Epidemiol. Infect. (2012), **140**, 137–145. © Cambridge University Press 2011
doi:10.1017/S0950268811000744

Carbapenem-resistant *Acinetobacter baumannii*: diversity of resistant mechanisms and risk factors for infection

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(*Accepted 21 March 2011; first published online 18 April 2011*)

SUMMARY

Carbapenem-resistant *Acinetobacter baumannii* (CRAB) are an increasing infectious threat in hospitals. We investigated the clinical epidemiology of CRAB infections *vs*. colonization in patients, and examined the mechanisms of resistance associated with elevated minimum inhibitory concentrations (MICs) for carbapenems. From January to June 2009, 75 CRAB strains were collected. CRAB infection was significantly associated with malignancy and a high APACHE II score. The most dominant resistance mechanism was IS*Aba1* preceding OXA-51, producing strains with overexpression of efflux pump. Strains carrying $bla_{\text{OXA-23}}$-like enzymes had higher carbapenem MICs than those carrying $bla_{\text{OXA-51}}$-like enzymes; however, the presence of multiple mechanisms did not result in increased resistance to carbapenems. There was no difference in the resistance mechanisms in strains from infected and colonized patients. The majority of strains were genetically diverse by DNA macrorestriction although there was evidence of clonal spread of four clusters of strains in patients.

Key words: *Acinetobacter baumannii*, antibiotic resistance, infection, molecular epidemiology, risk factors.

INTRODUCTION

Acinetobacter baumannii is an opportunistic pathogen and is emerging as a cause of numerous global outbreaks in hospitals [1, 2] and several widespread strains of the species are increasingly resistant to many classes of antibiotics. Carbapenems have been widely used to treat serious multidrug-resistant *A. baumannii* infections; however, carbapenem-resistant strains are commonly reported worldwide [3], raising serious concerns about the limited antimicrobial treatment options remaining.

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Resistance to carbapenems in *A. baumannii* occurs due to the accumulation of various resistance mechanisms including β-lactamase production, the loss of outer-membrane protein (OMP) and/or overexpression of efflux pumps or more rarely with penicillin-binding protein alterations [4]. There are few reports of combinations of different resistance mechanisms in single isolates of *A. baumannii*, but Bratu *et al.* showed a correlation of antimicrobial resistance with β-lactamase production and porin and efflux pump, but this was not limited to carbapenem-resistant strains of *A. baumannii* [5].

There are marked geographical differences in the prevalence of the various mechanisms of carbapenem resistance in *A. baumannii*. Various different carbapenemases, for example OXA-58 oxacillinase in

Turkey and VIM-2 in South Korea, have been described in Asia and Europe [4, 6, 7]. Here, we evaluated the epidemiology of carbapenem-resistant *A. baumannii* (CRAB) infections in a Korean hospital and investigated various mechanisms of carbapenem resistance in isolates and the effect of combinations of mechanisms on the degree of carbapenem resistance. In addition, we compared the clinical characteristics, outcomes and genotypic analyses of the carbapenem-resistant mechanism of *A. baumannii* in infected *vs.* colonized patients.

MATERIALS AND METHODS

Patient selection and case definition

From January to June 2009, a total of 105 non-repetitive *A. baumannii* strains in clinical samples were collected from a 1200-bed tertiary hospital in Korea. Demographic characteristics, various risk factors and outcome were compared between patients classified as infected or colonized. Risk factors included co-morbidity, prior admission to hospital in the preceding month, prior surgery, the length of stay before isolation of *A. baumannii*, APACHE (acute physiological and chronic health evaluation) II score, ventilator support, recent invasive procedures, and prior antibiotic therapy in the month before isolation of *A. baumannii*.

Infection was assessed according to Center for Disease Control (CDC) criteria, and defined by the isolation of *A. baumannii* from a sterile site in patients with definite clinical signs of infection [8]. Colonization was defined as the growth of *A. baumannii* from sputum, open wounds or other non-sterile sites, without clinical signs or symptoms of infection. Approval for the study was obtained from the institutional review boards of our hospital.

