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Dissemination of multidrug-resistant, class 1 integron-carrying *Acinetobacter baumannii* isolates in Taiwan

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ABSTRACT

In this study, 283 multidrug-resistant *Acinetobacter baumannii* (MDR-AB) bloodstream isolates were collected between 1996 and 2004, from three teaching hospitals located in different regions of Taiwan. Susceptibility data showed that strains carrying class 1 integrons were significantly more resistant ($p < 0.01$) to all tested antibiotics (except aztreonam and chloramphenicol) than strains lacking integrons, Seven types of gene cassette were identified among these strains, including two that have not been previously reported. The vast majority of the cassettes encoded aminoglycoside resistance genes, including *aacA4*, *aacC1*, *aac(6')*-II, *aadA1*, *aadA2*, *aadA4* and *aadDA1*. Sixteen distinct ribotypes were identified in MDR-AB isolates carrying class 1 integrons. Only one strain was found to produce an extended-spectrum β-lactamase, i.e. VEB-3. In the 18 imipenem-resistant strains, two carbapenenmase genes, $bla_{\text{VIM-11}}$ and $bla_{\text{OXA-58}}$, were found concomitantly in one isolate. An island-wide epidemic clone and an endemic clone from a hospital located in the northern region were identified by ribotyping. On the basis of the susceptibility data among the different ribogroups, the epidemic clone was associated more significantly with resistance to cefepime and ampicillin–sulbactam than was the endemic clone. In conclusion, the presence of class 1 integrons was significantly associated with resistance in MDR-AB, and the epidemic, class 1 integron-carrying MDR-AB clone was found to be widespread in Taiwan.

Keywords *Acinetobacter baumannii*, epidemic clone, integron-associated, multidrug-resistant, ribotype

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INTRODUCTION

Acinetobacter baumannii is an important cause of nosocomial infections, especially in intensive-care units, and has been documented to be the major cause of ventilator-associated pneumonia and bacteraemia [1,2]. Its rapid acquisition of a wide variety of antibiotic resistance genes, as well as its ability to survive in various harsh environments, has caused difficulties in the control and eradication of the pathogen [3]. This extremely rapid development of resistance has caused serious therapeutic problems worldwide [4,5].

Mobile elements, including plasmids, transposons and integrons, are the most efficient genetic elements promoting acquisition and dissemination of resistance determinants. Integrons carrying diverse arrays of resistance gene cassettes [6,7] are particularly useful for the epidemiological study of *A. baumannii* [8]. Currently, five distinct classes of integrons have been described [6]. Class 1 integrons are commonly found in clinical isolates of Gram-negative bacilli, including

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A. baumannii [8,9]. In Taiwan, integron-associated multidrug resistance in *A. baumannii* has been investigated only in a single hospital [10,11]. However, no large-scale study has been carried out concerning the role of integrons in multidrug-resistant *A. baumannii* (MDR-AB) isolates in Taiwan. In this study, a comparison of integron-carrying and non-integron-carrying MDR-AB strains was made to assess the differences in their drug susceptibility and clonal dissemination.

MATERIALS AND METHODS

Isolates

Between July 1996 and December 2004, a total of 283 non-repetitive clinical blood isolates of MDR-AB were collected from three medical centres located in different regions of Taiwan. Among these, 195 isolates were obtained from the northern region (Taipei Veterans General Hospital), 50 from the central region (Taichung Veterans General Hospital) and 38 from the southern region (Kaohsiung Medical University Hospital). *A. baumannii* isolates resistant to three or more different classes of antibiotics, including at least one extended-spectrum β-lactam antibiotic, were defined as MDR-AB [12]. All clinical isolates were identified using the API 32GN system (bioMerieux, Marcy-l'Etoile, France). Species identification was further confirmed using a multiplex PCR based on the intergenic spacer region of the 16S–23S rRNA genes [13]. An endemic strain (R-25) identified in a previous study [11] was used for comparison with the isolates of this study. Bacteria were stored at −70°C using a Microbank system (Prolab Diagnostics, Neston, UK).

Integron detection and typing

Detection of the class 1 integrase gene (integrase gene PCR) and integron cassettes (integron PCR) were performed as described previously [9]. Class 2 and class 3 integrons were detected using PCR as described by Poly *et al.* [14]. A long-range PCR assay for integron cassettes was performed with Blend Taq Plus DNA polymerase (Toyobo, Osaka, Japan) with the representatives of each PCR–restriction fragment length polymorphism pattern to confirm the presence of large integrons. Integron PCR products were purified using Microspin S-300 HR columns (GE Healthcare, Uppsala, Sweden). The typing of the integron gene cassettes was done by restriction analysis using *Alu*I (New England Biolabs, Beverly, MA, USA). The fragments, after restriction digestion, were resolved with 2% agarose gel electrophoresis in TBE buffer at 100 V and examined after ethidium bromide staining. Integron PCR products with different PCR–restriction fragment length polymorphism patterns were selected, purified and subcloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) for sequencing. Additional primers were designed on the basis of sequences obtained to complete the entire sequence of the gene cassettes. Sequence analysis and comparison were carried out using the BLAST program supported by the NCBI database (http://www.ncbi.nlm.nih.gov).

Antimicrobial susceptibility testing

The antimicrobial agents used in this study include chloramphenicol, ampicillin–sulbactam (2 : 1; w/w), piperacillin, piperacillin with a fixed concentration of tazobactam at 4 mg/L, aztreonam, ceftazidime, ceftriaxone, cefepime, imipenem, gentamicin, tobramycin, amikacin and ciprofloxacin. All drugs were incorporated into Mueller–Hinton broth (TREK Diagnostic Systems Ltd, East Grinstead, UK). The concentration of drugs used was from 0.03 to 128 mg/L, with two-fold increments. Two control strains, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853, were included to determine the MIC of the drugs. Antimicrobial susceptibility was determined by automated SensiTitre Susceptibility Plate (Trek Diagnostic Systems Ltd) according to the CLSI recommendations [15].

