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Molecular characterization of metallo-β-lactamase-producing *Acinetobacter baumannii* and *Acinetobacter* genomospecies 3 from Korea: identification of two new integrons carrying the bla_{VIM-2} gene cassettes

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Carbapenem-resistant *Acinetobacter* spp. used to be rare, but are increasingly isolated in Korea. Among 28 isolates of imipenem-resistant *Acinetobacter* spp. found in a Korean hospital in 1998 and 1999, 14 produced metallo-β-lactamases. The bla_{VIM-2} gene was detected, by PCR, in 11 and two isolates of *Acinetobacter baumannii* and *Acinetobacter* genomospecies 3, respectively, and bla_{IMP-1} in one isolate of *A. baumannii*. The MICs of imipenem for the isolates were 8–32 mg/L. PFGE analysis of *Sma*I-digested genomic DNA gave identical patterns in eight of 11 bla_{VIM-2}-positive *A. baumannii* isolates from respiratory specimens of ICU patients. The bla_{VIM-2} gene cassettes in the isolates are identical to those from *Pseudomonas aeruginosa* isolates in Europe, but are inserted into new class I integrons In105 and In106. The *attC* site of the last cassette of the array in In106 is interrupted by the insertion of a putative class II intron. This is the first report of VIM-2 β-lactamase-producing *A. baumannii* and *Acinetobacter* genomospecies 3. Production of the VIM-2 enzyme presents an emerging threat of carbapenem resistance among *Acinetobacter* spp. in Korea.

Introduction

Carbapenems are very useful for the treatment of infections caused by Gram-negative bacilli that are resistant to other β-lactam antibiotics. However, carbapenem-resistant Gram-negative bacilli are being isolated with increasing frequency from clinical sources. *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and a number of species of *Enterobacteriaceae* producing IMP-type metallo-β-lactamases have been found in several hospitals in Japan since the early 1990s, and identical or related enzymes have subsequently been detected elsewhere.[1] More recently, the emergence of new metallo-β-lactamases VIM-1 and VIM-2 in *P. aeruginosa* isolates from Europe was reported,[2] and *Pseudomonas* spp. carrying bla_{VIM-2} have been reported to be in circulation in Korea.[3] Both bla_{VIM} and bla_{IMP} are found on mobile gene cassettes inserted in the variable region of integrons.[1,2] Therefore, horizontal spread of these resistance determinants, in addition to clonal spread of individual strains, can be anticipated. In this paper, we report the detection of VIM-2 and IMP-1 metallo-β-lactamase-producing *Acinetobacter* spp. in Korea, and the characteristics of novel bla_{VIM-2}-carrying integrons.

Materials and methods

Bacterial strains and in vitro susceptibility testing

Twenty-eight non-repetitive isolates of imipenem-resistant *Acinetobacter* spp. were obtained from patients in a tertiary care hospital in Seoul, Korea, in 1998 and 1999. The species

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were identified by the conventional method[4] and the ATB 32 GN System (bioMérieux SA, Marcy 1'Étoile, France). For these isolates, resistance to imipenem was detected by disc diffusion test.[5] MICs of antimicrobial agents were determined by an agar dilution method.[5]

β-*Lactamase assays*

The modified Hodge and EDTA–disc synergy tests[3] were used to screen for metallo-β-lactamase production by imipenem-resistant isolates. β-Lactamase activities in crude cell sonicates were determined using 100 μM β-lactams in 50 mM phosphate buffer (pH 7.0) at 30°C using a UV1601 spectrophotometer (Shimadzu, Tokyo, Japan). Enzyme activities were also determined after incubation of cell extracts with 10 mM EDTA or 50 μM clavulanate for 30 min at 30°C.[2] Analytical isoelectric focusing (IEF) was carried out following the manufacturer's instructions using precast gels with pH gradient 3–10, and a ThermoFlow Electrophoresis Temperature Control System (Novex Experimental Technologies, San Diego, CA, USA). Detection of β-lactamase bands, EDTA inhibition and bioassays were carried out as described previously with minor modification.[1]

