

# Detection and characterization of class 1 integrons among carbapenem-resistant isolates of *Acinetobacter* spp. in Malaysia

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**Background and purpose:** To detect and characterize class 1 integrons among carbapenem-resistant strains of *Acinetobacter* spp. at University Malaya Medical Centre (UMMC), Kuala Lumpur, Malaysia.

**Methods:** Thirty nine carbapenem-resistant *Acinetobacter* strains were obtained from UMMC from August 2003 to March 2004 and analyzed for the presence of *bla*<sub>IMP</sub> genes and class 1 integrons.

**Results:** Class 1 integrons were detected in 31 of 39 strains. Two *Acinetobacter calcoaceticus* strains harbored an integron-borne *bla*<sub>IMP-4</sub> metallo- $\beta$ -lactamase, 1 of which was located on a 36-kb plasmid. Two different amplified products were found in the 31 isolates with 3 restriction pattern profiles 1, 2, and 3. Correlation was observed between carriage of class 1 integrons and genomic relatedness among these isolates, indicating that particular mechanisms of carbapenem resistance could have been acquired by genotypically distinct clinical isolates of *Acinetobacter* spp.

**Conclusion:** Although class 1 integrons are widely disseminated among clinical isolates of *Acinetobacter* spp. they do not play a major role in the spread of carbapenem resistance.

**Key words:** *Acinetobacter*; beta-lactamase IMP-4; Electrophoresis, gel, pulsed-field; Integrons

## Introduction

*Acinetobacter* spp. are important opportunistic pathogens that are responsible for a wide range of infections, including bacteremia, pneumonia, and surgical wound and urinary tract infections [1,2]. Although imipenem and meropenem are the drugs of choice for the treatment of infections caused by resistant strains of *Acinetobacter* spp., there is a steadily increasing incidence of carbapenem resistance among these pathogens [3,4]. Carbapenem resistance has been intensified by the acquisition and dissemination of resistance genes by mobile genetic elements. Many studies have demonstrated the presence of carbapenemase genes in *Acinetobacter* spp. on plasmids, transposons, and

integrons and, more recently, on a resistance island [5-8]. Integrons are genetic elements that integrate via site-specific recombination and harbor gene cassettes, most of which contain genes that encode for antibiotic resistance. To date, there are more than 9 classes of integrons, with the class 1 integrons being the most documented and well characterized [9]. Class 1 integrons consist of 3 different segments. The 5' conserved segment (5'CS) contains an *intI* gene encoding an integrase and an *attI* recombination site, and the 3'CS contains a combination of *qacE* (antiseptic-resistance gene); *sulI* (sulfonamide-resistance gene) and *orf5* (an open reading frame of unknown function). A variable region is situated between 5'CS and 3'CS [10-12]. Since integron is an element that possesses a site-specific recombination system, it enables the insertion, deletion, and rearrangement of discrete genetic cassettes within this variable region, often comprising antibiotic-resistance genes.

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The main enzymatic mechanism of carbapenem resistance among *Acinetobacter* spp. is the production of  $\beta$ -lactamase enzymes, particularly the Ambler class D (oxacillinases) and class B (*bla*<sub>IMP</sub>, *bla*<sub>VIM</sub>). There have been increasing reports of *Acinetobacter* spp. producing *bla*<sub>IMP</sub>- and *bla*<sub>VIM</sub>-type metallo- $\beta$ -lactamases (MBLs) [13-16]. However, most of the carbapenem resistance in these pathogens is due to the presence of the OXA-type  $\beta$ -lactamases, which have weak activity against carbapenems [17]. The primary aim of this study was to determine the role of integrons in carbapenem resistance in *Acinetobacter* spp. A carbapenem-resistant strain of *A. calcoaceticus* producing increased membrane permeability (IMP)-4  $\beta$ -lactamase contained in a class 1 integron, which is located on a plasmid, is also reported.