β-Lactamase detection

Extended-spectrum β-lactamase (ESBL) production screening was performed using the double-disk synergy test with cefepime, ceftazidime and ticarcillin–clavulanic acid on Mueller–Hinton agar and also on plates containing cloxacillin (200 μg/mL) to inhibit the naturally occurring AmpC-type β-lactamases in *A. baumannii* [16]. PCR testing of synergy-positive isolates for known ESBL genes was done using specific primers for bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, bla_{VEB} and bla_{PER} [17,18]. The amplicons obtained were identified by sequencing. Primers VEB-Fc and VEB-Rc [19] were used for amplification of the entire bla_{VEB} gene. In imipenem-resistant MDR-AB strains, the genes of the carbapenem-hydrolysing oxacillinases OXA-23 and OXA-58 were also detected by PCR as previously described [20].

Molecular typing

Ribotyping was used to determine the epidemiological relationship among *A. baumannii* isolates harbouring class 1 integrons. Ribotyping was performed using the automated Riboprinter Microbial Characterization System (Qualicon, Wilmington, DE, USA) according to the manufacturer's instructions and the protocols described by Wu *et al.* [11]. To reveal ribotype polymorphism, each sample was analysed by the Molecular Analyst Fingerprinting, Fingerprinting Plus and Fingerprinting DST Software (Bio-Rad Laboratories, Richmond, CA, USA). The grouping method was used to generate a dendrogram from the matrix using the unweighted pair group method using arithmetic average (UPGMA) clustering technique after calculation of similarities using Pearson correlation coefficients between every pair of organisms.

Pulsed-field gel electrophoresis (PFGE) was used for further subtyping of the isolates of each major ribopattern. Total DNA was prepared and PFGE was performed as described previously [21]. *Apa*I (New England Biolabs, Beverly, MA, USA) was used, and restriction fragments were separated by PFGE in 1% SeaKem Gold agarose gels (Cambrex Bio Science, Rockland, ME, USA) in 0.5 × TBE buffer (45 mM Tris, 45 mM boric acid, 1.0 mM EDTA, pH 8.0) using a Bio-Rad CHEF-Mapper apparatus (Bio-Rad Laboratories). Gels were stained with ethidium bromide and photographed under ultraviolet light. Band patterns were compared and classified as indistinguishable (clonal), closely related (clonal variants, with ≤3 band differences), possibly related (four to six band differences) or unrelated, using previously described criteria [22]. Dendrograms showing percentage similarity were prepared

with the Molecular Analyst Fingerprinting, Fingerprinting Plus and Fingerprinting DST Software (Bio-Rad Laboratories) and compared using the UPGMA clustering method.

Statistical analysis

The susceptibility differences among MDR-AB stains were analysed using the EPI INFO 6 programme (CDC, USA), available at (http://www.cdc.gov/epiinfo/Epi6/ei6.htm). The chi-square test was used to determine the significance of differences. Yate's correction was used when any parameter to be compared had a frequency of less than five. A difference was considered statistically significant if the p value was less than 0.05. Fisher's exact test was performed when the sample number was less than five.

Nucleotide accession numbers

The sequences of the variable regions of the integrons of representative strains K43 (type 2 cassette) and K46 (type 7 cassette) were deposited in GenBank under the accession numbers EF127959 and EF116550, respectively.

RESULTS

Antimicrobial susceptibility testing

Among the 283 isolates analysed by PCR, 202 isolates were found to have the class 1 integrase gene (*intI*1$^+$), whereas the rest did not (*intI*1$^-$). Among the *intI*1$^+$ isolates, 120 (59.4%) were found in northern Taiwan, 49 (24.3%) in the central region, and 33 (16.3%) in the southern region. MDR-AB isolates with *intI*1 were resistant to almost all of the tested antibiotics, with the exception of imipenem, and were significantly more resistant to all of the antibiotics tested (except aztreonam and chloramphenicol) than *intI*1$^-$ MDR-AB isolates (p <0.001) (Table 1).

Detection and typing of class 1 integrons

Among the 202 (71.38%) class 1 integron-carrying isolates, five different types of gene cassettes in segments of approximately 2.5, 2.0, 1.4, 1.2 and 1.0 kb, between the 5′-conserved segment (CS) and 3′-CS regions, were amplified by PCR. To further differentiate among these five gene cassette amplicons, all were subjected to *Alu*I restriction analysis, and their product sizes were compared. Three additional subtypes were identified in the 2.5-kb cassette sequences, resulting in a total of seven cassette types. Isolates carrying one of these seven types were randomly selected for sequencing. Sequencing results for each of the seven amplicons revealed that these cassette arrays carried various open reading frames (ORFs) (Fig. 1). The ORFs of the three subtypes in the *c.* 2.5-kb sequences included: type 1, *aacC1-orfX-orfX′-aadDA1*; type 2, $bla_{\text{IMP-8}}$-*aac(6′)*-II-*addA4*; and type 3, *aacA4-cat8-aadA1*. These were 2542, 2507 and 2381 bp long, respectively. When the *c.* 2.0-kb, 1.4-kb, 1.2-kb and 1.0-kb cassette sequences were determined, the ORFs identified included: type 4, *dhfrXII-orfF-aadA2* (1873 bp); type 5, *arr-3-accA4* (1395 bp); type 6, *dfrA1-orfC* (1242 bp); and type 7, $bla_{\text{VIM-11}}$ (1062 bp) (Table 2). The cassettes of type 3 accounted for 71.7% of all cassettes, making it the most common type in the integron-carrying MDR-AB isolates. Four cassette types (types 1, 2, 5 and 7) were identified among the strains isolated from the hospital located in the southern region, and the others, i.e. cassette types 3, 4 and 6, were found in isolates from at least two regions of Taiwan. Two

Table 1. Susceptibility testing results of *intI*1$^+$ and *intI*1$^-$ *Acinetobacter baumannii* isolates from three medical centres in northern, central and southern Taiwan