Molecular analysis techniques

The presence of bla_{VIM-2} and bla_{IMP-1} was detected by PCR. Previously described bla_{VIM-2} primers[2] and bla_{IMP-1} primers IMP1-F (5′-CATGGTTTGGTGGTTCTTGT-3′) and IMP1-R (5′-ATAATTTGGCGGACTTTGGC-3′) were used. PCR mixes contained l μL of heat-extracted template DNA, 1 μL (20 pmol) of each primer and PCR Premix containing 1 U of *Taq* DNA polymerase (Bioneer, Cheongwon, Korea) in a final volume of 20 μL. The thermocycle protocol used was: an initial denaturation step at 94°C for 5 min, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, elongation at 72°C for 45 s, plus a final extension step at 72°C for 7 min.

Gene cassette arrays inserted into class I integrons were amplified using the primers of Riccio *et al.*[1] The amplication protocol was that of Levesque & Roy[6] with minor modification. Briefly, the reaction was carried out in 100 μL, using 10 μL of heat-extracted template DNA, 20 pmol of each primer and 3 U of LA *Taq* DNA polymerase (Takara, Shiga, Japan).

PCR-generated amplicons were separated by agarose gel electrophoresis and recovered using a DNA extraction kit (Qiagen, Hilden, Germany). When necessary, the amplicon was inserted into the pGEM-T-easy vector (Promega, Madison, WI, USA) and cloned in *Escherichia coli* DH5α.[7]

Southern hybridization was carried out using a digoxigenin (DIG)-labelled bla_{VIM-2}-containing PCR amplicon. Labelling and detection of the probe were carried out using a DIG DNA labelling and detection kit (Roche Diagnostics, Mannheim, Germany).

Sequencing was carried out by the dideoxynucleotide-chain termination method with an automatic DNA sequencer (ABI 3700; Perkin-Elmer, Foster City, CA, USA) and custom-

Table 1. Antimicrobial susceptibilities of metallo-β-lactamase-producing *A. baumannii* and *Acinetobacter* genomospecies 3 isolates. MICs of the same drugs for eight randomly selected imipenem-resistant isolates that did not produce metallo-β-lactamase are shown for comparison

	MIC (mg/L)[a] for isolates						
	bla_{VIM-2} positive (n = 13)				metallo-β-lactamase negative (n = 8)		
β-Lactam(s)	range	50%	90%	bla_{IMP-1} positive (n = 1)	range	50%	90%
Ampicillin	64–>128	>128	>128	>128	>128	>128	>128
Piperacillin	16–64	32	64	128	256–>256	256	256
Piperacillin + TAZ[b]	16–32	32	32	4	128	128	128
Cefalothin	>128	>128	>128	>128	>128	>128	>128
Cefotaxime	64–>128	128	128	>128	>128	>128	>128
Cefoxitin	64–>128	128	>128	>128	>128	>128	>128
Ceftazidime	16–64	32	32	>128	128–>128	>128	>128
Imipenem	8–32	8	16	16	8–32	32	32
Meropenem	4–8	4	8	32	16–64	64	64
Aztreonam	16–32	32	32	16	128	128	128

[a]50% and 90%, MICs at which 50% and 90% of isolates are inhibited, respectively.
[b]TAZ, tazobactam (4 mg/L fixed concentration).

designed primers. Both strands of two independently cloned PCR amplicons originating from each isolate were sequenced.

PFGE

Genomic DNAs of the *Acinetobacter* isolates were digested with *Sma*I, as suggested by the manufacturer. The fragments were separated using a CHEF-DR II System (Bio-Rad, Hercules, CA, USA). The band patterns were interpreted according to the recommendations of Tenover *et al*.[8]

Nucleotide sequence accession numbers

The nucleotide sequences of the bla_{VIM-2}-carrying integrons have been assigned the GenBank accession nos AF324464 for that from *A. baumannii* YMC 98/7/363, and AF369871 for that from *Acinetobacter* genomospecies 3 YMC 99/11/160.

