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Spread of Integron-Associated VIM-Type Metallo-β-Lactamase Genes among Imipenem-Nonsusceptible *Pseudomonas aeruginosa* Strains in Greek Hospitals

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Fifty-eight imipenem-nonsusceptible (MIC ≥ 8 μg/ml) *Pseudomonas aeruginosa* strains isolated during May 2001 in 15 Greek hospitals were studied. Thirty-six isolates derived from nine hospitals carried VIM-type metallo-β-lactamase genes, as found by PCR. In 34 isolates, *bla*$_{VIM}$ was associated with class 1 integrons of various sizes. DNA sequencing indicated the presence of *bla*$_{VIM-2}$ gene cassettes in a variety of integron structures. Random amplified polymorphic DNA typing suggested diversity of the *bla*$_{VIM}$-positive strains. Synergy between 2-mercaptoacetic acid and imipenem indicated carbapenemase activity in 26 *bla*$_{VIM}$-positive strains.

Carbapenems exhibit potent antipseudomonal activity. Intensive use of these antibiotics, however, has facilitated the emergence of resistance in *Pseudomonas aeruginosa*. The mechanisms include decreased outer membrane permeability, overproduction of AmpC, up-regulation of multidrug efflux pumps (7), and production of carbapenem-hydrolyzing metallo-β-lactamases (MBLs) that belong to two types, IMP and VIM (8). The spread of MBL-producing *P. aeruginosa* strains has been reported mostly in the Far East (6, 17) and the Mediterranean region (3, 5, 12–14, 18).

In 2000 an outbreak of VIM-2-producing *P. aeruginosa* was described in a hospital in Thessaloniki, Greece (10, 18). Also, data from the National Surveillance System for Antimicrobial Resistance (WHONET-Greece) indicated an average frequency of imipenem-nonsusceptible (IPM-NS) (MIC ≥ 8 μg/ml, or ≤15-mm zone diameter in the disk diffusion test) *P. aeruginosa* isolates of 12% for the year 2000 (www.mednet.gr/whonet). This study was undertaken to assess the contribution of MBLs in this resistance. For this purpose, 18 hospital laboratories participating in WHONET-Greece were asked to contribute all IPM-NS *P. aeruginosa* clinical strains isolated during May 2001.

MICs of ceftazidime (CAZ), aztreonam (ATM), piperacillin-tazobactam (PTZ), IPM, and meropenem (MER) were determined by the Etest method (AB Biodisk, Solna, Sweden). Susceptibility to other antibiotics was assessed by disk diffusion (11). *P. aeruginosa* ATCC 27853 was used as a control.

VIM-type genes were detected by PCR using the primers VIM-F (5′-AGTGGTGAGTATCCGACAG-3′) and VIM-R (5′-ATGAAAGTGCGTGGAGAC-3′) (10). Detection of *bla*$_{IMP}$ by PCR was performed as described previously (16). Class 1 integrons were detected by PCR using the 5′CS and 3′CS oligonucleotides (15). Association of integrons with MBL genes was confirmed by PCR using combinations of *bla*- and integron-specific primers. Partial nucleotide sequences of selected PCR products were determined with an ABI Prism 377 DNA sequencer (Perkin Elmer, Applied Biosystems Division, Foster City, Calif.) using 5′CS, 3′CS and *bla*$_{VIM}$-specific primers.

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TABLE 1. Isolation frequencies of IPM-NS *P. aeruginosa* strains in 18 hospitals during 2001

Hospital (location[a])	No. of isolates in:			
	January-December 2001		May 2001	
	Total	IPM-NS (%)	Studied	*bla*$_{VIM}$ positive[c]
A (Patra, SW)	ND[b]	ND	10	7
B (Ioannina, NW)	317	114 (36)	10	10
C (Athens)	ND	ND	5	5
D (Alexandroupolis, NE)	65	25 (38)	1	0
E (Thessaloniki)	411	219 (53)	5	5
F (Athens)	389	143 (37)	2	1
G (Athens)	165	45 (27)	5	0
H (Heraklion, S)	ND	ND	3	3
I (Thessaloniki)	43	11 (26)	2	2
J (Athens)	403	76 (19)	2	0
K (Athens)	222	20 (9)	2	0
L (Athens)	129	33 (26)	5	0
M (Volos, E)	88	16 (18)	2	2
N (Xanthi, N)	92	12 (13)	2	0
O (Athens)	180	55 (31)	3	1
P (Thessaloniki)	85	9 (11)	0	
Q (Thessaloniki)	29	2 (7)	0	
R (Athens)	38	7 (18)	0	

[a] SW, southwest; NW, northwest; NE, northeast; S, south; E, east; N, north.
[b] ND, not determined.
[c] As determined by *bla*$_{VIM}$-specific PCR assays.