## Methods

### Bacterial strains and antimicrobial susceptibility testing

Thirty nine carbapenem-resistant isolates of *Acinetobacter* spp. were obtained from patients admitted to the University Malaya Medical Centre (UMMC), Kuala Lumpur, Malaysia, from August 2003 to March 2004. Almost 30% of the *Acinetobacter* spp. strains were isolated from patients in the intensive care unit. The sites of infection included respiratory tract (61%), surgical wound (20%), intravenous catheter (9%), blood (6%), and urine (4%). Bacterial identification was carried out using the conventional biochemical tests and the API 20 NE system (bioMérieux SA, Marcy-1'Etoile, France) [18]. Growth at 44°C was performed to differentiate between *A. baumannii* and *A. calcoaceticus* [19]. Susceptibility to imipenem, meropenem, ceftazidime, cefotaxime, and aztreonam was determined by the agar dilution method according to the Clinical and Laboratory Standards Institute guidelines [20].

### Metallo- $\beta$ -lactamase screening

MBLs were detected using the double-disc synergy test and modified Hodge test as previously described [21,22].

### Isoelectric focusing

An overnight culture of bacteria was harvested, and cell-free lysates were prepared by sonication [23]. The isoelectric point (pI) values were determined by isoelectric focusing of cell extracts on polyacrylamide gels containing ampholytes with a pH range of 3.5 to

9.5 (Ampholine PAGplate; Pharmacia Biotech, Uppsala, Sweden) using the PhastSystem electrophoresis system (Pharmacia Biotech). The  $\beta$ -lactamases were detected by staining the gel with nitrocefin (0.5 mM).

### Polymerase chain reaction amplification of carbapenemase, OXA, and integron genes

The presence of carbapenemase genes was detected by polymerase chain reaction (PCR), as previously described for *bla*<sub>IMP</sub> [24], *bla*<sub>VIM</sub> [16] and *bla*<sub>OXA</sub> [17]. The amplified products were resolved on a 2% agarose gel and stained with ethidium bromide prior to visualization under ultraviolet light. An additional set of primers, IMP-F (5'-GCCCTAAAACAAAGTTAG-3') and IMP-R (5'-GCTGCAACGACTTGTTAGAAAT-3'), flanking the entire coding region of the *bla*<sub>IMP</sub> gene were designed and used to amplify the 795-bp *bla*<sub>IMP</sub> gene prior to nucleotide sequencing. An internal reverse primer (IRP; 5'-AGCTTGAACCTTACCGTC-3') was designed to complete the sequencing. PCR amplification of the class 1 integrons was carried out using the primers and amplification conditions described previously by Houang et al [24] and Lévesque et al [25]. The amplified products were digested using *AluI* and the resulting restriction bands were analyzed.

### Hybridization studies

Plasmid DNA was extracted according to the modified protocol of Sambrook and Russell [26]. *Escherichia coli* 39R861 containing plasmids of sizes 147 kb, 63 kb, 36 kb, and 7 kb was used to determine the size of the plasmids. To investigate the genetic location of the *bla*<sub>IMP-4</sub> gene in carbapenem-resistant isolates, Southern hybridization was performed on plasmid DNA after its transfer to a nylon membrane. A *bla*<sub>IMP-4</sub> PCR product was labeled with digoxigenin and used as a probe according to the reagent manufacturer's instruction (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). In addition, to detect the presence of the *bla*<sub>IMP-4</sub> gene in class 1 integrons, Southern blot hybridization was performed on integron PCR products using the digoxigenin-labeled *bla*<sub>IMP-4</sub> probe.

### Molecular typing with pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) fingerprints were generated by a contour-clamped homogeneous electric field electrophoresis apparatus (CHEF DRII apparatus; Bio-Rad Laboratories, Hercules, CA, USA) as described in a previous study [27]. The restriction

endonuclease *ApaI* was used for the in situ digestion of intact *Acinetobacter* genomic DNA embedded in 1.6% low melting agarose gel blocks prepared according to previously described methods [11]. Samples were loaded into 1.2% agarose gels and electrophoresed with 0.5X Tris-borate-ethylenediaminetetraacetic acid (EDTA) buffer with an electric field of 200 V, an included angle of 120°, and a pulse time of 5 to 35 sec over 32 h at 14°C. Images of ethidium bromide-stained gels were digitized by use of a gel documentation system, GelCompar II (Alpha InnoTech System; Alpha Innotech Corp, San Leandro, CA, USA). The criteria used in the dendrogram analysis were based on those of Tenover et al [28].