	*intI*1$^+$ Isolates (n = 202), MIC (mg/L)				*intI*1$^-$ Isolates (n = 81), MIC (mg/L)			
Antibiotic	Range	MIC_{50}	MIC_{90}	Resistance (%)	MIC_{50}	MIC_{90}	Resistance (%)	p value[a]
PIP	≤4 to ≥128	≥128	≥128	198 (98.0)	≥128	≥128	61 (75.3)	<0.001
PIP/TAZ	≤2 to ≥128	≥128	≥128	187 (92.6)	64	≥128	41 (50.6)	<0.001
AZT[b]	≤1 to ≥32	≥32	≥32	198 (98.0)	≥32	≥32	77 (95.1)	0.336
CRO	≤2 to ≥64	≥64	≥64	199 (98.5)	≥64	≥64	70 (86.4)	<0.001
CAZ	≤1 to ≥64	≥64	≥64	196 (97.0)	32	≥64	47 (58.0)	<0.001
FEP	≤0.5 to ≥32	≥32	≥32	169 (83.7)	16	≥32	37 (45.7)	<0.001
IMP	≤0.25 to ≥64	2	4	17 (8.4)	0.5	1	1 (1.2)	0.049
AMP/SUL	≤0.5 to ≥32	≥32	≥32	155 (76.7)	16	≥32	28 (34.6)	<0.001
GEN	≤0.25 to ≥16	≥16	≥16	192 (95.0)	≥16	≥16	49 (60.5)	<0.001
AMK	≤1 to ≥64	≥64	≥64	190 (94.1)	16	≥64	33 (40.7)	<0.001
TOB	≤0.5 to ≥16	≥16	≥16	189 (95.6)	≥16	≥16	43 (53.1)	<0.001
CHL[b]	≤2 to ≥32	≥32	≥32	202 (100)	≥32	≥32	80 (98.8)	0.063
CIP	≤0.03 to ≥4	≥4	≥4	175 (86.6)	2	≥4	36 (44.4)	<0.001

PIP, piperacillin; PIP/TAZ, piperacillin with a fixed concentration of tazobactam at 4 mg/L; AZT, aztreonam; CRO, ceftriaxone; CAZ, ceftazidime; FEP, cefepime; IMP, imipenem; AMP/SUL, amplicillin–sulbactam at a 2 : 1 ratio; GEN, gentamicin; AMK, amikacin; TOB, tobramycin; CHL, chloramphenicol; CIP, ciprofloxacin.
[a]A p-value of <0.05 is considered to indicate a significant difference in resistance between *intI*1$^+$ and *intI*1$^-$ isolates.
[b]The drug was not listed in the CLSI informational supplement [47] for the *Acinetobacter* species after 2006.

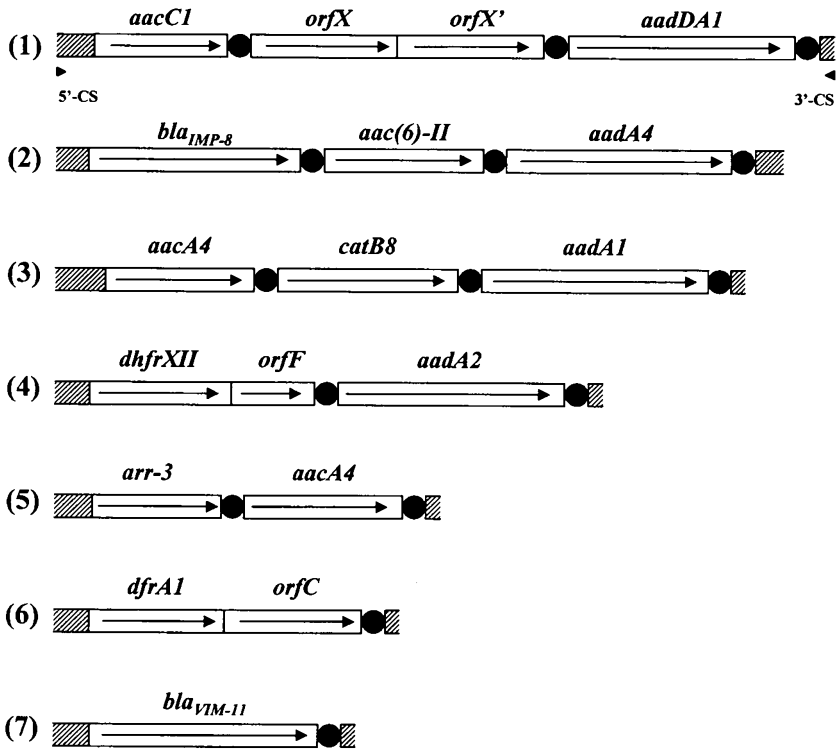

Fig. 1. Schematic representation of the variable regions identified in multidrug-resistant *Acinetobacter baumannii* (MDR-AB) isolates. Cassette types (1–7) are arranged as identified in Table 2. Gene cassettes are shown as boxes, with arrows indicating the orientation of transcription and black circles indicating the 59-base elements. Hatched rectangles indicate 5′- and 3′-conserved segments (CSs). The 5′-CS and 3′-CS amplification primers are indicated by arrowheads.

Table 2. Relationships among cassette types, amplicon size of conserved regions, gene arrays, resistance patterns and associated ribotypes

Cassette type (no. of isolates)	Amplicon size (bp)	Resistance gene(s)	Resistance phenotype	Accession no.	Ribotype (no. of isolates)
1 (4)	2542	*aacC1*, *orfX*, *orfX'*, *aadDA1*	Gen, Sis, Ast, Spt, Str	AY307113	R7(3), R3
2 (3)	2507	bla_{IMP-8}, *aac(6′)*-II, *aadA4*	Imp, Tob, Net, Gen, Spt, Str	EF127959[a]	R4, R15, R16
3 (145)	2381	*aacA4*, *catB8*, *aadA1*	Amk, Net, Tob, Chl, Spt, Str	AY922989	R5(130), R6(5), R7(4), R9(3), R2, R8, R15
4 (34)	1873	*dhfrXII*, *orfF*, *aadA2*	Tmp, Spt, Str	DQ995286	R6(32), R9, R15
5 (1)	1395	*arr-3*, *aacA4*	Rif, Amk, Net, Tob	AY038837	R10
6 (13)	1242	*dfrA1*, *orfC*	Tmp	DQ123842	R15(5), R14(2), R1, R5, R6, R9, R11, R13
7 (2)	1062	bla_{VIM-11}	Imp	EF116550[a]	R12(2)