Results and discussion

Acinetobacter spp. are often resistant to most β-lactams, but are generally susceptible to carbapenems. Of the 28 imipenem-resistant *Acinetobacter* spp. isolated in the hospital study, 14 were positive for metallo-β-lactamases, as determined by both modified Hodge and EDTA–disc synergy tests. Enzyme assays of crude cell extracts confirmed the presence of carbapenemase activity, which was susceptible to inhibition by EDTA, but not clavulanate (data not shown). This finding indicates that metallo-β-lactamase production is an important mechanism of carbapenem resistance in *Acinetobacter* spp. in Korea.

The bla_{IMP-1} and bla_{VIM-2} determinants were detected by PCR in one and 13 isolates, respectively. IMP-1-producing *Acinetobacter* spp. have been reported in Japan, but this is the first report of a VIM-2 enzyme in *Acinetobacter* spp. Bands of β-lactamase activity, approximate pIs 5.3 and >8.5, were detected by analytical IEF in cell extracts of bla_{VIM-2}- and bla_{IMP-1}-positive isolates, respectively. These enzymes were susceptible to EDTA inhibition and active against carbapenems in bioassays. Additional β-lactamases with different pIs and resistant to EDTA inhibition were also present in these isolates (data not shown).

The MICs of imipenem were 8–32 mg/L for both bla_{VIM-2}-positive and metallo-β-lactamase-negative isolates (Table 1). The MIC range of imipenem for bla_{VIM-2}-positive *Acinetobacter* spp. was lower than that reported for bla_{VIM-2}-positive *P. aeruginosa*, 8–>128 mg/L, at our hospital. It is significant that 50% of VIM-2-producing *Acinetobacter* spp. were found to be inhibited by 8 mg/L imipenem (intermediate category). All of the isolates were either susceptible or displayed

