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VIM-1 Metallo-β-Lactamase-Producing *Klebsiella pneumoniae* Strains in Greek Hospitals

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Seventeen *Klebsiella pneumoniae* clinical isolates carrying the bla_{VIM-1} metallo-β-lactamase gene were collected in the intensive care units of three hospitals in Athens, Greece, in 2002. They exhibited various carbapenem resistance levels (Etest MICs of imipenem ranged from 4 to 32 μg/ml). All isolates gave positive results by the imipenem-EDTA synergy Etest. The isolates were classified into four main types by pulsed-field gel electrophoresis; the majority of the isolates (5 and 10 isolates) belonged to two types. The bla_{VIM-1} gene cassette was part of the variable region of a class 1 integron that also included *aac6*, *dhfrI*, and *aadA*. This structure was carried by transferable plasmids.

The metallo-β-lactamases (MBLs) of the IMP and VIM types are increasingly important clinically. These enzymes are active against most β-lactams, including carbapenems, and have been found in various gram-negative clinical isolates mostly in the Far East and the Mediterranean region (12). The wide spread of integron-borne bla_{VIM} genes in *Pseudomonas aeruginosa* strains in Greek hospitals has been documented (2, 9, 16). Additionally, a sporadic strain of *Escherichia coli* exhibiting resistance to imipenem due to production of a plasmid-mediated VIM-1 MBL was recently isolated in a Greek hospital (10). In the present study, we describe VIM-1-producing *Klebsiella pneumoniae* clinical strains isolated in three hospitals in Athens, Greece.

Seventeen *K. pneumoniae* clinical isolates were studied. They were derived from patients hospitalized from September to December 2002 in the intensive care units of three teaching hospitals (I, II, and III) located in Athens, Greece (Table 1). At least 12 of the isolates were considered causes of infection. Isolates were retained in the respective hospital laboratories because they exhibited resistance or decreased susceptibility to imipenem (a MIC of ≥8 μg/ml or an inhibition zone of <16 mm in diameter by the disk diffusion test) using the automated systems VITEK 2 (bioMerieux S.A.) (hospital I) and Wider I (Francisco Soria Melguizo S.A.) (hospital II) and the disk diffusion test (11) (hospital III).

Species identification was confirmed by using API 20E strips (bioMerieux). MICs of β-lactams were determined by the Etest (AB Biodisk). Susceptibility to other antimicrobial agents was assessed by the disk diffusion test (11). The Etest strip containing imipenem plus EDTA was used to detect MBL production.

Typing was performed by pulsed-field gel electrophoresis (PFGE) of *Xba*I-restricted genomic DNA as described previously (1). Restriction fragments were separated through a 1% agarose gel using a contour-clamped homogeneous electric field DRIII apparatus (Bio-Rad). Classification of the isolates into PFGE types was based on the criteria proposed by Tenover et al. (15).

Conjugal transfer of antibiotic resistance was performed in mixed broth cultures as described previously (17) using the *E. coli* strain 14R (Nal^r *lac*) as a recipient. Transconjugant clones were selected in Mueller-Hinton agar containing nalidixic acid (100 μg/ml) plus ampicillin (50 μg/ml). Plasmid DNA preparations obtained by an alkaline lysis technique (13) were digested with *Pst*I and electrophoresed in 0.8% agarose gels.

VIM-type genes were detected by PCR using primers VIM-F (5′-AGTGGTGAGTATCCGACAG-3′) and VIM-R (5′-ATGAAAGTGCGTGGAGAC-3′) corresponding to nucleotides 1339 to 1357 and 1599 to 1582, respectively, of the bla_{VIM-1} integron (6) (GenBank accession no. Y18050). These primers were also used to amplify a 261-bp bla_{VIM-1} fragment from the *E. coli* plasmid p541 (10). The 261-bp bla_{VIM-1} fragment was labeled with digoxigenin with commercially available reagents (Roche Diagnostics) and used as a bla_{VIM-1} probe in hybridization experiments. Mapping of class 1 integrons was performed by PCR using primers 5′CS and 3′CS (7) and a set of primers specific for various resistance genes, including *aacA*, *dhfrI*, and *aadA*. Nucleotide sequences of the PCR products were determined on both strands with an ABI Prism 377 DNA sequencer (Applied Biosystems).