Gen, gentamicin; Sis, sisomicin; Ast, astromicin; Spt, spectinomycin; Str, streptomycin; Imp, imipenem; Tob, tobramycin; Net, netilmicin; Amk, amikacin; Chl, chlorampenicol; Tmp, trimethoprim; Rif, rifampin.
[a]In this study.

novel integron cassette arrays were identified, which comprised the metallo-β-lactamase (MBL) genes bla_{IMP-8} and bla_{VIM-11}, and also encoded resistance to aminoglycosides, chloramphenicol, folate pathway inhibitors, carbapenems and ansamycins. The vast majority of cassettes encoded various aminoglycoside resistance genes (Table 2). Neither a class 2 nor a class 3 integron was detected in the other isolates. With regard to the imipenem susceptibility of the MBL gene cassette-carrying MDR-AB isolates, three isolates with a bla_{IMP-8} cassette showed varying susceptibilities (MICs: 4, 8 or 32 mg/L) and two isolates with a bla_{VIM-11} cassette showed high-level imipenem resistance (MIC: 32 mg/L).

β-Lactamase detection

With the exception of the C2 strain (ribotype (R)15) (Fig. S1), all strains yielded negative results for ESBL production using either Mueller–Hinton agar or cloxacillin plates. PCR with primers for known ESBL genes in the C2 strain was positive for $bla_{VEB.}$ DNA sequencing of the full-length bla_{VEB} gene revealed the bla_{VEB-3} variant. Among 18 imipenem-resistant strains (Table 3), OXA-23

Table 3. Characteristics of imipenem-resistant multidrug-resistant *Acinetobacter baumannii* blood isolates examined in the present study

		MIC (mg/L)						Resistance determinant identified			
Isolate	Date of isolation	IMP	CAZ	PIP/TAZ	AZT	FEP	Ribotype	*intI1*	MBL cassette	CHDL	ESBL
1	15 May 1998	32	≥64	≥128	≥32	≥32	R14	+	–	–	–
2	16 September 2001	≥64	8	64	≥32	8	ND	–	–	–	–
3	12 January 2004	16	≥64	≥128	≥32	≥32	R9	+	–	+ (OXA-58)	–
4	18 February 2004	16	≥64	≥128	≥32	≥32	R15	+	–	+ (OXA-58)	–
5	14 July 2004	16	≥64	≥128	≥32	≥32	R5	+	–	–	–
6	2 August 2004	≥64	≥64	≥128	≥32	≥32	R5	+	–	+ (OXA-58)	–
7	18 August 2004	16	≥64	≥128	≥32	≥32	R15	+	–	–	–
8	27 August 2004	≥64	≥64	≥128	≥32	≥32	R5	+	–	–	–
9	3 September 2004	16	≥64	≥128	≥32	≥32	R5	+	–	–	–
10	3 October 2004	32	≥64	≥128	≥32	≥32	R16	+	+ (IMP-8)	–	–
11	12 October 2004	32	≥64	≥128	≥32	≥32	R13	+	–	–	–
12	13 October 2004	32	≥64	≤2	8	16	R12	+	+ (VIM-11)	–	–
13	17 October 2004	32	≥64	≥128	≥32	≥32	R15	+	–	–	–
14	1 December 2004	32	≥64	≥128	≥32	≥32	R6	+	–	+ (OXA-58)	–
15	1 December 2004	16	≥64	64	≥32	≥32	R15	+	–	–	–
16	5 December 2004	16	≥64	≥128	≥32	≥32	R5	+	–	–	–
17	7 December 2004	32	≥64	64	≥32	≥32	R12	+	+ (VIM-11)	+ (OXA-58)	–
18	17 December 2004	32	≥64	≥128	≥32	≥32	R15	+	–	+ (OXA-58)	–

IMP, imipenem; CAZ, ceftazidime; PIP/TAZ, piperacillin with a fixed concentration of tazobactam at 4 mg/L; AZT, aztreonam; FEP, cefepime; *intI1*, class 1 integrase gene; MBL cassette, metallo-β-lactamase gene-carrying cassette; CHDL, carbapenem-hydrolysing class D β-lactamase; ESBL, extended-spectrum β-lactamase; ND, not determined. ND, not done.

and OXA-58 carbapenemase genes were also detected by PCR. Six strains contained $bla_{\text{OXA-58}}$, but $bla_{\text{OXA-23}}$ was not detected. Two carbapenemase genes, $bla_{\text{VIM-11}}$ and $bla_{\text{OXA-58}}$, were found concomitantly in one isolate.

Genotyping of integron-carrying MDR-AB isolates

Sixteen distinct ribotypes were identified among 202 class 1 integron-associated MDR-AB isolates. The predominant clone in all three regions (represented by 131 (64.8%) of the 202 isolates) belonged to R5. The R5 clone members were identical to the control strain R-25 described by Wu *et al.* [11], representing a major clone in another hospital in Taiwan. Approximately 20% of the R5 isolates were randomly selected from three hospitals for further subtyping by PFGE (Fig. 2). PFGE profiles of randomly selected R5 strains revealed that the isolates were clonally related. Apart from the R5 isolates, the R6 isolates were found to be high in prevalence in the northern region and rarely found in other regions. Only one isolate from the central region and three isolates from the southern region were observed to be of the R6 type. Further subtyping of the R6 strains by PFGE showed clonal spread of R6 only among the isolates from the northern hospital, indicating endemic spread in that hospital (Fig. 2). Taken together, R5 isolates belonged to an island-wide epidemic clone, whereas R6 isolates belonged to a hospital-endemic clone found in the northern region. Two ribotypes, R9 and R15, were also found in (at least) two hospitals. PFGE showed that R9 and R15 were non-clonal (data not shown). A limited diversity of gene cassette arrays was revealed in 16 ribotypes (Table 2). All 131 R5 isolates carried the type 3 cassette arrangement, and all except one carried the type 6 cassette.