### **β-Lactamase fractionation and carbapenem hydrolysis**

Isolates S26 and S90, both carrying the IMP-4 gene, were used in the kinetic studies. log-Phase cells were harvested from a 10-L broth culture, washed, and resuspended in phosphate buffer. Cells were disrupted by sonication and the cell debris removed by ultracentrifugation at 10,000 rpm for 20 min. The resulting supernatant containing the β-lactamases was purified using the Rotofor system (Bio-Rad Laboratories), which enabled the collection of fractions based on pI values. The β-lactamase activity was determined spectrophotometrically by measuring the hydrolysis of oxacillin, imipenem, or meropenem as substrates. The enzyme was incubated with inhibitor for 10 min at 37°C before the addition of the substrate. The relative maximum rates of reaction ( $V_{max}$ ) were determined from the Hanes plots generated.

## **Results**

### **Bacterial identification and antimicrobial susceptibility patterns**

All 39 strains were identified as *A. baumannii-calcoaceticus* complex using the API 20 NE system. Growth at 44°C distinguished 36 strains of *A. baumannii* from 3 strains of *A. calcoaceticus* [19]. All strains were resistant to all antimicrobials tested, including imipenem and meropenem (Table 1).

### **Metallo-β-lactamase production in carbapenem-resistant *Acinetobacter* spp.**

Of 39 imipenem-resistant strains screened by the IMP-EDTA-double-disk synergy test and modified Hodge test, only 2 strains, S26 and S90, were positive for the

presence of MBLs, although these enzymes were not inhibited by EDTA or 2-mercaptopropanoic acid. Both strains had similar minimal inhibitory concentration (MIC) values for imipenem, cefotaxime, and aztreonam at 32, 512, and 64 μg/mL, respectively (Table 1). Isoelectric focusing analysis showed that both strains had a band of pI 8.0, which corresponded to that of IMP-4, while an additional band of pI 7.1 was present in strain S90 (Fig. 1).

### **Identification of *bla*<sub>IMP-4</sub> and plasmid analysis of increased membrane permeability-4 producing strains**

To confirm the mechanism of carbapenem resistance, PCR was performed using primers specific to *bla*<sub>IMP</sub>, *bla*<sub>VIM</sub>, and *bla*<sub>OXA</sub>. Of the 39 carbapenem-resistant strains of *Acinetobacter* spp., only 2 strains, S26 and S90, were PCR-positive for *bla*<sub>IMP</sub>, while the remaining 37 harbored *bla*<sub>OXA</sub>. None of the 39 isolates harbored *bla*<sub>VIM</sub>. Amplification and subsequent nucleotide sequencing of the entire coding region of *bla*<sub>IMP</sub> confirmed the identity of the *bla*<sub>IMP</sub> amplicon to be *bla*<sub>IMP-4</sub>. Plasmid analysis showed that only 2 imipenem-resistant MBL-producing strains, S26 and S90, carried 147, 63, 36 plasmids in both strains with an additional 7-kb plasmid in S26 (Fig. 2A). Southern blot hybridization showed that the *bla*<sub>IMP-4</sub> gene was located on the 36-kb plasmid in strain S26 (Fig. 2B).

### **Detection of class D β-lactamases**

Amplification of *bla*<sub>OXA</sub> gene gave rise to an amplicon of 1058 bp. Among the 39 carbapenem-resistant strains, 37 were positive for the presence of *bla*<sub>OXA</sub>. Nucleotide sequencing of the purified PCR product showed that this gene has 96% amino acid sequence homology to *bla*<sub>OXA-23</sub>.