Bacterial strains and antimicrobial susceptibility testing

Species were identified using the VITEK® 2 system (bioMérieux, France). The $bla_{\text{OXA-51}}$-like gene was detected by PCR to confirm species of *A. baumannii* [9]. Minimum inhibitory concentrations (MICs) were determined by agar dilution for the following antimicrobial agents: amikacin (Donga Pharmaceutical Co., Korea), cefepime (Boryung Pharmaceutical Co., Korea), piperacillin (Yuhan Co., Korea), ceftazidime (SK Chemical Co., Korea), ampicillin/sulbactam (Shinpoong Co., Korea), levofloxacin (Ildong Pharmaceutical Co., Korea), imipenem (Choongwae Pharmaceutical Co., Korea), meropenem (Yuhan Co.), tigecycline (Wyeth Pharmaceuticals Inc., USA) and colistin (Sigma Aldrich, USA). *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853 were used for quality assurance. Antimicrobial susceptibility was interpreted according to Clinical and Laboratory Standards Institute guidelines [10], except for tigecycline for which susceptibility/resistance breakpoints were interpreted according to the US Food and Drug Administration criteria (susceptible $\leqslant 2$ mg/l, intermediate 4 mg/l, resistant $\geqslant 8$ mg/l) [11]. Seventy-five meropenem or imipenem non-susceptible, including intermediate resistant, *A. baumannii* strains were selected for further study.

DNA typing

*Apa*I digests of genomic DNA from strains were resolved by pulsed-field gel electrophoresis (PFGE) as described previously [12]. Cluster analysis by the unweighted pair-group method with mathematical averaging was performed using the Dice coefficient and a similarity cut-off of $\geqslant 70\%$ on the dendrogram was used to define related strains within a cluster.

Detection of OXA genes, and genes encoding metallo-β-lactamases

Detection of the four main groups of OXA-carbapenemase genes ($bla_{\text{OXA-23}}$-like, $bla_{\text{OXA-24}}$-like, $bla_{\text{OXA-51}}$-like, $bla_{\text{OXA-58}}$-like) was performed using a previously described multiplex PCR assay [13]. The insertion sequence IS*Aba1* upstream of the $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-51}}$ genes was sought using combinations of the IS*Aba1*F primer and the OXA23-R/OXA51-R primer [14]. PCR analysis was performed to confirm the presence of MBL genes with primers specific for the bla_{IMP}, bla_{VIM}, bla_{SIM}, $bla_{\text{SPM-1}}$ and $bla_{\text{GIM-1}}$ genes [15]. Searches and alignments for the nucleotide sequences were performed with the BLAST program (http://www.ncbi.nlm.nih. gov/BLAST).

Analysis of and amplication of *carO* gene

OMPs of strains were analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) by a standard protocol [16]. *A. baumannii* ATCC 19606 was used as a positive control for CarO expression. Target mRNA was transcribed into cDNA using oligo$(dT)_{15}$ primer and optiscript reverse

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transcriptase (Intron, Korea). PCRs were performed with a 50 μl reaction mixture containing 10 mM Tris–HCl (pH 8·4), 50 mM KCl, 1·5 mM $MgCl_2$, 200μM of each deoxynucleoside triphosphate, 1 μM each of forward (5′-CAT ATG AAA GTA TTA CGT GTT TTA GTG -3′) and reverse (5′-GGT ACC TTA CCA GTA GAA GTT TAC ACC-3′) primers, 50 ng target cDNA and 2 U *Taq* polymerase (Enzynomics, Korea). An initial denaturation step at 94 °C for 3 min was followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C with a final extension step of 10 min at 72 °C. The *carO* gene product was sequenced and homology searches in the GenBank database were performed using the BLAST program.

Detection of efflux pump

To determine the presence of efflux mechanism, strains were tested for susceptibility to carbapenems by the agar dilution method with Mueller–Hinton agar plates with and without 100 μg/ml of the efflux pump inhibitor, 1-(1-naphthylmethyl)-piperazine (NMP, Sigma, USA) [5, 17]. The positive criterion for phenotypic detection of efflux pump was at least a fourfold reduction in the MICs of carbapenem in the presence of NMP [18]. Efflux pump-encoding genes *adeA, adeB* and *adeC*, and regulatory genes (*adeR, adeS*) were detected with primers as described previously [19].