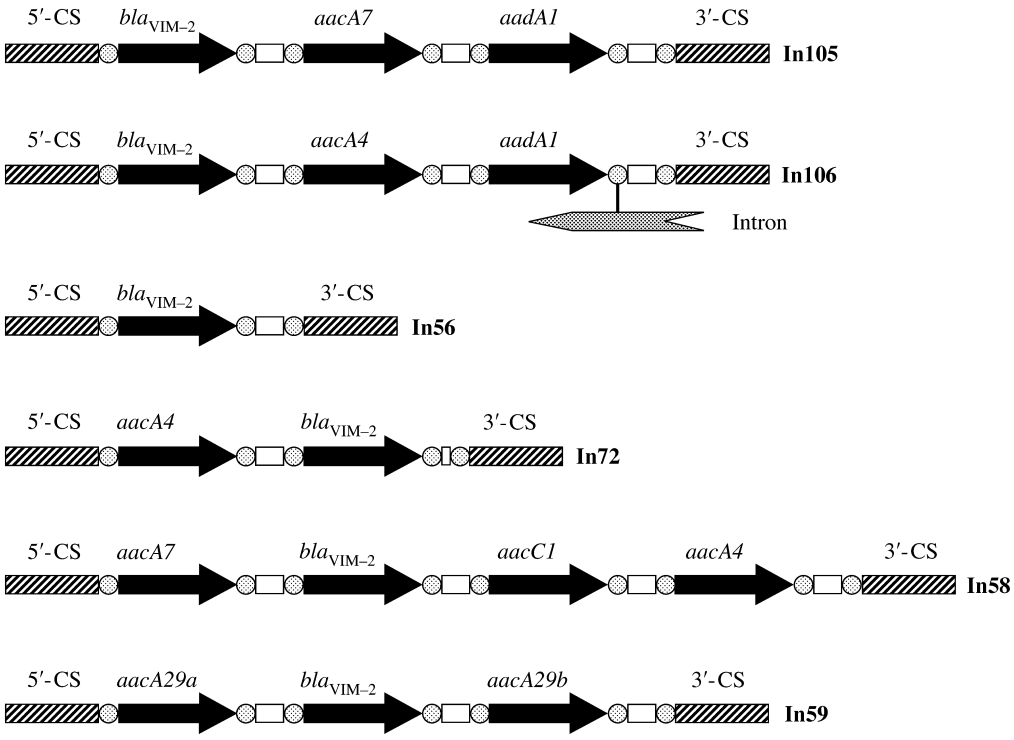

Figure 1. Comparison of the structures of the variable regions of In105 and In106 with those of other bla_{VIM-2} cassette-containing class I integrons: In56 (AF191564), In72 (AF302086), In58 (AF263519) and In59 (AF263520). Hatched rectangles, 5′- and 3′-conserved segments (5′-CS and 3′-CS); black arrows, cassette-borne resistance genes; empty rectangles, *attC* sites of the cassettes; grey circles, recombination core sites and inverse core sites. The insertion and orientation of the putative class II intron in the *attC* site of the last cassette of In106 is indicated.

intermediate resistance to meropenem when results were interpreted according to the criteria of the National Committee for Clinical Laboratory Standards.[5] Relatively low-level carbapenem resistance is not uncommon among metallo-β-lactamase-producing bacteria and underscores the need for reliable screening procedures to detect such isolates.

All 11 bla_{VIM-2}-positive *A. baumannii* isolates were from respiratory specimens, while two bla_{VIM-2}- and one bla_{IMP-1}-positive *Acinetobacter* genomospecies 3 were from urine specimens. PFGE showed that among the 11 *A. baumannii* isolates, eight gave identical fragmentation patterns and one had a closely related pattern and had been isolated from ICU patients. The two bla_{VIM-2}-positive *Acinetobacter* genomospecies 3 isolates were unrelated (data not shown).

The VIM-2 gene has been found on a class I integron.[2] PCR products of *c.* 3 kb from 12 *A. baumannii* isolates and of *c.* 5 kb from one *Acinetobacter* genomospecies 3 isolate were obtained using integron-directed primers. These products hybridized to a bla_{VIM-2} probe in Southern hybridization experiments (data not shown). *Eae*I digestion of the 3 kb amplicons from the 12 isolates yielded identical six fragment band patterns, indicating that these isolates have identical or very similar integron structures.

The 3 and 5 kb amplicons obtained from isolates of *A. baumannii* (YMC 98/7/363) and *Acinetobacter* genomospecies 3 (YMC 99/11/160), respectively, were cloned into pGEM-T-easy. The inserts recovered were sequenced, revealing two new gene cassette arrays inserted between the 5′- and 3′-conserved segments of class I integrons. The 3 kb integron, named In105, carries the bla_{VIM-2}, *aacA7* and *aadA1* cassettes (Figure 1). The 5 kb integron, named In106, carries the bla_{VIM-2}, *aacA4* and *aadA1* cassettes, and a putative class II intron inserted into the *attC* site of the last cassette of the array (Figure 1). This finding indicates that this type of mobile genetic element can provide a target for retro-homing of class II introns.[9] The two bla_{VIM-2} cassettes were identical to those of *P. aeruginosa* isolates from Europe.[2] The sequences of the 5′-conserved segments of the two integrons were identical, except for a putative G to A transition at position 10 in the smaller integron (GenBank accession No. AF324464). The integrons contain two putative promoters, a strong-type P_c and a non-functional P_2.[10]

In conclusion, this is the first report of the VIM-2 metallo-β-lactamase in *Acinetobacter* spp. The bla_{VIM-2} gene cassette was found to be part of the two new integrons In105 and In106. Furthermore, the *attC* site of the last cassette of the array in In106 is interrupted by the insertion of a putative class II intron. Production of the VIM-2 β-lactamase presents an emerging threat of carbapenem resistance among *Acinetobacter* spp. in Korea.

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