Relationship between molecular types and antimicrobial susceptibility patterns

In order to investigate the relationship between the antibiotic susceptibility differences among $intI^+$ MDR-AB isolates, the molecular types were divided into three groups: group 1 (R5), island-wide epidemic clone; group 2 (R6), hospital endemic clone in the northern region; and group 3 (ribotypes other than R5 and R6), sporadic isolates. On comparison of group 1 strains to group 2 strains, group 1 strains were significantly more resistant to cefepime and ampicillin–sulbactam. Further comparison of the three groups of isolates showed that both group 1 and group 2 isolates were significantly more resistant than group 3 isolates to all β-lactam–β-lactamase inhibitor combinations (piperacillin–tazobactam and ampicillin–sulbactam), aminoglycosides (gentamicin, amikacin and

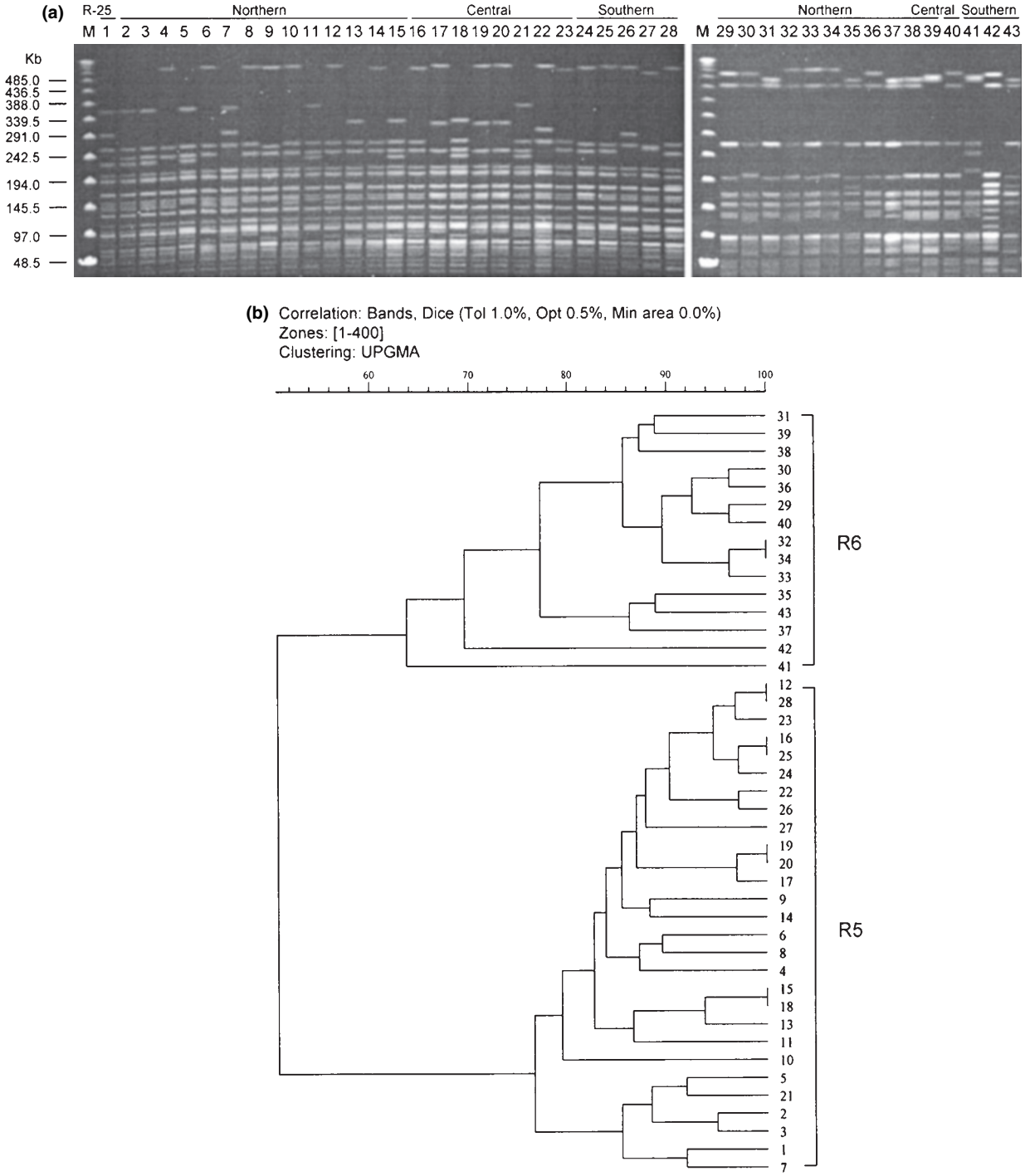

Fig. 2. (a) Pulsed-field gel electrophoresis (PFGE) analysis by *Apa*I restriction enzyme digestion of 43 *Acinetobacter baumannii* isolates with ribotype (R)5 (left panel) and R6 (right panel). Isolates from different geographical areas are shown as follows: lane 1, R25 control endemic clone from another hospital in Taiwan [11]; lanes 2–15, northern Taiwan R5 isolates; lanes 16–23, central Taiwan R5 isolates; lanes 24–28, southern Taiwan R5 isolates; lanes 29–39, northern Taiwan R6 isolates; lane 40, central Taiwan R6 isolate; lanes 41–43, southern Taiwan R6 isolates; lane M, molecular weight marker. (b) Dendrogram generated by UPGMA clustering. Isolates with similarity of >90% were considered to be identical; isolates with <70% similarity (>3 band difference) were considered to be non-clonally related. Tol, tolerance; OPT, optimum; Min, minimum; UPGMA, unweighted pair group method using arithmetic averages.

