheira *et al.*, 2004; Lee *et al.*, 2005; Pitout *et al.*, 2005), as well as Ambler class D carbapenemase-encoding genes $bla_{OXA\text{-}23\text{-}like}$, $bla_{OXA\text{-}24\text{-}like}$, $bla_{OXA\text{-}51\text{-}like}$ and $bla_{OXA\text{-}58}$ (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 β-lactamase-encoding genes (bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, bla_{VEB} and bla_{PER}) (Hopkins *et al.*, 2006; Naas *et al.*, 2006) and Ambler class C *Acinetobacter*-derived cephalosporinase-encoding genes (bla_{ADC}) (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 3130*xl* 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) and compared with existing GenBank sequences using the BLAST programme (www.ncbi.nih.gov/blast). The sequences of our isolates were submitted to GenBank, National Center for Biotechnology Information (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 *Apa*I 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)

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

*Based on the Etest method; no isolate had an MIC of 8 or 16 mg l^{-1}.

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 $\geqslant 32$ mg l^{-1}) (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 $\leqslant 4$ mg l^{-1}). 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, cefoxitin, meropenem, imipenem and ciprofloxacin., while all isolates had colistin MICs of $\leqslant 0.5$ mg l^{-1}. Ratios of $\geqslant 8$ 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 $\leqslant 0.5$ mg l^{-1}, while the *A. baumannii* isolate had a tigecycline MIC of 2 mg l^{-1}.

Carbapenem-resistance genes and their genetic surroundings

The resistance genes of the various isolates are summarized in Table 3. PCR was negative for bla_{VIM}, bla_{IMP}, bla_{SPM}, bla_{GIM}, bla_{SIM}, $bla_{OXA\text{-}24\text{-}like}$ and $bla_{OXA\text{-}58}$. 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^{-1})	I (16 mg l^{-1})	R (128–$\geqslant$256 mg l^{-1})	R ($\geqslant$256 mg l^{-1})
Ceftazidime	S (4–8 mg l^{-1})	S (8 mg l^{-1})	I/R (16–32 mg l^{-1})	R ($\geqslant$256 mg l^{-1})
Cefepime	I/R (16–64 mg l^{-1})	R (32 mg l^{-1})	R (64–128 mg l^{-1})	R ($\geqslant$256 mg l^{-1})
Trimethoprim–sulfamethoxazole	S ($\leqslant$1/19 mg l^{-1})	R ($\geqslant$32/608 mg l^{-1})	R ($\geqslant$32/608 mg l^{-1})	R ($\geqslant$32/608 mg l^{-1})
Gentamicin	S (0.25–1 mg l^{-1})	R (128 mg l^{-1})	R (32 mg l^{-1})	R ($\geqslant$256 mg l^{-1})
Amikacin	S (0.5–2 mg l^{-1})	S (1 mg l^{-1})	S (0.5–2 mg l^{-1})	R ($\geqslant$256 mg l^{-1})
Types of isolates (no.)	AG3 (7)	*A. johnsonii* (1)	AG3 (3)	*A. baumannii* (1)
Isolate identifier	S659, A629, S448, S748, S165, A594, A616	A868	A540, A277, A357	U437