### **Detection and characterization of class 1 integrons**

PCR amplification of class 1 integrons resulted in 2 bands of 2.5 kb and 3 kb in 31 of 39 carbapenem-resistant strains of *Acinetobacter* spp. Restriction enzyme analysis with *AluI* showed 3 fingerprint patterns, which were designated profiles 1, 2, and 3 (Fig. 3). Profiles 1 and 2 had similar fingerprint patterns of 5 bands, with the exception of an additional band of 200 bp in profile 2 (Fig. 3). Subsequent sequencing of the entire gene cassette representatives of each profile showed that strains with profile 1 (2.5 kb) contained the *aacC1* gene, whereas the larger (3 kb) integron cassette (profile 2) was identical to that of profile 1

**Table 1.** Minimal inhibitory concentrations (MICs) of  $\beta$ -lactam antibiotics for metallo- $\beta$ -lactamases (MBL)-producing and non-MBL-producing *Acinetobacter* strains.

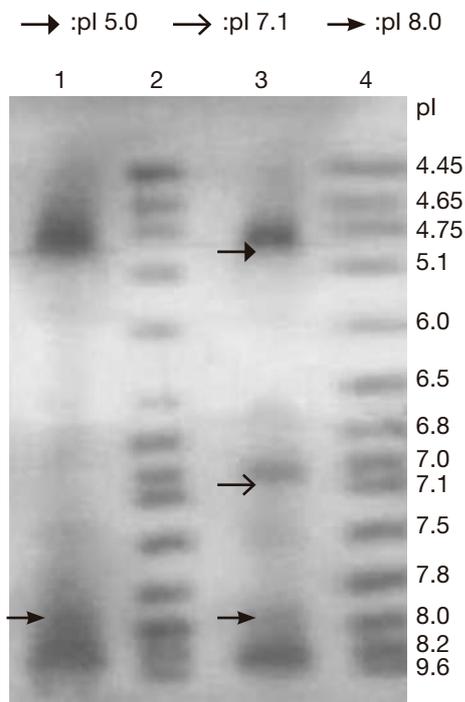
$\beta$ -Lactam antibiotics	MIC ( $\mu$ g/mL)		
	MBL-producing strains (n = 2)		Non-MBL-producing strains (n = 37)
	S26	S90	
Imipenem	32	32	16-64
Meropenem	12	64	32-128
Ceftazidime	25	512	8-512
Cefotaxime	512	512	32-512
Aztreonam	64	64	64-512

with the exception of an additional copy of the *orfX* gene. Both *bla*<sub>IMP-4</sub>-producing *A. calcoaceticus*, S26 and S90, had a unique profile 3. Nucleotide sequencing of the inserted gene cassettes showed identical genes: *bla*<sub>IMP-4</sub>, *qacG*, *aacA4*, and *catB3*. Southern blot hybridization using a *bla*<sub>IMP-4</sub> probe showed positive hybridization on the class 1 integron.

### Correlation of integron profiles with genotyping

Analysis of the dendrogram generated from PFGE patterns showed that 39 carbapenem-resistant strains of *Acinetobacter* spp. could be classified into 12 groups designated A to L (Fig. 4). Data were subsequently

analyzed to correlate the observed minor variations in integron profiles within each genotype with macro-restriction profiles generated by PFGE. Analysis showed that 9 of 10 isolates with profile 1 (2.5 kb) had identical PFGE patterns, designated genotype B, whereas 11 of 19 isolates with profile 2 had a PFGE pattern of genotype C (Table 2). The remaining isolates, 1 with integron profile 1 and 8 with integron profile 2, had unique PFGE profiles and were classified as sporadic isolates. However, both *bla*<sub>IMP-4</sub>-producing *A. calcoaceticus* strains, S26 and S90, with integron profile 3 gave distinct PFGE profiles that differed from each other by more than 7 bands.