Statistical analysis

All statistical analyses were performed using SPSS software, version 13·0 (SPSS Inc., USA), and a P value $<0{\cdot}05$ was considered statistically significant. Statistical significance was assessed via χ^2 test or Fisher's exact test for categorical variables and Student's t test or the Mann–Whitney U test for continuous variables. Risk factors associated with carbapenem non-susceptible *A. baumannii* infection were analysed using logistic regression analyses. Variables with a P value $<0{\cdot}1$ in the univariate analyses were included in a logistic regression model for multivariate analysis.

RESULTS

Characteristics of bacterial isolates

We collected 105 clinical isolates of *A. baumannii*, of which 75 (71·4%) were intermediate or resistant to carbapenems. These strains were recovered from sputum (47), wound (11), catheter tip (6), blood (5), bile/intra-abdominal drainage (4) and urine (2); 49 were from patients in intensive-care units (ICUs), and the remainder from patients in general wards.

Clinical characteristics of patients with CRAB

A comparison of the clinical characteristics and outcomes of the 75 patients colonized or infected with CRAB is shown in Table 1. The mean age was $58{\cdot}3 \pm 23{\cdot}9$ years and 68% were male. Twenty-four patients were defined as infected and 51 patients as colonized. More patients with infection had malignancies and longer duration of hospital stays as well as higher APACHE II scores. The use of carbapenems and glycopeptides prior to *A. baumannii* isolation was more frequent in the infected group and both the all-cause in-hospital mortality and the *A. baumannii*-attributable mortality were higher in this group. The presence of invasive devices such as central venous catheter, urinary catheter, or surgical drainage was not a significant risk factor for *A. baumannii* infection. Univariate analysis showed that the risk factors associated with infection were malignant disease, duration of hospital stay, APACHE II score, and prior use of carbapenems and glycopeptides. Of these, malignancy as underlying disease and high APACHE II score (>15) were significantly associated with *A. baumannii* infection on multivariate analysis ($P< 0{\cdot}05$).

Antimicrobial susceptibility

The susceptibility patterns of the 75 *A. baumannii* strains are shown in Table 2. Most exhibited resistance (>95%) across different classes of agents notably ampicillin/sulbactam, ceftazidime, levofloxacin and piperacillin. Six isolates showed carbapenem discordant susceptibility (intermediate or resistant to meropenem, but susceptible to imipenem). All strains were susceptible to colistin but 38·7% were intermediate or fully resistant to tigecycline.

DNA typing

Eighteen distinct clusters of strains were identified by PFGE. Four clusters (E, I, L, N) accounted for 64% of the strains with pattern E represented by 27 strains (Fig. 1).