Determination of imipenem MICs with the Etest confirmed the results reported from the hospital laboratories for 13 of the 17 isolates. The remaining four isolates (Kp5, Kp11, Kp15, and Kp16), however, exhibited an imipenem Etest MIC equal to 4 μg/ml, which is within the susceptibility range. The possible causes of this discrepancy were not investigated. All the iso-

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TABLE 1. Characteristics of 17 bla_{VIM-1}-containing *K. pneumoniae* clinical isolates and 6 *E. coli* transconjugants

Isolate[a]	Hospital	Isolation date (day-mo-yr)	PFGE type	Etest MICs (μg/ml) of β-lactams[b]								Other resistance markers[c]
				PIP	TZP	CTX	CAZ	ATM	IPM	IPM-E	MEM	
Kp1	I	17-09-02	A	>256	256	128	256	256	32	<1	32	Gm, Net, Tb, An, Sxt, C
Ec(trc-1)				>256	128	128	>256	2	1	<1	0.25	Gm, Net, Tb, An, Sxt, C
Kp2	I	18-10-02	A	>256	256	>256	>256	>256	8	<1	4	Gm, Net, Tb, An, Sxt, C
Ec(trc-2)				128	128	128	32	2	0.5	<1	0.12	Gm, Net, Tb, An, Sxt, C
Kp3	I	06-11-02	A	128	128	128	256	128	16	<1	16	Gm, Net, Tb, An, Sxt, C
Kp4	I	10-11-02	A	>256	256	64	128	64	32	<1	32	Gm, Net, Tb, An, Sxt, C
Kp5	I	17-11-02	B	>256	256	64	>256	>256	4	<1	1	Gm, Net, Tb, An, Sxt
Kp6	I	12-12-02	A	256	128	128	256	128	8	<1	4	Gm, Net, Tb, An, Sxt, C
Kp7	II	10-10-02	C	>256	>256	256	256	0.12	32	<1	16	Net, Tb, An, Sxt
Kp8	II	11-10-02	C	>256	>256	128	256	0.25	32	<1	>32	Net, Tb, An, Sxt
Ec(trc-8)				>256	256	64	>256	0.12	4	<1	0.5	Net, Tb, An, Sxt
Kp9	II	22-10-02	C	>256	256	64	>256	0.5	16	<1	8	Net, Tb, An, Sxt
Kp10	II	16-11-02	C	>256	256	64	256	0.5	32	<1	8	Net, Tb, An, Sxt
Kp11	II	27-11-02	C1	>256	>256	256	>256	0.12	4	<1	1	Net, Tb, An, Sxt
Ec(trc-11)				>256	256	32	>256	0.06	2	<1	0.25	Net, Tb, An, Sxt
Kp12	III	09-11-02	D	>256	>256	128	>256	0.5	16	<1	8	Net, Tb, An, Sxt
Kp13	III	28-11-02	C	>256	256	64	>256	0.12	8	<1	2	Net, Tb, An, Sxt
Ec(trc-13)				>256	128	32	>256	0.06	2	<1	0.5	Net, Tb, An, Sxt
Kp14	III	05-12-02	C	>256	256	32	>256	0.5	8	<1	8	Net, Tb, An, Sxt
Kp15	III	05-12-02	C	>256	256	64	256	0.25	4	<1	2	Net, Tb, An, Sxt
Kp16	III	05-12-02	C	>256	256	128	>256	32	4	<1	2	Net, Tb, An, Sxt
Ec(trc-16)				256	128	32	256	0.12	2	<1	0.25	Net, Tb, An, Sxt
Kp17	III	15-12-02	C	256	128	64	128	0.25	8	<1	8	Net, Tb, An, Sxt
E. coli 14R				0.5	0.5	<0.03	0.06	<0.03	0.03		<0.03	

[a] *K. pneumoniae* clinical isolates Kp1 to Kp17 and six *E. coli* transconjugants [Ec(trc-1), Ec(trc-2), etc.] and *E. coli* strain 14R.
[b] PIP, piperacillin; TZP, piperacillin-tazobactam (inhibitor fixed at 4 μg/ml); CTX, cefotaxime; CAZ, ceftazidime; ATM, aztreonam; IPM, imipenem; IPM-E, imipenem-EDTA (inhibitor fixed at 320 μg/ml); MEM, meropenem.
[c] Gm, gentamicin; Tb, tobramycin; Net, netilmicin; An, amikacin; Sxt, co-trimoxazole; C, chloramphenicol.

lates examined were multidrug resistant. MICs of imipenem ranged from 4 to 32 μg/ml. In four isolates, the MICs of imipenem were two or more doubling dilutions higher than those of meropenem. In one isolate, the MIC of meropenem exceeded that of imipenem. The MICs of other β-lactams also varied. Seven isolates were highly resistant to aztreonam, while the MICs of aztreonam were significantly lower for the remaining isolates. The isolates were also resistant to various non-β-lactam antibiotics, including aminoglycosides, co-trimoxazole, and chloramphenicol (Table 1). Ciprofloxacin was active against all 17 isolates.