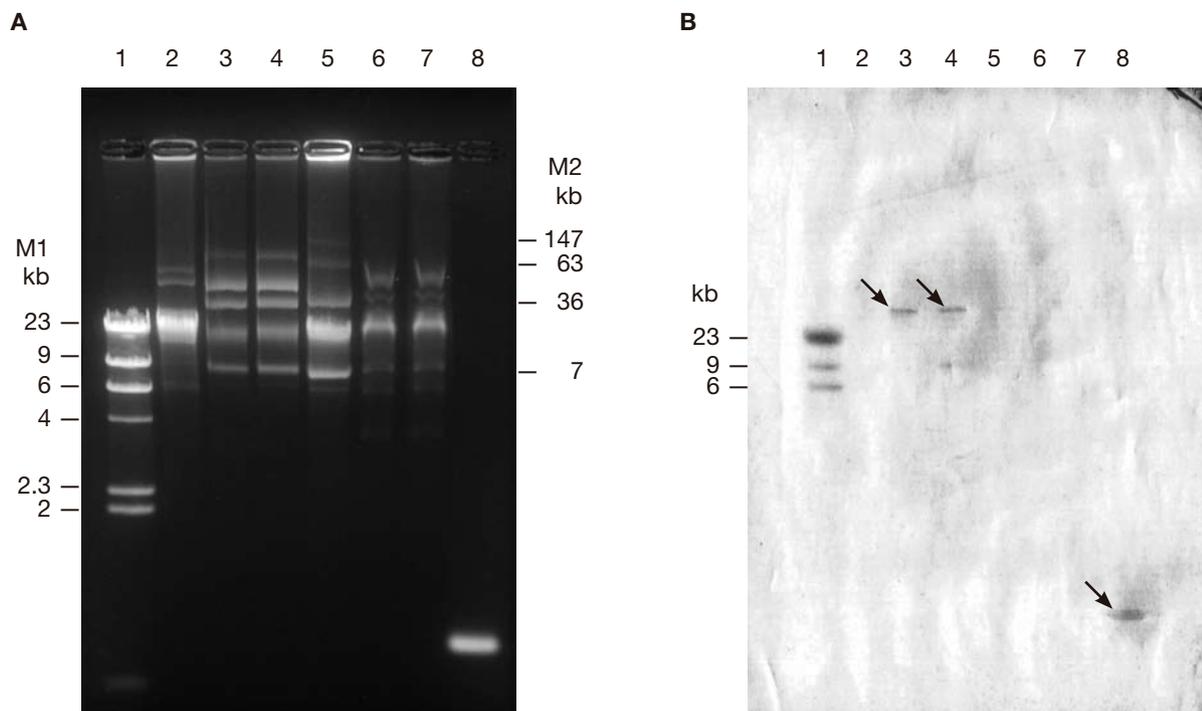
**Fig. 1.** Isoelectric focusing of  $\beta$ -lactamases produced by carbapenem-resistant strains of *Acinetobacter calcoaceticus*. Lane 1, strain 26, isoelectric point (pI) value 8.0; lane 2, pI marker; lane 3, strain 90, pI values 7.1 and 8.0; lane 4, pI marker.

### $\beta$ -Lactamase purification and kinetics

The purified IMP-4 enzyme from strains S26 and S90 had a wide range of activity against several antibiotics, including carbapenems, cephalosporins, and aztreonam. The relative  $V_{\max}$  rates were obtained for imipenem (0.82-0.97 mmol/L/h), meropenem (0.97-1.07 mmol/L/h), oxacillin (0.78-0.79 mmol/L/h), and aztreonam (1.11-1.24 mmol/L/h) [Table 3]. The 50% inhibitory concentration of EDTA when the inhibitor was incubated with the enzyme was 0.01 mM.