Table 4. Comparison of the rates of resistance to antimicrobial agents among isolates of different epidemicity ribogroups

	Group I	Group II	Group III	p-value[a]	
Antibiotics	R5 (n = 131)	R6 (n = 34)	Others (n = 33)	Group I vs. group II	Group II vs. group III
PIP	130	34	30	0.794	0.114
PIP/TAZ	128	33	22	0.606	0.003
AZT	128	34	32	0.498	0.492
CRO	131	34	30	ND	0.113
CAZ	130	32	30	0.108	0.485
FEP	120	23	23	<0.001	0.934
IMP	5	0	11	0.310	<0.001
AMP/SUL	119	25	7	0.011	<0.001
GEN	131	34	23	ND	0.004
AMK	125	34	28	0.244	0.025
TOB	130	34	22	0.794	<0.001
CHL	131	34	33	ND	ND
CIP	130	33	8	0.371	<0.001

PIP, piperacillin; PIP/TAZ, piperacillin with a fixed concentration of tazobactam at 4 mg/L; AZT, aztreonam; CRO, ceftriaxone; CAZ, ceftazidime; FEP, cefepime; IMP, imipenem; AMP/SUL, ampicillin–sulbactam at a 2 : 1 ratio; GEN, gentamicin; AMK, amikacin; TOB, tobramycin; CHL, chloramphenicol; CIP, ciprofloxacin; ND, not determined.
[a]A p-value <0.05 is considered to indicate a significant difference in resistance between ribogroups.

tobramycin) and ciprofloxacin (Table 4). All R5 strains except one contained the type 3 cassettes, but their antimicrobial susceptibility profiles varied. Although the presence of *aacA4* in R5 strains was expected to correlate well with aminoglycoside resistance, six R5 isolates showed susceptibility to amikacin or tobramycin, despite carrying *aacA4*.

DISCUSSION

This study showed that MDR-AB isolates carrying integrons were significantly more resistant to all antibiotics tested (except aztreonam and chloramphenicol) than non-integron-carrying isolates. Integrons comprise one type of the gene acquisition elements that drive horizontal gene exchange [6,7]. It could be postulated that integrons confer a selective advantage to strains in environments under intense antibiotic pressure [8]. Integron-carrying MDR-AB isolates have been observed in geographical regions other than Taiwan, including China (52.8%, 56/106) [23] and Spain (84%, 59/70) [24]. R15 MDR-AB strains carrying different gene cassettes (integron cassette types 2, 3, 4, and 6) were found to have similar multidrug resistance profiles, demonstrating the association of integrons and multidrug resistance (Table S1).

Among all integron carriers, seven integron cassette types were identified on the basis of their restriction fragment profiles (Fig. 1; Table 2). Type 1, 3, 4 and 6 cassettes have been previously documented in *A. baumannii* worldwide [8,23,25,26]. The type 3 cassette was the most prevalent (71.7%) in this study, as well as in a report from China (55.5%, 30/54) [23]. The type 4 cassette has appeared in Gram-positive clinical isolates of *Staphylococcus aureus* and *Enterococcus faecalis* in China [27]. The type 6 cassette has recently been identified in an epidemic MDR-AB strain, AYE, in France [28], within a 86-kb island of multidrug resistance genes [29]. The type 5 cassette has been reported only in *Acinetobacter* strains in Hong Kong [30]. Two novel MBL cassettes, of types 2 and 7, which carry the IMP-8 and VIM-11 genes, have been identified only in *Klebsiella pneumoniae* [31] and *A. haemolyticus* (GenBank accession number DQ022682) in Taiwan. Taken together, the presence of integron cassettes may indicate horizontal antibiotic resistance gene transfer as well as the dissemination of carbapenem resistance.

Although gene cassettes encoding resistance to a wide range of antibiotics have been reported in *A. baumannii*, aminoglycoside resistance determinants, including *aacA4*, *aacC1*, *aac(6′)*-II, *aadA1*, *aadA2*, *aadA4* and *aadDA1*, were predominantly found in this study. The high prevalence of aminoglycoside resistance genes in the *A. baumannii* integrons described here was comparable to that observed in other studies [8,23,32,33]. Several MDR-AB isolates (6/132; 4.55%) harbouring *aacA4* did not confer resistance to amikacin and tobramycin. Similar findings were reported by Houang *et al.* [30].

Further studies need to be carried out to determine whether point mutations are occurring inside the gene [34] or if changes in the sequences upstream of the cassette affect its expression [35].

Although all of the *intI1*$^{+}$ MDR-AB isolates carried the chloramphenicol acetyltransferase cassette gene (*catB8*), the resistance rate among the *intI1*$^{-}$ MDR-AB isolates was higher, at 98.8% (80/88).

Genes involved in chloramphenicol resistance that are not encoded by integrons, e.g. the gene encoding the intrinsic efflux pump *ade*ABC [36], may also be involved. Various profiles of antibiotic resistance in the absence of integron-associated genes suggest that other antibiotic resistance mechanisms may be involved in MDR-AB resistance [37]. ESBL production screening yielded negative results for all except one isolate, indicating that its broad-spectrum β-lactam resistance may result from other mechanisms [38]. However, this is the first time that an ESBL-producing, in this case VEB-3-producing, isolate has been reported in Taiwan. One intriguing finding is that the MBL gene cassette carrying $bla_{\text{IMP-8}}$ and those carrying $bla_{\text{OXA-58}}$ confer various susceptibilites to imipenem (Table 3), demonstrating the involvement of different carbapenem resistance mechanisms.

Furthermore, the concomitant presence of two carbapenemases, VIM-11 and OXA-58, was identified in an imipenem-resistant MDR-AB strain (Table 3); this had similarly been reported previously in Greece [39]. In *A. baumannii*, plasmid-encoded OXA-58 was the most widespread carbapenem-hydrolysing oxacillinase. The insertion sequences associated with $bla_{\text{OXA-58}}$ may contribute a strong promoter as well as an acquisition system for resistance [40]. The association of $bla_{\text{OXA-58}}$ with an insertion sequence element or the presence of two carbapenemases in these isolates are possibly the major causes of high-level carbapenem resistance.

In this study, identical integron cassettes appeared in clonally unrelated strains, whereas clonally related strains appeared to carry different integron cassettes (Table 2), supporting the notion that the integron cassettes in MDR-AB are neither genotype-specific nor genus-specific [41,42]. It is generally considered that the acquisition of entire integrons may be easier than that of individual cassette genes [43,44].