Table 1. *Clinical characteristics and outcomes of patients with* A. baumannii

	Total ($n=75$)	Infection group ($n=24$)	Colonization group ($n=51$)	P value
Age, years, mean ± S.D.	58·3 ± 23·9	58·5 ± 21	58·2 ± 25·4	0·19
Male sex	51 (68·0%)	18 (75·0%)	33 (64·7%)	0·37
Comorbidity				
Diabetes mellitus	12 (16·0%)	4 (16·7%)	8 (15·7%)	0·91
Hypertension	19 (25·3%)	7 (29·2%)	12 (23·5%)	0·77
Dialysis	6 (8·0%)	2 (8·3%)	4 (7·8%)	0·08
Malignancy	20 (26·7%)	11 (45·8%)	9 (17·6%)	0·01
Related to hospitalization				
Prior hospitalization in 1 month	34 (45·3%)	7 (19·2%)	27 (52·9%)	0·15
Prior surgery in 1 month	40 (53·3%)	9 (37·5%)	31 (60·8%)	0·08
Length of stay before isolation, days, mean ± S.D.	24·4 ± 22·7	30·1 ± 23·9	21·1 ± 21·6	0·48
Duration of hospital stay, days, mean ± S.D.	57·8 ± 48·7	65·8 ± 58·3	12·5 ± 5·5	0·05
Invasive procedure				
Central venous catheter	52 (69·3%)	18 (75·0%)	34 (66·6%)	0·78
Drainage	43 (57·3%)	17 (70·8%)	26 (50·9%)	0·13
Foley catheter	55 (73·3%)	17 (70·8%)	38 (74·5%)	0·28
Levine tube	50 (66·7%)	14 (58·3%)	36 (70·5%)	0·61
Tracheostomy	27 (36·0%)	8 (33·3%)	19 (37·3%)	0·80
Mechanical ventilation	36 (48·0%)	14 (58·3%)	22 (43·1%)	0·32
APACHE II score, mean ± S.D.	14·4 ± 7·0	18·1 ± 8·3	12·5 ± 5·5	0·004
Prior antibiotics				
Cephalosporin	42 (56·0%)	12 (50·0%)	30 (58·8%)	0·61
β-lactam	36 (48·0%)	8 (33·3%)	28 (54·9%)	0·09
Quinolone	24 (32·0%)	10 (41·6%)	14 (27·5%)	0·28
Aminoglycoside	23 (30·7%)	6 (25·0%)	17 (33·3%)	0·59
Carbapenem	31 (41·3%)	15 (62·5%)	16 (31·4%)	0·01
Glycopeptide	36 (48·0%)	16 (66·7%)	20 (39·2%)	0·04
All-cause in-hospital mortality	25 (32·8%)	14 (60·9%)	11 (21·6%)	0·001
Death due to *A. baumannii*	15 (20·0%)	14 (58·3%)	1 (1·9%)	<0·001

Table 2. *Susceptibility patterns of 75 carbapenem non-susceptible* A. baumannii *strains*

Antibiotics	MIC range (mg/l)	Number (%) of strains		
		S	I	R
Amikacin	2 to >256	21 (28)	5 (6·7)	49 (65·3)
Ampicillin/ sulbactam	8 to >256	1 (1·3)	2 (2·7)	72 (96)
Cefepime	4 to >256	3 (4·0)	3 (4·0)	69 (92)
Ceftazidime	16 to >256	0	1 (1·3)	74 (98·7)
Colistin	0·5 to 4	75 (100)	0	0
Imipenem	2 to 64	6 (8)	7 (9·3)	62 (82·7)
Levofloxacin	0·5 to 64	3 (4·0)	1 (1·3)	71 (94·7)
Meropenem	8 to 128	0	1 (1·3)	74 (98·7)
Piperacillin	128 to >256	0	0	75 (100)
Tigecycline	0·5 to 8	46 (61·3)	26 (34·7)	3 (4·0)

MIC, Minimum inhibitory concentration; S, susceptible; I, intermediate resistant; R, resistant.