TABLE 2. Characteristics of 36 bla_{VIM}-containing IMP-NS *P. aeruginosa* isolates

Hospital	Isolate	Result of MAA test	VIM integron size (kb)	RAPD type	β-Lactam resistance phenotype[a]				
					IPM	MER	CAZ	ATM	PTZ[b]
A	A1	+	1.5	1	R	R	R	R	R
	A2	+	1.5	1	R	R	R	R	R
	A3	+	1.5	1	R	R	R	R	R
	A4	−	1.5	1	R	R	R	R	R
	A5	+	1.5	2	R	R	R	I	R
	A6	+	1.5	3	R	R	I	S	R
	A7	+	1.5	3	R	R	S	S	S
B	B1	+	1.8	3	R	R	I	S	R
	B2	+	1.8	3	R	R	S	S	R
	B3	+	1.8	3	I	R	R	I	R
	B4	+	1.8	3	R	R	S	S	R
	B5	+	1.8	3	R	R	S	S	R
	B6	−	1.8	3	R	R	S	S	R
	B7	+	1.5	4	R	R	I	S	R
	B8	+	1.5	4	R	R	I	S	S
	B9	+	1.5	4	R	R	S	I	S
	B10	+	1.5	5	R	R	S	S	R
C	C1	+	1.5	6	R	R	I	S	R
	C2	+	1.5	6	R	R	I	S	S
	C3	+	ND[c]	3	R	R	I	I	S
	C4	−	1.5	7	R	R	R	I	R
	C5	−	1.5	8	R	R	R	I	R
E	E1	−	1.5	9	R	I	S	I	R
	E2	−	1.5	10	I	I	I	I	R
	E3	−	ND	11	R	S	I	S	S
	E4	+	2.0	12	R	R	I	S	R
	E5	−	1.5	13	R	I	R	I	R
F	F1	−	3.0	ND	I	R	S	I	R
H	H1	+	1.5	10	R	R	R	I	R
	H2	+	1.5	10	R	R	R	R	R
	H3	+	1.5	10	R	R	R	R	R
I	I1	+	3.0	14	R	S	I	S	R
	I2	+	3.0	14	R	R	I	S	R
M	M1	+	1.3	15	R	R	R	I	R
	M2	+	1.3	15	R	R	R	I	R
O	O1	−	1.5	16	R	R	S	S	R

[a] R, resistant; I, intermediate; S, susceptible.
[b] The inhibitor was fixed at 4 μg/ml.
[c] ND, not determined.

A synergy test using disks of CAZ (30 μg) and IPM (10 μg) combined with in-house-prepared disks containing mercaptoacetic acid (MAA) (3 μl per disk) was employed to detect strains producing MBLs as described previously (1).

Molecular typing was carried out by random amplified polymorphic DNA (RAPD) fingerprinting (9). Genomic DNA was extracted as described previously (4) and amplified by PCR using the oligonucleotide primer 208 (5′-AGCGGGCCAA-3′). Amplification products were separated in 1.5% agarose. RAPD patterns were compared as suggested by Campbell et al. (2).

Frequencies of IPM-NS *P. aeruginosa* isolates in the participating hospitals during 2001 ranged from 7 to 53%. Fifty-eight IPM-NS *P. aeruginosa* strains isolated in May 2001 were obtained from 15 hospitals. Three hospitals reported that no IPM-NS isolates were recovered during the study period (Table 1).

Etest confirmed the reported status of susceptibility to IPM (all MICs were ≥8 μg/ml). In 53 isolates, levels of resistance to IPM and to MER were similar (differences of ≤2 doubling dilutions). For four isolates, MICs of IPM were significantly higher than those of MER; one isolate was more resistant to MER than to IPM. Frequencies of resistance to CAZ and ATM were 71 and 64%, respectively. Only nine (16%) and three (5%) isolates were susceptible to PTZ and ticarcillin-clavulanate, respectively. Extensive cross-resistance to non-β-lactam drugs was also observed. Fifty-one (88%) isolates were resistant to ciprofloxacin, and 53 (91%) were resistant to at least one aminoglycoside.