An epidemic clone, R5, was identified as being widespread in Taiwan (with a prevalence of 64.85%). In addition, an endemic clone (R6) was found mostly in isolates from the northern hospital. Both the epidemic and the endemic clones were significantly more resistant than the sporadic isolates (Table 4). The ribopattern of the R5 clone was identical to that of the previously described R-25 clone, which was isolated in another Taiwanese hospital [11]. With the exception of one strain, all R5 strains carrying the type 3 cassettes have been reported to be associated with the worldwide MDR-AB outbreak clone [8]. The circulation of R5 strains harbouring this specific cassette arrangement may be associated with unknown epidemicity properties. One specific feature of the R5 clone is its higher resistance to various antibiotics than that of other integron-carrying clones, suggesting that AmpC β-lactamase [45] and penicillin-binding protein changes [46] may be involved.

In conclusion, integrons appear to comprise one of the common features among MDR-AB isolates in Taiwan, and are associated with a high prevalence of antibiotic resistance. An epidemic integron-carrying MDR-AB clone of a specific ribotype and carrying a specific cassette arrangement (*aacA4*, *catB8*, *aadA1*) was identified and was significantly more resistant than other MDR-AB clones.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Fig. S1. Agarose gel electrophoresis of different PCR amplicons of the C2 strain.

Table S1. Relationship between the cassette types and antibiotic resistance profiles in ribotype R15.

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REFERENCES

1. Garnacho-Montero J, Ortiz-Leyba C, Fernandez-Hinojosa E *et al.* *Acinetobacter baumannii* ventilator-associated pneumonia: epidemiological and clinical findings. *Intensive Care Med* 2005; **31**: 649–655.
2. Cisneros JM, Rodriguez-Bano J. Nosocomial bacteremia due to *Acinetobacter baumannii*: epidemiology, clinical features and treatment. *Clin Microbiol Infect* 2002; **8**: 687–693.
3. Fournier PE, Richet H. The epidemiology and control of *Acinetobacter baumannii* in health care facilities. *Clin Infect Dis* 2006; **42**: 692–699.
4. McGowan JE Jr. Resistance in nonfermenting gram-negative bacteria: multidrug resistance to the maximum. *Am J Infect Control* 2006; **34** (suppl): 29–37.
5. Bergogne-Berezin E, Towner KJ. *Acinetobacter* spp. as nosocomial pathogens: microbiological, clinical, and epidemiological features. *Clin Microbiol Rev* 1996; **9**: 148–165.
6. Mazel D. Integrons: agents of bacterial evolution. *Nat Rev Microbiol* 2006; **4**: 608–620.
7. Depardieu F, Podglajen I, Leclercq R *et al.* Modes and modulations of antibiotic resistance gene expression. *Clin Microbiol Rev* 2007; **20**: 79–114.
8. Turton JF, Kaufmann ME, Glover J *et al.* Detection and typing of integrons in epidemic strains of *Acinetobacter baumannii* found in the United Kingdom. *J Clin Microbiol* 2005; **43**: 3074–3082.
9. Koeleman JG, Stoof J, van der Bijl MW *et al.* Identification of epidemic strains of *Acinetobacter baumannii* by integrase gene PCR. *J Clin Microbiol* 2001; **39**: 8–13.
10. Liu SY, Lin JY, Chu C *et al.* Integron-associated imipenem resistance in *Acinetobacter baumannii* isolated from a regional hospital in Taiwan. *Int J Antimicrob Agents* 2006; **27**: 81–84.
11. Wu TL, Ma L, Chang JC *et al.* Variable resistance patterns of integron-associated multidrug-resistant *Acinetobacter baumannii* isolates in a surgical intensive care unit. *Microb Drug Resist* 2004; **10**: 292–299.
12. Falagas ME, Koletsi PK, Bliziotis IA. The diversity of definitions of multidrug-resistant (MDR) and pandrug-resistant (PDR) *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. *J Med Microbiol* 2006; **55**: 1619–1629.
13. Chen TL, Siu LK, Wu RC *et al.* Comparison of one-tube multiplex PCR, automated ribotyping and intergenic spacer (ITS) sequencing for rapid identification of *Acinetobacter baumannii*. *Clin Microbiol Infect* 2007; **13**: 801–806.
14. Ploy MC, Denis F, Courvalin P *et al.* Molecular characterization of integrons in *Acinetobacter baumannii*: description of a hybrid class 2 integron. *Antimicrob Agents Chemother* 2000; **44**: 2684–2688.
15. Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial susceptibility testing*, 15th informational supplement. M100-S15. Wayne, PA: CLSI, 2005.
16. Poirel L, Menuteau O, Agoli N *et al.* Outbreak of extended-spectrum beta-lactamase VEB-1-producing isolates of *Acinetobacter baumannii* in a French hospital. *J Clin Microbiol* 2003; **41**: 3542–3547.
17. Poirel L, Le TI, Naas T *et al.* Biochemical sequence analyses of GES-1, a novel class A extended-spectrum beta-lactamase, and the class 1 integron In52 from *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 2000; **44**: 622–632.
18. Brasme L, Nordmann P, Fidel F *et al.* Incidence of class A extended-spectrum beta-lactamases in Champagne-Ardenne (France): a 1 year prospective study. *J Antimicrob Chemother* 2007; **60**: 956–964.
19. Pasteran F, Rapoport M, Petroni A *et al.* Emergence of PER-2 and VEB-1a in *Acinetobacter baumannii* strains in the Americas. *Antimicrob Agents Chemother* 2006; **50**: 3222–3224.
20. Heritier C, Poirel L, Lambert T *et al.* Contribution of acquired carbapenem-hydrolyzing oxacillinases to carbapenem resistance in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 2005; **49**: 3198–3202.
21. Seifert H, Dolzani L, Bressan R *et al.* Standardization and interlaboratory reproducibility assessment of pulsed-field gel electrophoresis-generated fingerprints of *Acinetobacter baumannii*. *J Clin Microbiol* 2005; **43**: 4328–4335.
22. Tenover FC, Arbeit RD, Goering RV *et al.* Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* 1995; **33**: 2233–2239.
23. Gu B, Tong M, Zhao W *et al.* Prevalence and characterization of class I integrons among *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolates from patients in Nanjing, China. *J Clin Microbiol* 2007; **45**: 241–243.
24. Gallego L, Towner KJ. Carriage of class 1 integrons and antibiotic resistance in clinical isolates of *Acinetobacter baumannii* from northern Spain. *J Med Microbiol* 2001; **50**: 71–77.
25. Chiu CH, Su LH, Chu CH *et al.* Detection of multidrug-resistant *Salmonella enterica* serovar typhimurium phage types DT102, DT104, and U302 by multiplex PCR. *J Clin Microbiol* 2006; **44**: 2354–2358.
26. Levings RS, Lightfoot D, Partridge SR *et al.* The genomic island SGI1, containing the multiple antibiotic resistance region of *Salmonella enterica* serovar Typhimurium DT104 or variants of it, is widely distributed in other *S. enterica* serovars. *J Bacteriol* 2005; **187**: 4401–4409.
27. Shi L, Zheng M, Xiao Z *et al.* Unnoticed spread of class 1 integrons in gram-positive clinical strains isolated in Guangzhou, China. *Microbiol Immunol* 2006; **50**: 463–467.
28. Fournier PE, Vallenet D, Barbe V *et al.* Comparative genomics of multidrug resistance in *Acinetobacter baumannii*. *PLoS Genet* 2006; **2**: e7.
29. Roberts MC. Multidrug-resistant genes are associated with an 86-kb island in *Acinetobacter baumannii*. *Trends Microbiol* 2006; **14**: 375–378.
30. Houang ET, Chu YW, Lo WS *et al.* Epidemiology of rifampin ADP-ribosyltransferase (*arr-2*) and metallo-beta-lactamase (bla_{IMP-4}) gene cassettes in class 1 integrons in *Acinetobacter* strains isolated from blood cultures in 1997 to 2000. *Antimicrob Agents Chemother* 2003; **47**: 1382–1390.
31. Yan JJ, Ko WC, Wu JJ. Identification of a plasmid encoding SHV-12, TEM-1, and a variant of IMP-2 metallo-beta-lactamase, IMP-8, from a clinical isolate of *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 2001; **45**: 2368–2371.
32. Valenzuela JK, Thomas L, Partridge SR *et al.* Horizontal gene transfer in a polyclonal outbreak of carbapenem-resistant *Acinetobacter baumannii*. *J Clin Microbiol* 2007; **45**: 453–460.