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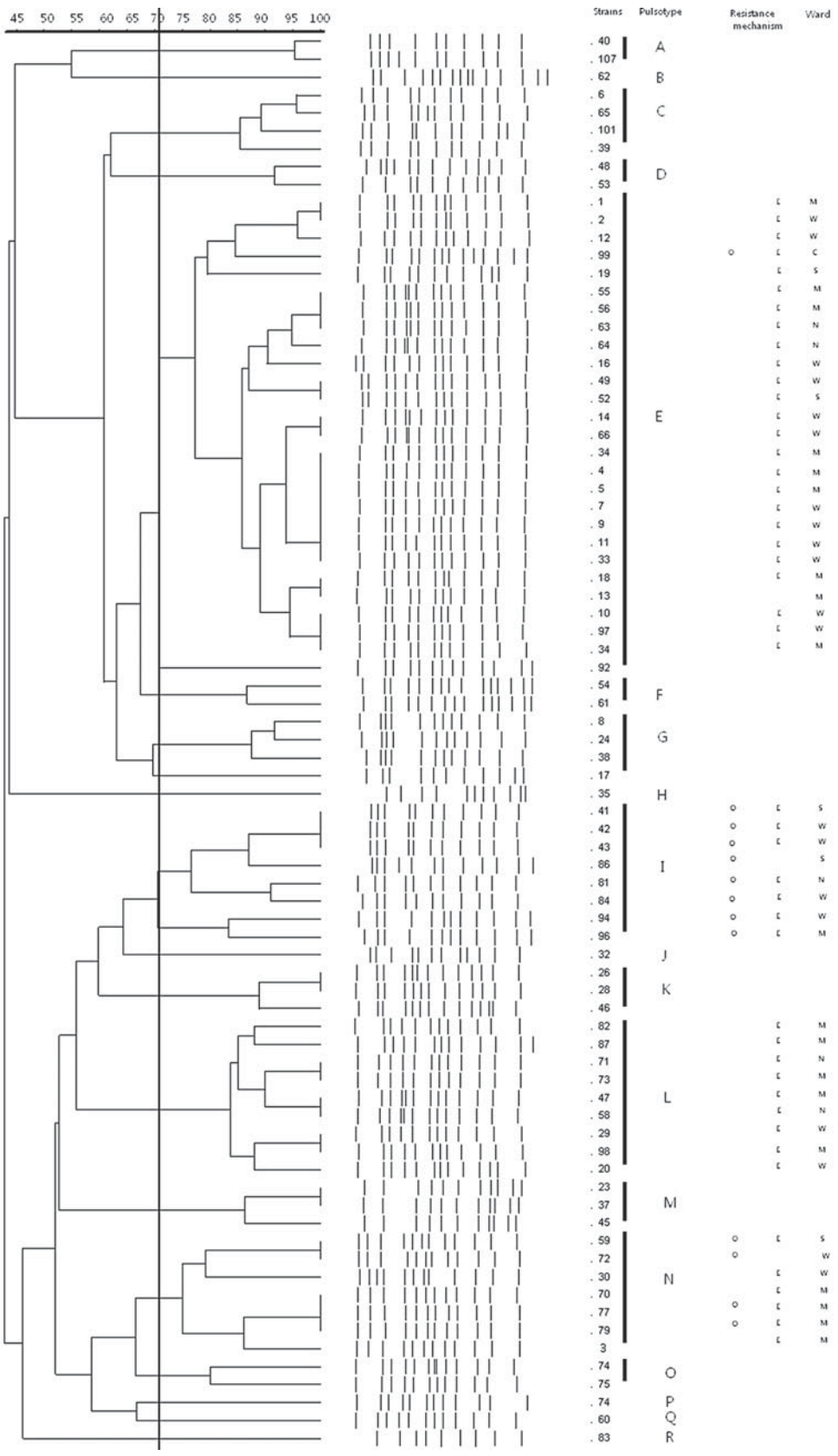

Fig. 1. Dendrogram of PFGE types of carbapenem-resistant *A. baumannii* strains. Strains producing IS*Aba1*-enhanced $bla_{\text{OXA-23}}$-like (O); efflux pump overexpression (E); surgical intensive-care unit (ICU) (S); medical ICU (M); neurosurgery ICU (N); coronary care unit (C); general wards (W).

https://doi.org/10.1017/S0950268811000744 Published online by Cambridge University Press

Table 3. *Effect on carbapenem minimum inhibitory concentrations (MICs) of resistance mechanism in* A. baumannii

