Infections due to multidrug- and carbapenem-resistant isolates of *Acinetobacter baumannii* were frequent among patients treated in the Tzannion General Hospital in Piraeus, Greece, throughout the period 2005 to 2006. Six of these isolates, derived from blood cultures of patients in three different wards (four were from patients in the intensive care unit) and taken at least 1 month apart from each other, were studied. Species identification, initially performed with the API 20NE system (bioMérieux, Marcy l’Etoile, France), was confirmed by sequencing of 16S rRNA genes.

Antimicrobial MICS were determined by agar dilution. Screening for metallo-β-lactamases (MBLs) was performed with imipenem-EDTA synergy tests: (i) the MBL Etest (AB Biodisk), (ii) the double-disc synergy test (3), and (iii) the combined-disc test (1). Other types of β-lactamases were studied by isoelectric focusing of crude cell extracts derived by sonication. The carbapenamase activity of β-lactamase-containing extracts was assessed by spectrophotometry using imipenem (100 μM) as the reporter substrate and is expressed as units (1 U was the amount of enzyme hydrolyzing 1 μmol of imipenem per min per mg of protein). Reaction mixtures were supplemented with ZnSO₄ (50 μM) (6). Imipenem hydrolysis rates were also determined in the presence of EDTA at a final concentration of 100 μM. Preliminary experiments using extracts from *Escherichia coli* laboratory strains containing various β-lactamases showed that EDTA at 100 μM inhibited VIM-1 activity by >95%, while the activities of OXA, TEM, and AmpC enzymes were virtually unaffected. The *bla*<sub>OXA</sub> and MBL genes were identified by PCR as described previously (10) and by sequencing. The association of *bla*<sup>OXA</sup> genes with *IsaA* elements was studied by PCR (11). *bla*<sub>VIM-1</sub>-carrying integrons were characterized by the sequencing of overlapping PCR products based on published sequences (7, 8). The isolates were typed by pulsed-field gel electrophoresis (PFGE) (9). The location of the VIM-1-encoding integrons was determined by an I-CeuI-based method (5).

The isolates were highly resistant to piperacillin-tazobactam, cefotaxime, ceftazidime, cefepime, and aztreonam (MICs were ≥ 128 μg/mL). Imipenem and meropenem MICs ranged from 16 to 32 μg/mL. The isolates were also resistant to ampicillin-sulbactam, gentamicin, amikacin, tobramycin, and ciprofloxacin. Only one isolate was MBL positive by the double-disc synergy test, though it tested negative by the MBL Etest and the combined-disc test. The remaining five isolates appeared to be negative by all three tests. Notwithstanding the minor phenotypic differences, the isolates exhibited similar PFGE patterns (>80% relatedness), suggesting a clonal relationship.

The isolates carried the carbapenemase genes *bla*<sub>OXA-51</sub> and *bla*<sub>OXA-58</sub>. *IsaA*<sub>ba</sub> was found by PCR upstream of *bla*<sub>OXA-58</sub>. Moreover, *IsaA*<sub>ba</sub>1 was adjacent to *bla*<sub>OXA-51</sub>, probably providing the promoter for this gene (11). Other types of *bla*<sub>OXA</sub> genes were not identified. Unlike with the results of the MBL detection tests, all six isolates contained the *bla*<sub>VIM-1</sub> MBL gene.

The results of the isoelectric focusing experiments were in line with the *bla* gene content. The isolates produced β-lactamases with isoelectric points (pIs) of approximately 6.5 and 7.0, consistent with OXA enzymes, as well as an enzyme with an alkaline pI (~9.0), probably representing the chromosomal cephalosporinase of this species. A β-lactamase with a pI equal to that of VIM-1 (5.1) that was inhibited in situ by EDTA was also detected, indicating MBL expression. The levels of imipenem hydrolysis by the extracts of five *bla*<sub>VIM-1</sub>-positive isolates were similar, ranging from 28 to 36 U. The remaining *bla*<sub>VIM-1</sub>-positive isolate (TZ42/166) exhibited significantly higher activity against imipenem (90 U). These activities were at least 10-fold higher than those of 10 MBL-negative, carbapenem-resistant OXA-58 producers (obtained from two hospitals in Athens, Greece, in 2007) tested in parallel. EDTA reduced imipenem hydrolysis at levels similar to those of the *bla*<sub>VIM-1</sub>-negative isolates (1.5 to 3 U), suggesting that carbapenemase activities were largely due to VIM-1.