A. baumannii was positive for both $bla_{\text{OXA-23-like}}$ and $bla_{\text{OXA-51-like}}$ genes, while the other 11 isolates were positive for the $bla_{\text{OXA-23-like}}$ gene only. Nucleotide sequencing of the $bla_{\text{OXA-23-like}}$ amplicons revealed that all 12 isolates carried the classical $bla_{\text{OXA-23}}$ 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 IS*Aba1* was found immediately upstream of $bla_{\text{OXA-23}}$ in all 12 isolates (separated by 17 nucleotides); while the ATPase-encoding gene was found downstream of it. The regions between IS*Aba1* and $bla_{\text{OXA-23}}$ of all 12 isolates were identical in sequence, as were the regions between $bla_{\text{OXA-23}}$ and ATPase-encoding gene (104 nucleotides in length). IS*Aba1* was not present upstream of the $bla_{\text{OXA-51-like}}$ gene in the *A. baumannii* isolate. Hence the β-lactamase-encoding gene contributing to carbapenem resistance for all 12 isolates from St James's Hospital was $bla_{\text{OXA-23}}$, since IS*Aba1* was shown to possess promoter sequences that upregulated expression of the β-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 β-lactamase-encoding genes (bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, bla_{VEB} and bla_{PER}) 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 $bla_{\text{ADC-18}}$ (>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	IS*Aba1*+OXA-23	ADC-18	IS*Aba1*+*aacC2*	−
S659	AG3	IS*Aba1*+OXA-23	ADC-18	−	−
A629	AG3	IS*Aba1*+OXA-23	ADC-18	−	−
S448	AG3	IS*Aba1*+OXA-23	ADC-18	−	−
S748	AG3	IS*Aba1*+OXA-23	ADC-18	−	−
A594	AG3	IS*Aba1*+OXA-23	ADC-18	−	−
A616	AG3	IS*Aba1*+OXA-23	ADC-18	−	−
A277	AG3	IS*Aba1*+OXA-23	ADC-18	IS*Aba1*+*aacC2*	−
A357	AG3	IS*Aba1*+OXA-23	ADC-18	IS*Aba1*+*aacC2*	−
S165	AG3	IS*Aba1*+OXA-23	ADC-18	−	−
U437	*A. baumannii*	OXA-51 group; IS*Aba1*+OXA-23	IS*Aba1*+ADC-25	*aphA6*	+
A868	*A. johnsonii*	IS*Aba1*+OXA-23	?*	*aacC2*	−

+, Present; −, absent

*PCR product not obtained with study primers.

baumannii isolate was positive for bla_{ADC-25} (>99 % concordance with GenBank accession number EF016355). We were unable to obtain an amplicon for the *A. johnsonii* isolate with the bla_{ADC} 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 β-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. IS*Aba1* was found upstream of the *A. baumannii* bla_{ADC-25}, but not of the bla_{ADC-18} 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). IS*Aba1* was found upstream of the *aacC2* gene in all three isolates, with a truncated 3′ end of insertion sequence IS*1133* found between IS*Aba1* and *aacC2* (GenBank accession number EU839488). The *aacC2* gene was also found in the *A. johnsonii* isolate, but without IS*Aba1* upstream of it. This study demonstrates the role of IS*Aba1* in the regulation of various β-lactamase-encoding genes in *A. baumannii* and also in other *Acinetobacter* species. Not only was it found upstream of the bla_{OXA-23} of various species, but was also found upstream of the bla_{ADC-25} gene in *A. baumannii.* Upregulation of bla_{ADC-25} 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 IS*Aba1* upstream of bla_{ADC-18}. The role of IS*Aba1* in the regulation of genes encoding aminoglycoside-modifying enzymes was less clear. Gentamicin MICs of AG3 isolates possessing IS*Aba1* upstream of *aacC2* were lower than MICs observed in *A. baumannii* and *A. johnsonii* where IS*Aba1* was absent upstream of *aphA6* and *aacC2*, respectively. This would suggest that IS*Aba1* 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 ⩾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_{OXA-23}, 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_{OXA-23} 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_{OXA-23} for the 40 MDa bands in both isolates, implying that bla_{OXA-23} 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_{OXA-23} 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_{OXA-23} 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	*A. johnsonii*	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 granulomatosus, diabetes mellitus
U437	*A. baumannii*	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 β-lactam/β-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 co-morbidities (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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Detection of *bla*$_{OXA-58}$ and *bla*$_{OXA-23-like}$ genes in carbapenem-susceptible *Acinetobacter* clinical isolates: should we be concerned?