Resistance mechanism		MIC (μg/ml)					
		4	8	16	32	64	128
$bla_{\text{OXA-23}}$-like only ($n=7$)	Imipenem	0	0	4	1	2	0
	Meropenem	0	0	2	2	2	1
bla_{OXA23}-like + IS*Aba1* enhanced $bla_{\text{OXA-51}}$-like ($n=2$)	Imipenem	0	0	1	1	0	0
	Meropenem	0	0	0	2	0	0
$bla_{\text{OXA-23}}$-like + efflux pump ($n=18$)	Imipenem	0	0	7	11	0	0
	Meropenem	0	0	8	10	0	0
bla_{OXA23}-like + IS*Aba1* enhanced $bla_{\text{OXA-51}}$-like + efflux pump ($n=1$)	Imipenem	0	0	0	1	0	0
	Meropenem	0	0	1	0	0	0
IS*Aba1* enhanced $bla_{\text{OXA-51}}$-like only ($n=5$)	Imipenem	2	3	0	0	0	0
	Meropenem	0	0	5	0	0	0
IS*Aba1* enhanced $bla_{\text{OXA-51}}$-like + loss of *carO* ($n=2$)	Imipenem	2	0	0	0	0	0
	Meropenem	0	1	1	0	0	0
IS*Aba1* enhanced $bla_{\text{OXA-51}}$-like + efflux pump ($n=34$)	Imipenem	1	2	29	1	1	0
	Meropenem	0	0	34	0	0	0
IS*Aba1* enhanced $bla_{\text{OXA-51}}$-like + loss of carO + efflux pump ($n=2$)	Imipenem	0	2	0	0	0	0
	Meropenem	0	0	2	0	0	0
MBL + loss of *carO* ($n=1$)	Imipenem	0	0	1	0	0	0
	Meropenem	0	0	1	0	0	0

$bla_{\text{OXA-23}}$-like was consistently associated with IS*Aba1*.

Resistance mechanisms

All strains carried the OXA-51-like gene characteristic of *A. baumannii*, and among them 46 isolates had IS*Aba1* upstream of the $bla_{\text{OXA-51}}$-like genes. The $bla_{\text{OXA-23}}$-like genes were carried by 28 strains preceded upstream by IS*Aba1*. Coexistence of $bla_{\text{OXA-23}}$-like and IS*Aba1*-activated $bla_{\text{OXA-51}}$-like genes was detected in three strains. Only one strain harboured bla_{VIM}-like genes, and sequencing of bla_{VIM} amplicons identified a $bla_{\text{VIM-2}}$ allele. All strains were negative for the other MBL genes (bla_{IMP}, bla_{SPM}, bla_{SIM}, bla_{GIM}).

Five strains did not yield a *carO* PCR product with a size of 750 bp, and of these three gave a product of ~1700 bp. The *carO* sequence of *A. baumannii* ATCC 19606 was identical to GenBank accession no. FJ652396.1, which was used as a positive control. The ~1700 bp DNA sequence of the PCR fragment corresponding to the IS*Aba825*-interrupted *carO* gene was deposited in the GenBank database under accession no. AY751532. SDS–PAGE of the OMP profiles of the five strains confirmed the absence of a protein of ~25 kDa.

PCR amplication showed that 74 strains were positive for *adeA* and *adeB* genes and all but one carried *adeC*, *adeR* and *adeS* genes. The efflux pump inhibition test using NMP showed that the meropenem MICs decreased by fourfold or greater in 56 strains. Of the 69 imipenem non-susceptible *A. baumannii* strains, the imipenem MICs decreased by a similar margin in 58 strains.

Influence of resistance mechanism

The MICs of imipenem and meropenem associated with the various resistance mechanisms of the strains are shown in Table 3. Strains carrying $bla_{\text{OXA-23}}$-like genes had higher MICs for both agents than strains with $bla_{\text{OXA-51}}$ (median, 32 *vs.* 16, respectively, $P<0{\cdot}05$). Furthermore, the coexistence of multiple resistance mechanisms did not lead to higher carbapenem MICs than those of strains with a single mechanism. Nine mechanisms of resistance were identified but there was no significant difference in mechanisms of strains from infected and colonized patients. Coexistence of IS*Aba1*-activated $bla_{\text{OXA-51}}$-like genes and overexpression of efflux pump were the most prevalent mechanisms in both groups (54·2% *vs.* 41·2%, respectively).

DISCUSSION

A. baumannii is usually a healthcare-associated pathogen, particularly in the subset of critically ill

https://doi.org/10.1017/S0950268811000744 Published online by Cambridge University Press

patients in ICUs. The emergence of CRAB in the hospital setting is a serious problem worldwide, mainly because of limited therapeutic options for treating this infection. There are several factors that lead to the emergence of multidrug resistance in *A. baumannii* and include the acquisition of class D carbapenem-hydrolysing oxacillinases and/or class B metallo-β-lactamases, decreased membrane permeability due to loss of porins, and multidrug efflux systems [4, 20–23].