Results of the imipenem-EDTA synergy tests indicated that all 17 isolates produced MBLs. In 10 isolates, a phantom zone of inhibition between the two gradient sections of the imipenem-EDTA Etest strip was considered a positive result (18).

The isolates were classified into four main types (A to D) by analyzing the results of PFGE. Five of six isolates from hospital I exhibited highly similar PFGE patterns (type A). Chromosomal type C accounted for the majority of the isolates from hospitals II and III. Types B and D were represented by one isolate each (Table 1 and Fig. 1).

Conjugal transfer of resistance was attempted with seven isolates representing the three hospitals and three PFGE types (A, B, and C). bla_{VIM}-positive transconjugants were obtained from all but one isolate (Kp5, PFGE type B). Carbapenem resistance levels were significantly lower in the *E. coli* clones than in the donors (Table 1). Cotransfer of resistance to non-β-lactams was also observed, indicating involvement of multiresistant plasmids. Results of plasmid DNA analysis were in line with the typing results. The plasmids derived from isolates Kp1 and Kp2 (PFGE type A) were similar (plasmid type 1).

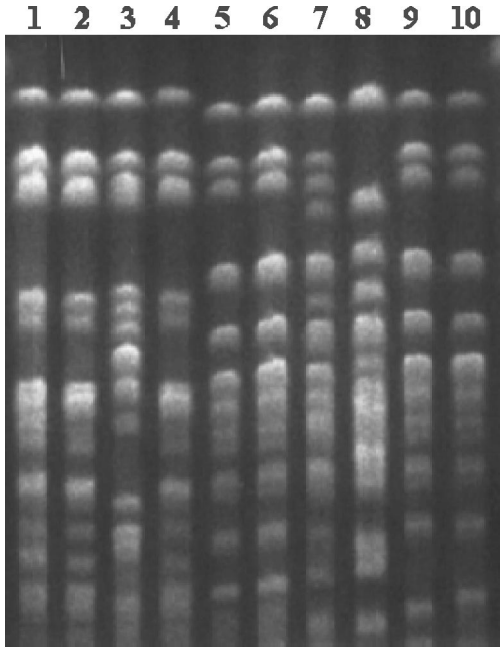

FIG. 1. PFGE patterns of *K. pneumoniae* isolates carrying VIM-type MBL genes. Lanes 1 and 2, isolates Kp1 and Kp2 (pattern A); lane 3, isolate Kp5 (pattern B); lane 4, isolate Kp6 (pattern A); lanes 5 and 6, isolates Kp7 and Kp8 (pattern C); lane 7, isolate Kp11 (pattern C1); lane 8, isolate Kp12 (pattern D); lanes 9 and 10, isolates Kp13 and Kp16 (pattern C).