Acquisition of OXA carbapenemases has emerged as one of the predominant mechanisms for carbapenem resistance in *Acinetobacter* spp. in many parts of the world. Four main groups of OXA carbapenemase-encoding genes are found in this genus, namely, *bla*$_{OXA-23-like}$, *bla*$_{OXA-40-like}$, *bla*$_{OXA-51-like}$ and *bla*$_{OXA-58}$ genes. *Acinetobacter baumannii* is the source of *bla*$_{OXA-51-like}$ genes, while more recently the reservoir for *bla*$_{OXA-23-like}$ genes has been identified as being *Acinetobacter radioresistens* (Turton *et al.*, 2006; Poirel *et al.*, 2008). However, carbapenem resistance is only manifested in isolates possessing strong promoters upstream of the *bla*$_{OXA}$ genes, such as insertion sequence IS*Aba1* (Turton *et al.*, 2006). We undertook a study to investigate the silent carriage of *bla*$_{OXA-58}$, *bla*$_{OXA-23-like}$ and *bla*$_{OXA-40-like}$ genes in carbapenem-susceptible isolates of uncommon *Acinetobacter* spp. and to characterize the genetic environments of such genes.

Non-duplicate *Acinetobacter* clinical isolates were collected in the Central Pathology Laboratory, St James's Hospital, from May 2005 to October 2007. They were speciated using sequencing of the ribosomal polymerase B subunit-encoding gene *rpoB* and flanking regions (La Scola *et al.*, 2006). Detection of *bla*$_{OXA}$ genes and characterization of the genetic surroundings of such genes were performed by PCR of whole-cell DNA extracts of isolates using a PCR core kit (Qiagen). The primers are shown in Supplementary Table S1 (available with the online journal). Sequencing of amplicons was performed in the Genomics Core Facility, Queens University Belfast, Belfast, UK, using an ABI Prism 3130xl analyser (Applied BioSystems). To find out if the *bla*$_{OXA}$ genes were plasmid mediated, plasmid extraction was performed using a Qiagen midi kit and the plasmid was used for PCR with the appropriate *bla*$_{OXA}$ primers. PCR for the *rpoB* gene was also performed to rule out chromosomal DNA carry-over. To locate the actual plasmids carrying these genes, agarose gel electrophoresis was first used to visualize individual plasmids, and subsequently, DNA from individual plasmid bands was eluted using a gel extraction kit (Qiagen) for use in PCR with *bla*$_{OXA}$ primers. PCR for the *rpoB* gene was again performed to rule out chromosomal DNA carry-over. Susceptibility testing was also performed on *bla*$_{OXA}$-positive isolates using the Etest method (bioMérieux) for meropenem, imipenem, cefepime, cefotaxime, ceftazidime, piperacillin–tazobactam, amoxicillin–clavulanate and ampicillin. A standard inoculum of 0.5 McFarland and a heavier inoculum of 2 McFarland were used.

A total of 37 isolates of various *Acinetobacter* spp. were collected during the 30 month period (the number of isolates of each species is shown in parentheses): *Acinetobacter johnsonnii* (12), *Acinetobacter ursingii* (8), *Acinetobacter* genomospecies 9 (AG9) (5), *Acinetobacter* genomospecies 11 (4), *Acinetobacter lwoffii* (2), *A. radioresistens* (2), *Acinetobacter* genomospecies 10 (AG10) (1), *Acinetobacter haemolyticus* (1), *Acinetobacter schindleri* (1) and *Acinetobacter tjernbergiae* (1).