### Discussion

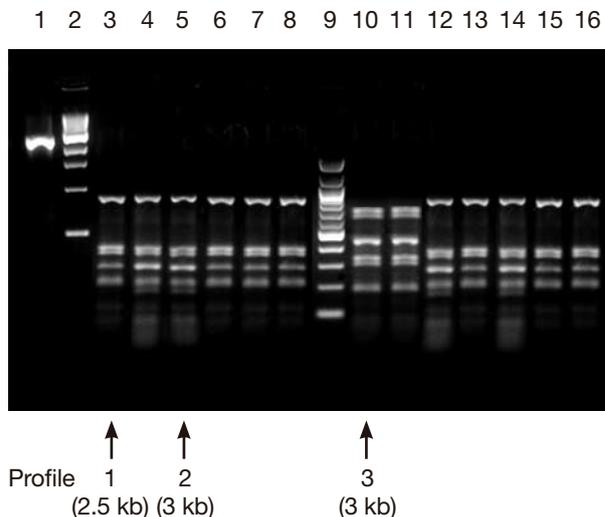
The incidence of carbapenem resistance in *Acinetobacter* spp. is increasing worldwide. There are several mechanisms of carbapenem resistance in these pathogens, which include the presence of Ambler class B or class D carbapenemases or loss of porins. Between August 2003 and March 2004, the UMMC isolated 39 strains of carbapenem-resistant *Acinetobacter* spp., which were confirmed biochemically to belong to the *A. baumannii-calcoaceticus* complex. The study showed that, of 39 carbapenem-resistant isolates of *Acinetobacter* spp., only 2 harbored a *bla*<sub>IMP-4</sub> carbapenemase,



**Fig. 2.** (A) Plasmid profiles of *bla*<sub>IMP-4</sub>-producing strains of *Acinetobacter* spp. (B) Plasmid DNA hybridization using *bla*<sub>IMP-4</sub> probe. Lane 1,  $\beta$  *Hind*III marker (M1); lane 2, *Acinetobacter baumannii* American Type Culture Collection 15308; lanes 3 and 4, strain 26; lane 5, *Escherichia coli* 39R861 standard plasmid marker (M2); lanes 6 and 7, strain 90; lane 8, *bla*<sub>IMP-4</sub> polymerase chain reaction product (*Acinetobacter* spp.).

<sup>a</sup>Arrows in lanes 3 and 4 indicate positive hybridization with *bla*<sub>IMP-4</sub> probe on plasmids; arrow in lane 8 indicates polymerase chain reaction product.

which was inhibited by EDTA. The remaining 37 isolates were positive for a *bla*<sub>OXA</sub> via PCR amplification, which was subsequently confirmed to be *bla*<sub>OXA-23</sub> via nucleotide sequencing. Susceptibility testing using the agar dilution method showed that the MBL-producing strains (S26 and S90) generally had higher MICs to all the antibiotics tested compared with the non-MBL-producing strains, and higher MICs to meropenem than to imipenem (Table 1). Further analysis of IMP-4-producing strains showed that both enzymes had similar kinetic properties and pls with the exception of an additional band in strain S90 (Fig. 1). From the kinetic data, the IMP-4 enzyme from S26 appeared to be slightly more active compared with the enzyme from S90. Both enzymes had higher hydrolyzing activity against meropenem than imipenem, which correlated with the MICs obtained. However, these findings contrast with previous studies, whereby the IMP-type carbapenemases were shown to hydrolyze imipenem more rapidly than meropenem [13]. In addition, the  $V_{max}$  rates for aztreonam were higher than for ceftazidime, ampicillin, and gentamycin, which contrasts with other reports [13]. This difference could



**Fig. 3.** Restriction enzyme profiles of class 1 integrons digested with *Alu*I from clinical isolates of *Acinetobacter* spp. Lane 1, strain 6 (undigested polymerase chain reaction product); lane 2, 1 kb; lane 3, strain 6; lane 4, strain 12; lane 5, strain 13; lane 6, strain 14; lane 7, strain 15; lane 8, strain 22; lane 9, 100 bp; lane 10, strain 26; lane 11, strain 90; lane 12, strain 29; lane 13, strain 30; lane 14, strain 33; lane 15, strain 35; lane 16, strain 45.

<sup>a</sup>Arrows indicate the different restriction profiles (1, 2, and 3).