Our study showed that multiple factors contribute to carbapenem resistance in *A. baumannii*, and the most dominant was IS*Aba1* preceding OXA-51, producing strains with overexpression of efflux pump, followed by OXA-23 with overexpression of efflux pump. OXA-51-like enzymes are capable of hydrolysing carbapenems but their activity is extremely weak and requires an insertional element as a strong transcriptional promoter [4, 14]. Forty-six and 28 of the 75 carbapenem-resistant strains studied here carried respectively IS*Aba1*-activated $bla_{\text{OXA-51}}$-like and $bla_{\text{OXA-23}}$-like genes. The $bla_{\text{OXA-23}}$-like genes have been documented in strains associated with outbreaks of CRAB in Asia, Europe and South America [4, 6, 24, 25], and the presence of IS elements upstream of this gene may also play a role in gene expression by providing promoter sequences [4].

There is limited information on the role of OMPs in multidrug-resistant *A. baumannii*; however, resistance to carbapenems has been associated with the loss of a 29 kDa OMP, designated as CarO [22, 26], and IS*Aba1* is reported to cause insertional inactivation of CarO. We showed disrupted expression of *carO* in five strains and the loss of an OMP of ~25 kDa in these strains compared to a positive control [23]. However, further research on the outer membrane permeability in CRAB is necessary.

A. baumannii possesses an efflux system, adeABC, that belongs to the resistance-nodulation-division family of transporters [27, 28]. An increased level of *adeABC* expression has been shown to correlate with a reduced level of susceptibility to gentamicin or tigecycline [27–29]. We demonstrated that the addition of an efflux pump inhibitor (NMP) led to a fourfold or greater reduction in the MICs of meropenem and imipenem [57/75 (76%) vs. 59/69 (85·6%), respectively], which suggests but does not confirm that an efflux mechanism may be involved in carbapenem resistance in this species. However, we did not determine the expression level of *adeB* by RT–PCR, and did not confirm that the observed reduction of carbapenem MICs in the presence of the inhibitor was associated with expression of *adeABC* or the regulatory genes. Indeed, sequencing of *adeRS* revealed several point mutations which did not correlate with adeABC (data not shown).

Our data indicate that the presence of multiple mechanisms in strains such as production of carbapenemase, loss of CarO and efflux pump overexpression did not result in increased carbapenem resistance compared to strains with a single mechanism. It appears that IS*Aba1*-activated bla_{OXA51}-like enzymes have weaker hydrolytic activity than enzymes encoded with $bla_{\text{OXA-23}}$, but no direct evidence for this is provided.

Several case-control studies have identified risk factors associated with the acquisition of *A. baumannii*, and these include prolonged hospitalization, ICU admission, recent surgical procedures, antimicrobial agent exposure, central venous catheter use and prior hospitalization [30–33]. However, most of these studies lacked a standard methodology, and designated carbapenem-susceptible *A. baumannii* or non-*A. baumannii* as a control group [31, 32, 34]. We determined that the risk factors for infection in patients with CRAB were malignancy as an underlying disease and high APACHE II score and showed that resistance mechanisms did not differ between infected and colonized groups. Molecular typing showed that although one clone accounted for about one-third of the strains, the remainder exhibited considerable diversity. Further study using multilocus sequence typing may allow us to compare the prevalent PFGE types with those from epidemics worldwide.

The main limitation of this study is that it was confined to a single centre and it would be valuable to extend the geographical origin of the strains. In addition, we were unable to follow patients colonized with *A. baumannii* who went on to develop infection; however, we tried to overcome this bias by including strains from distinct individuals.

In conclusion, CRAB bearing a class D carbapenemase, and OXA-51-like or OXA-23-like enzymes, are relatively widespread in a tertiary hospital in Seoul, Korea and various factors, including loss of OMP or efflux pump, probably contribute to carbapenem resistance. Multiple resistance mechanisms did not confer higher resistance and the underlying clinical status of patients may play a more important role in the development of CRAB infection.

DECLARATION OF INTEREST

None.

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