All isolates were negative for *bla*$_{OXA-40-like}$ genes. The two *A. radioresistens* isolates (strains B472 and B374) were positive for *bla*$_{OXA-23-like}$ genes. In both isolates, a putative *O*-sialoglycoprotein endopeptidase-encoding (*gcp*) gene was present upstream of *bla*$_{OXA-23-like}$ (98.4 % sequence concordance with GenBank accession no. EU571228), while downstream of the *bla*$_{OXA-23-like}$ gene, the ATPase-encoding gene was present in both isolates [99 % (B472) and 100 % (B374) sequence concordance with GenBank accession no. EU131372]. Sequencing of the entire *bla*$_{OXA-23-like}$ genes revealed that B374 carried *bla*$_{OXA-23}$ while B472 carried a novel OXA gene *bla*$_{OXA-134}$. *bla*$_{OXA-134}$ differed from *bla*$_{OXA-23}$ by two amino acid substitutions (Δ10, valine→phenylalanine; Δ33, valine→alanine). *bla*$_{OXA-58}$ was found in two isolates, A361 (AG9) and S1055 (AG10). In both isolates, an IS*Aba3*-like transposase was present upstream of *bla*$_{OXA-58}$ (Fig. 1). The amino acid sequence of its C-terminus differed from the corresponding 23 amino acids of the IS*Aba3* transposase. Insertion sequences IS*Aba2*, IS*Aba1* and IS*18* were absent in our isolates. Downstream of *bla*$_{OXA-58}$, the insertion sequence IS*Aba3*, as well as a transcriptional regulator-encoding gene (*araC1*), were found in both isolates. The gene encoding the threonine efflux protein (*lysE*) was found downstream of the *araC1* gene in AG10 but not in AG9. Additional genes (putative esterase-encoding *est* and transcriptional regulator-encoding *araC2* genes) reported in the *A. baumannii* MAD strain were not found in either of our isolates (Poirel & Nordmann, 2006).

PCR of plasmid extracts of A361 and S1055 was positive for *bla*$_{OXA-58}$, indicating that these genes were plasmid-

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are: FJ195387 (*Acinetobacter radioresistens* strain B472 putative *O*-sialoglycoprotein endopeptidase-encoding (*gcp*) gene/*bla*$_{OXA-134}$ gene/ATPase-encoding gene); FJ195388 (*Acinetobacter radioresistens* strain B374 putative *O*-sialoglycoprotein endopeptidase-encoding (*gcp*) gene/*bla*$_{OXA-23}$ gene/ATPase-encoding gene); FJ195389 (*Acinetobacter* genomospecies 10 strain S1055 IS*Aba3*-like transposase-encoding gene/*bla*$_{OXA-58}$ gene/insertion sequence IS*Aba3*/*araC1* gene/*lysE* gene); FJ200197 (*Acinetobacter* genomospecies 9 strain A361 IS*Aba3*-like transposase-encoding gene/*bla*$_{OXA-58}$ gene/insertion sequence IS*Aba3*/*araC1* gene).

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

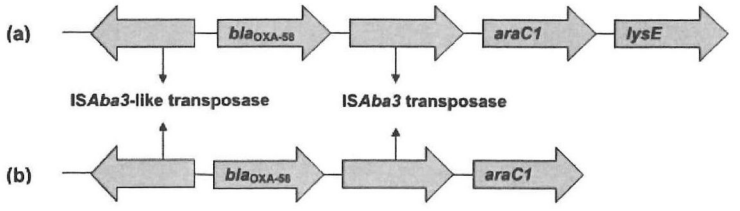

Fig. 1. Genetic environment of bla_{OXA-58} in carbapenem-susceptible AG10 (a) and AG9 (b) isolates.

mediated. PCR of plasmid extracts for $bla_{OXA-23-like}$ was negative for B374 and B472, suggesting the genes were chromosomally located. Four plasmids were visualized for S1055 (ranging from about 5 to 30 MDa), while six plasmids were visualized for A361 (ranging from about 1.5 to 25 MDa). The bla_{OXA-58} gene was found on the approximately 30 MDa plasmid and the approximately 22 MDa plasmid in S1055 and A361, respectively. Antimicrobial susceptibility results are shown in Table 1. All four isolates were susceptible to meropenem and imipenem, and no carbapenem-resistant subpopulations were observed even with the heavier inocula.