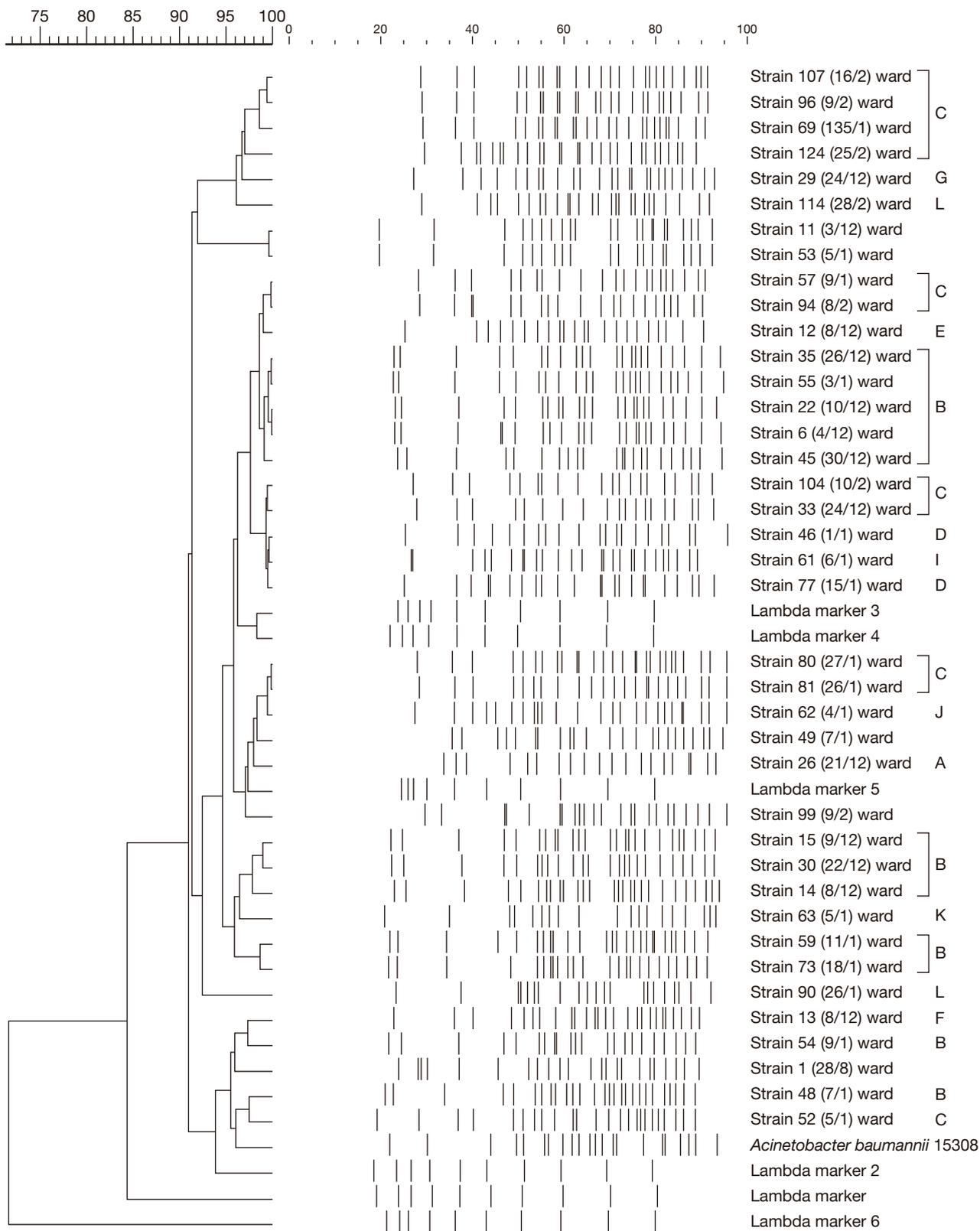


Fig. 4. Dendrogram analysis depicting the clonal relationship of the carbapenem-resistant strains of *Acinetobacter* spp.

**Table 2.** Correlation between class 1 integron profiles and the genotypes of clinical isolates of *Acinetobacter* spp.