33. Ruiz J, Navia MM, Casals C *et al.* Integron-mediated antibiotic multiresistance in *Acinetobacter baumannii* clinical isolates from Spain. *Clin Microbiol Infect* 2003; **9**: 907–911.
34. Rather PN, Munayyer H, Mann PA *et al.* Genetic analysis of bacterial acetyltransferases: identification of amino acids determining the specificities of the aminoglycoside 6′-*N*-acetyltransferase Ib and IIa proteins. *J Bacteriol* 1992; **174**: 3196–3203.
35. Lee MD, Sanchez S, Zimmer M *et al.* Class 1 integron-associated tobramycin–gentamicin resistance in *Campylobacter jejuni* isolated from the broiler chicken house environment. *Antimicrob Agents Chemother* 2002; **46**: 3660–3664.
36. Vila J, Marti S, Sanchez-Cespedes J. Porins, efflux pumps and multidrug resistance in *Acinetobacter baumannii*. *J Antimicrob Chemother* 2007; **59**: 1210–1215.
37. Bonomo RA, Szabo D. Mechanisms of multidrug resistance in *Acinetobacter* species and *Pseudomonas aeruginosa*. *Clin Infect Dis* 2006; **43** (suppl 2): 49–56.
38. Babic M, Hujer AM, Bonomo RA. What's new in antibiotic resistance? Focus on beta-lactamases. *Drug Resist Update* 2006; **9**: 142–156.
39. Tsakris A, Ikonomidis A, Pournaras S *et al.* VIM-1 metallo-beta-lactamase in *Acinetobacter baumannii*. *Emerg Infect Dis* 2006; **12**: 981–983.
40. Poirel L, Pitout JD, Nordmann P. Carbapenemases: molecular diversity and clinical consequences. *Future Microbiol* 2007; **2**: 501–512.
41. Martinez-Freijo P, Fluit AC, Schmitz FJ *et al.* Class I integrons in Gram-negative isolates from different European hospitals and association with decreased susceptibility to multiple antibiotic compounds. *J Antimicrob Chemother* 1998; **42**: 689–696.
42. Martinez-Freijo P, Fluit AC, Schmitz FJ *et al.* Many class I integrons comprise distinct stable structures occurring in different species of Enterobacteriaceae isolated from widespread geographic regions in Europe. *Antimicrob Agents Chemother* 1999; **43**: 686–689.
43. Leverstein-van Hall MA, Box AT, Blok HE *et al.* Evidence of extensive interspecies transfer of integron-mediated antimicrobial resistance genes among multidrug-resistant Enterobacteriaceae in a clinical setting. *J Infect Dis* 2002; **186**: 49–56.
44. Michael CA, Gillings MR, Holmes AJ *et al.* Mobile gene cassettes: a fundamental resource for bacterial evolution. *Am Nat* 2004; **164**: 1–12.
45. Corvec S, Caroff N, Espaze E *et al.* AmpC cephalosporinase hyperproduction in *Acinetobacter baumannii* clinical strains. *J Antimicrob Chemother* 2003; **52**: 629–635.
46. Fernandez-Cuenca F, Martinez-Martinez L, Conejo MC *et al.* Relationship between beta-lactamase production, outer membrane protein and penicillin-binding protein profiles on the activity of carbapenems against clinical isolates of *Acinetobacter baumannii*. *J Antimicrob Chemother* 2003; **51**: 565–574.
47. Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial susceptibility testing*, 16th informational supplement. M100-S16. Wayne, PA: CLSI, 2006.