Our *A. radioresistens* findings support the hypothesis that this species may be the source of $bla_{OXA-23-like}$ genes (Poirel *et al.*, 2008). Mobilization of such genes via plasmids has resulted in worldwide dissemination of bla_{OXA-23} in *Acinetobacter* spp. However, bla_{OXA-58} is mostly reported in *A. baumannii* isolates and also in an *Acinetobacter* genomospecies 3 isolate (Marti *et al.*, 2008; Poirel *et al.*, 2005; Poirel & Nordmann, 2006). Our isolates represent what are believed to be the first reported cases of bla_{OXA-58} carriage in AG9 and AG10 spp. Additional insertion sequences, IS*Aba2*, IS*Aba1* and IS*18*, are frequently present upstream of bla_{OXA-58} in carbapenem-resistant *Acinetobacter* isolates, but were absent in our isolates (Poirel & Nordmann, 2006). Instead, the bla_{OXA-58} in our isolates is bracketed by IS*Aba3*-like and IS*Aba3* transposases in a similar arrangement as that of a composite transposon. It is likely that a similar arrangement of genes preceded the insertion of IS*Aba2* in other *A. baumannii* isolates since direct repeats were present within the disrupted IS*Aba3*-like transposase immediately upstream and downstream of IS*Aba2* (Poirel & Nordmann, 2006). Since IS*Aba2*, IS*Aba1* and IS*18* have promoter sequences to upregulate bla_{OXA-58}, our study suggests that they are more effective than those found within the IS*Aba3*-like transposase, given the difference in carbapenem susceptibility patterns (Poirel & Nordmann, 2006). Alternatively, other factors, such as plasmid copy number and the number of bla_{OXA-58} genes per plasmid, may also play a role in determining the carbapenem susceptibility, although increasing the inoculum concentrations of our isolates did not increase the carbapenem MICs appreciably (Bertini *et al.*, 2007).

The presence of bla_{OXA-58} and $bla_{OXA-23-like}$ genes in carbapenem-susceptible *Acinetobacter* isolates highlights the threat of undetected reservoirs of carbapenemase-encoding genes, since laboratory detection of such genes and subsequent infection control measures in hospitals generally target phenotypically multidrug-resistant organisms. In *A. radioresistens*, $bla_{OXA-23-like}$ genes can be mobilized onto plasmids and transferred to other species, as exemplified by the prevalence of bla_{OXA-23} in carbapenem-resistant *Acinetobacter* isolates worldwide. Isolates carrying plasmid-mediated bla_{OXA-58} also have the potential of conferring carbapenem resistance if such genes are subsequently transferred to species possessing more potent insertion sequences. As such, standard infection control precautions targeting all organisms (such as hand hygiene and scrupulous environmental cleaning) should be stringently adhered to in order to reduce

Table 1. β-Lactam antimicrobial susceptibility of $bla_{OXA-23-like}$-positive and bla_{OXA-58}-positive carbapenem-susceptible *Acinetobacter* isolates from St James's Hospital, Dublin

Antimicrobial agent	MIC (mg l^{-1})							
	A. radioresistens B427		*A. radioresistens* B374		AG9 (A361)		AG10 (S1055)	
	0.5 McFarland turbidity	2 McFarland turbidity	0.5 McFarland turbidity	2 McFarland turbidity	0.5 McFarland turbidity	2 McFarland turbidity	0.5 McFarland turbidity	2 McFarland turbidity
Ampicillin	16	32	8	16	128	⩾256	128	⩾256
Amoxicillin–clavulanate	2/1	4/2	2/1	4/2	2/1	4/2	8/4	16/8
Piperacillin–tazobactam	1/4	2/4	0.25/4	1/4	⩽0.125/4	⩽0.125/4	4/4	8/4
Ceftazidime	4	8	2	4	1	2	2	4
Cefotaxime	16	32	4	8	2	4	4	8
Cefepime	4	16	2	4	1	2	2	4
Meropenem	0.5	1	0.25	0.5	0.125	0.25	1	2
Imipenem	0.5	1	0.5	1	0.5	1	2	4

the risk of transfer of such resistance genes. Judicious use of carbapenems must also be advocated, as widespread use of such agents may also create an environment favouring the inter-species transfer as well as the overt expression of these genes.

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