The isolates contained a class 1 integron designated In-Ab10a with a variable region of 1,725 bp comprising, from 5′ to 3′, *aacA4* and *bla*<sub>VIM-1</sub>. The promoter region included a strong P1 and the inactive form of P2. Similar integrons have been identified in a VIM-1-encoding plasmid from an *Escherichia coli* isolate also from Greece (8), as well as in VIM-4-producing *Pseudomonas aeruginosa* isolates from Hungary and Poland (4, 7). The *bla*<sub>VIM-1</sub> cassettes in In-Ab10a and the related integrons contain, between the coding sequence and 59-base, a 170-bp direct repeat of the 3′ end of *bla*<sub>VIM-1</sub>. It has been proposed that this unusual structure evolved through a partial deletion of a tandem *bla*<sub>VIM-1</sub> cassette (7). In addition to In-Ab10a, isolate TZ42/166 harbored a novel integron, In-Ab10b, including only the characteristic *bla*<sub>VIM-1</sub> cassette. The presence of two VIM-1-encoding integrons in TZ42/166 may explain the relatively higher carbapenemase activity; however, the higher carbapenemase activity did not seem to significantly affect β-lactam resistance levels. *bla*<sub>VIM-1</sub> was detected by hybridization in an I-CeuI fragment of approximately 250 kb (Fig. 1), documenting the chromosomal location of In-Ab10a and In-Ab10b.

The above findings indicate a protracted hospital outbreak caused by genetically related *A. baumannii* isolates producing VIM-1. *A. baumannii* isolates are routinely examined by EDTA-imipenem tests in Greek hospitals, where VIM enzymes are widespread. This approach has allowed the detection of sporadic strains carrying In-e541, a VIM-1-encoding integron that, like In-Ab10a, is also spread in enterobacteria (10). Additionally, a PCR-based screening in a hospital in northern Greece of 78 carbapenem-resistant isolates that were negative by imipenem-EDTA synergy tests revealed two *bla*<sub>VIM-1</sub> carriers (2). Taken together, these studies suggest that *bla*<sub>VIM-1</sub>-positive *A. baumannii*
strains have been established in this setting and that the conventional EDTA-based tests may not be suitable for their detection. The precise contribution of VIM-1 to the overall -lactam resistance was not assessed. Nevertheless, the failure of the phenotypic tests to detect VIM-1 in these isolates underscores the role of multiple mechanisms, such as the production of OXA enzymes in determining carbapenem resistance in this species.

Nucleotide sequence accession numbers. The sequences of the integrons In-Ab10a and In-Ab10b have been assigned GenBank accession numbers EF690695 and EF690696, respectively.

REFERENCES


FIG. 1. (A) Structure of the blaVIM-1 -carrying integron In-Ab10a. The direction of transcription is indicated by the arrows. The dotted rectangle to the left of the duplicated 3’ end of blaVIM-1 (Pseudo 59-be) represents the inverse core sequence of the cassette and the pseudo-core sequence located within the structural gene. The white-dotted rectangle on the right (VIM-1 59-be) represents 59-be of the blaVIM-1 cassette. (B to B”) PFGE of I-CeuI-digested genomic DNA of eight A. baumannii clinical isolates from the same setting. (B) TZ28 and TZ29/20 in the first two lanes represent carbapenem-resistant blaVIM-1-negative isolates used for control purposes. Genomic fragments of the six blaVIM-1-positive isolates are shown in the remaining lanes. Hybridizations with probes specific for 16S rRNA and blaVIM-1 genes are shown in panels B’ and B”, respectively.


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