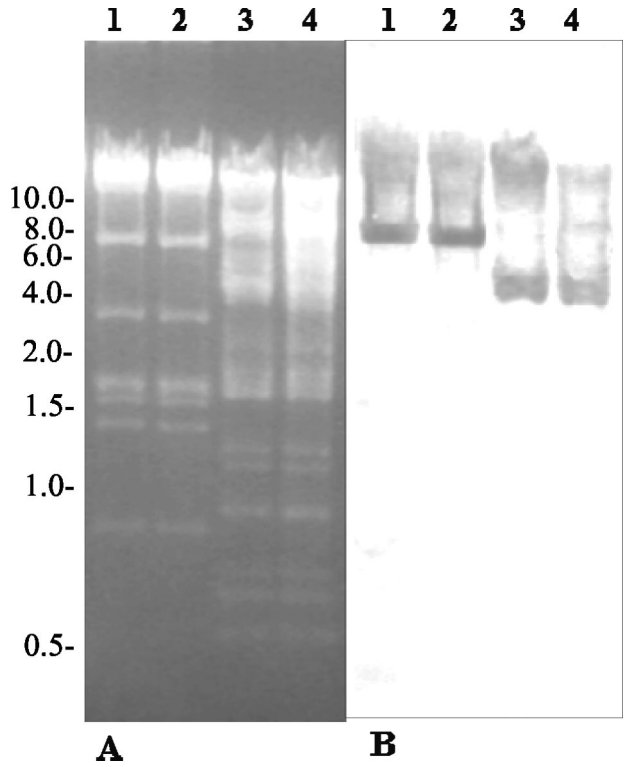

FIG. 2. (A) *Pst*I restriction profiles of the bla_{VIM-1}-carrying plasmids extracted from *E. coli* transconjugants trc-1, trc-2 (plasmid type 1), trc-8, and trc-13 (plasmid type 2) are presented in lanes 1 to 4, respectively. (B) Hybridization of the preparations shown in panel A with a bla_{VIM-1} probe.

Similar plasmids were also obtained from the PFGE type C isolates Kp8, Kp11, Kp13, and Kp16 (plasmid type 2). These two plasmid species were similar in size (approximately 50 kb) but exhibited different restriction profiles (Fig. 2A). In the digests of both plasmid types, a band (approximately 7.5 kbp for type 1 and 5 kbp for type 2) hybridized with the bla_{VIM-1} probe (Fig. 2B).

Integron mapping of seven *K. pneumoniae* isolates and the respective *E. coli* transconjugants by PCR (Table 1) indicated a common class 1 integron structure. The cassette region was approximately 3 kb in size and contained (from 5′ to 3′) bla_{VIM-1}, *aac6*, *dhfrI*, and *aadA*. DNA sequencing of various overlapping PCR amplicons from isolates Kp1, Kp8, and Kp16 and the respective *E. coli* transconjugants showed that the *aatI1* site of this class 1 integron was followed by bla_{VIM-1}. The bla_{VIM-1} cassette (including the 59-base element) was identical to the one in *P. aeruginosa* described previously (6) (GenBank accession no. Y18050) and *E. coli* (10).

The potential of IMP and VIM MBL genes to spread among clinical enterobacteria has also been shown previously (3–5, 14, 19–21). The results of this study, along with the recent isolation of a VIM-1-producing *E. coli* (10), indicate that VIM-type genes have been spread via transferable plasmids in the enterobacteria of the hospital flora in Greece. Also, acquisition of the VIM-1 integrons by distinct plasmids suggests a possible association with mobile elements. Therefore, containment of this resistance is expected to be difficult.

Decreased susceptibility to imipenem allowed recognition of the VIM-producing *K. pneumoniae* isolates in the hospital laboratories. Consequently, infection control measures, including isolation of these patients, were applied in a timely fashion. These measures probably accounted for the apparent decrease in the isolation frequency of imipenem-resistant *K. pneumoniae* strains, as indicated in the records of the three hospitals since January 2003. Typing results suggested spread of a limited number of strains. There were, however, substantial differences in the β-lactam resistance levels between isolates of the same chromosomal type. For instance, isolate Kp16 was resistant to aztreonam, while the rest of the type C isolates were susceptible to this antibiotic. It can be hypothesized that the similarity of the PFGE patterns reflected, in some cases, strains of common origin that evolved over time.

The isolates examined may represent a portion of the bla_{VIM-1}-containing strains circulating in these hospitals. MBL production in enterobacteria may not be able to substantially increase the MICs of carbapenems without the simultaneous operation of other mechanisms such as impaired permeability (5, 8). The differences in the imipenem MICs of the clinical isolates and transconjugants are compatible with this notion. Thus, a number of MBL-producing strains may pass unnoticed in routine susceptibility testing. Also, the imipenem-EDTA Etest strip may exhibit low sensitivity for MBL-producing enterobacteria with imipenem MICs lower than those observed here. Additionally, detection of MBL-producing enterobacteria may pose technical difficulties. As has been shown for MBL-producing nonfermenters, the composition of the medium significantly affects the MICs of carbapenems; this effect may be due to differences in zinc concentration (18). This could partly explain the discrepancies between the Etest and the methods employed in the hospitals. Therefore, standardization and evaluation of the performance of the susceptibility tests for MBL-producing enterobacteria are required.

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