Molecular characterization of metallo-β-lactamase-producing Acinetobacter baumannii and Acinetobacter genomospecies 3 from Korea: identification of two new integrons carrying the blaVIM-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 blaVIM-2 gene was detected, by PCR, in 11 and two isolates of Acinetobacter baumannii and Acinetobacter genomospecies 3, respectively, and blaIMP-1 in one isolate of A. baumannii. The MICs of imipenem for the isolates were 8–32 mg/L. PFGE analysis of SmaI-digested genomic DNA gave identical patterns in eight of 11 blaVIM-2-positive A. baumannii isolates from respiratory specimens of ICU patients. The blaVIM-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. More recently, the emergence of new metallo-β-lactamases VIM-1 and VIM-2 in P. aeruginosa isolates from Europe was reported, and Pseudomonas spp. carrying blaVIM-2 have been reported to be in circulation in Korea. Both blaVIM and blaIMP are found on mobile gene cassettes inserted in the variable region of integrons. 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 blaVIM-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
were identified by the conventional method and the ATB 32 GN System (bioMérieux SA, Marcy 1’Étoile, France). For these isolates, resistance to imipenem was detected by disc diffusion test. MICs of antimicrobial agents were determined by an agar dilution method.

**β-Lactamase assays**

The modified Hodge and EDTA–disc synergy tests 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.

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.

**Molecular analysis techniques**

The presence of blaVIM-2 and blaIMP-1 was detected by PCR. Previously described blaVIM-2 primers and blaIMP-1 Primers IMP1-F (5’-CATGGTTTGGTGTTCTTGTTGT-3’) and IMP1-R (5’-ATAATTTGGCGGACTTTGGC-3’) were used. PCR mixes contained 1 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. The amplification protocol was that of Levesque & Roy 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.

Southern hybridization was carried out using a digoxigenin (DIG)-labelled blaVIM-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 genospecies

<table>
<thead>
<tr>
<th>β-Lactam(s)</th>
<th>MIC (mg/L)* for isolates</th>
<th>MIC (mg/L)* for isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>blaVIM-2 positive (n = 13)</td>
<td>metallo-β-lactamase negative (n = 8)</td>
</tr>
<tr>
<td></td>
<td>range</td>
<td>50%</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>64–&gt;128</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>16–64</td>
<td>32</td>
</tr>
<tr>
<td>Cefalothin</td>
<td>&gt;128</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>64–&gt;128</td>
<td>128</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>64–&gt;128</td>
<td>128</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>16–64</td>
<td>32</td>
</tr>
<tr>
<td>Imipenem</td>
<td>8–32</td>
<td>8</td>
</tr>
<tr>
<td>Meropenem</td>
<td>4–8</td>
<td>4</td>
</tr>
</tbody>
</table>

*50% and 90%, MICs at which 50% and 90% of isolates are inhibited, respectively.

bTAZ, 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*<sub>VIM</sub>-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* genospecies 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*<sub>IMP</sub>-1 and *bla*<sub>VIM</sub>-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*<sub>VIM</sub>-2- and *bla*<sub>IMP</sub>-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*<sub>VIM</sub>-2- positive and metallo-β-lactamase-negative isolates (Table 1). The MIC range of imipenem for *bla*<sub>VIM</sub>-2-positive *Acinetobacter* spp. was lower than that reported for *bla*<sub>VIM</sub>-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

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**Figure 1.** Comparison of the structures of the variable regions of In105 and In106 with those of other *bla*<sub>VIM</sub>-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. 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_{IMP-1} and one bla_{VIM-2} 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. 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). EaeI 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 different six fragment band patterns, indicating that these isolates have identical or very similar integron structures.

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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References