Integron profile	5'- and 3'-CS amplicon size (kb)	Inserted resistance gene cassettes within 5'- and 3'-CS	PFGE genotype (no. of isolates)
1	2.5	<i>aacC1</i> , <i>aadA1a</i> , <i>orf</i>	B (9), J (1)
2	3.0	<i>aacC1</i> , <i>aadA1a</i> , <i>orfX</i> , <i>orfX'</i>	C (11), D (2), E (1), F (1), G (1), H (1), I (1), L (1)
3	3.0	<i>bla</i> <sub>IMP-4</sub> , <i>qacG</i> , <i>aacA4</i> , <i>catB3</i>	A (1), K (1)

Abbreviations: CS = conserved segment; PFGE = pulsed-field gel electrophoresis.

**Table 3.** Relative rate of hydrolysis of strains S26 and S90.

Enzyme	Original host	Relative rate of hydrolysis (mmol/L/h)								IC <sub>50</sub> for EDTA (μM)	Isoelectric point
		Imipenem	Meropenem	Oxacillin	Ceftazidime	Nitrocefin	Ampicillin	Gentamycin	Aztreonam		
IMP-4	S26	0.97	1.07	1.24	0.67	0.5	0.60	0.64	0.79	0.01	8.90
IMP-4	S90	0.82	0.97	1.11	0.75	0.48	0.63	0.75	0.78	0.01	8.90
AmpC	S90	0.61	0.81	0.85	1.13	0.82	0.87	0.84	0.87	0.01	7.05

Abbreviations: IC<sub>50</sub> = 50% inhibitory concentration; EDTA = ethylenediaminetetraacetic acid; IMP = increased membrane permeability.

be due to the presence of another β-lactamase, probably *Temoneira* gene-type. However, the  $V_{max}$  for both these enzymes was higher for oxacillin than any of the other antibiotics tested, which is in accordance with the findings of Afzal-Shah et al [17].

Integrons are widely distributed among clinical isolates of *Acinetobacter* spp. There have been several studies that have shown the association of antibiotic resistance, particularly aminoglycoside resistance, with integrons [29,30]. Most isolates tested carried a class 1 integron. PCR and subsequent nucleotide sequence analysis showed the presence of *aacC1* and *aadA1a* resistance genes in integron profiles 1 and 2 (genotypes B-J and L), which confer resistance to gentamicin (Table 2). However, the integrons in both MBL-producing strains contained a *bla*<sub>IMP-4</sub> allele along with *aacA4* and *catB3* alleles, both of which encode for resistance to aminoglycosides. In this study, *bla*<sub>IMP-4</sub> was identified within an integron that was located on a 36-kD plasmid in a clinical isolate of *A. calcoaceticus*. A similar finding of a *bla*<sub>IMP-1</sub> in a plasmid-borne class 1 integron in a clinical isolate of *A. baumannii* was first reported in 2006 by Liu et al [15]. Although identical cassette arrays were found in integrons of the IMP-4-producing strains, PFGE analyses showed that S26 and S90 had different genotypes (Table 2, Fig. 3). These results suggest that carbapenem resistance in these strains of *A. calcoaceticus* could have been acquired via horizontal gene transfer, which concurs with the findings of Gombac et al [31].

Although class 1 integrons are widely distributed among clinical isolates of *Acinetobacter* spp. at the UMMC, they do not seem to play a major role in the

dissemination of carbapenem resistance, as integron-associated MBL was only detected in 2 of 39 clinical isolates. However, the location of the integron-borne *bla*<sub>IMP-4</sub> gene on a plasmid is a cause for concern, as a previous study has demonstrated the ease of transmission of carbapenem resistance through plasmid conjugation [32]. Hence, this study indicates that precautionary monitoring of integron-associated *bla*<sub>IMP</sub> in clinical isolates of *Acinetobacter* spp. should be carried out, as they pose a potential threat for the spread of carbapenem resistance in these pathogens. Furthermore, clinicians treating infections caused by *Acinetobacter* spp. should consider using the inhibitory combination ampicillin-sulbactam instead of carbapenems with the intention of circumventing this potential threat.

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