Of the 58 IPM-NS isolates, 36 (62%), derived from nine

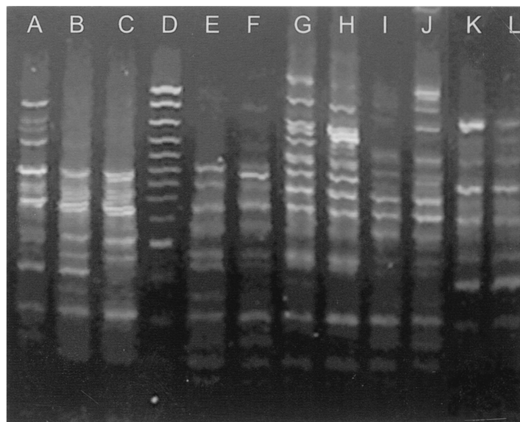

FIG. 1. RAPD patterns of *P. aeruginosa* isolates carrying bla_{VIM}. Lane A, RAPD type 6 (Table 2); lanes B and C, RAPD type 4; lanes E and F, RAPD type 10; lanes G and H, RAPD type 14; lanes I and J, RAPD type 1; lanes K and L, RAPD type 3. Molecular weight markers (100-bp DNA Ladder Plus; MBI Fermentas) are in lane D. The RAPD pattern similarity of the isolates in lanes I and J was confirmed in repeated experiments.

hospitals, were bla_{VIM} positive, producing an amplicon of the expected size (260 bp). bla_{IMP}-positive isolates were not detected. The presence of class 1 integrons was confirmed in 34 of the bla_{VIM}-positive isolates. The sizes of the regions encompassed by the 5′ and 3′ conserved sequences ranged from 1.3 to 3.0 kb (Table 2). For 22 isolates, a single product of 1.5 kb was observed. For seven isolates, the sizes of the products were 1.8 to 2.0 kb. For three isolates, a product of approximately 3.0 kb was observed. Two amplicons, 1.0 and 1.3 kb, were found in each of the remaining two isolates. By combining the primer 5′CS with VIM-R and 3′CS with VIM-F, colinearity of bla_{VIM} genes with class 1 integrons was indicated in all 34 integron-positive isolates. The sizes of the bla_{VIM}-carrying integrons are in Table 2.

Nucleotide sequencing of the 1.3-kb product from isolate M1 showed that the 5′CS-3′CS region included a single gene cassette identical to bla_{VIM-2} of integron In*56* (14) (GenBank accession no. AF191564). Partial sequencing of the 1.5-kb amplicons derived from isolates A4 and C5 indicated that both also contained bla_{VIM-2} preceded by an *aacA29* gene cassette. This structure resembles part of the VIM-2-encoding integron In*59* (GenBank accession no. AF263519), found recently in a *P. aeruginosa* clinical strain in France (13).

Results of the synergy test using CAZ and 2-MAA were equivocal and not reproducible for most IPM-NS isolates, including the bla_{VIM}-negative ones. The combination of IPM with 2-MAA performed better, giving clear synergy images for 26 of the 36 bla_{VIM}-carrying isolates (sensitivity, 72%) (Table 2); all 22 isolates that did not contain bla_{VIM} were negative in this test (specificity, 100%).

bla_{VIM}-positive isolates were typed by RAPD fingerprinting. The discriminatory power and reproducibility of the method were satisfactory. Sixteen distinct RAPD patterns were observed; six of them are presented in Fig. 1. RAPD typing did not indicate any significant spread of epidemic clones, though strains exhibiting similar patterns (RAPD types 3 and 10) were observed in more than one hospital (Table 2). However, patients' records did not provide indications for interhospital spread. Notably, in four hospitals (A, B, C, and E) there were more than two bla_{VIM}-carrying strains exhibiting distinct RAPD patterns (Table 2).

The diversity of RAPD types found here suggests the spread of bla_{VIM} genes among genetically distinct *P. aeruginosa* strains. This spread is likely facilitated by the carriage of the bla_{VIM} genes by integrons, which, though not mobile themselves, are frequently parts of transposons and/or transferable plasmids. DNA sequencing indicated that the predominant MBL gene type is bla_{VIM-2}. It is not known if the observed differences in the size and structure of the integrons reflect evolution of an index integron or acquisition of bla_{VIM} cassettes by different integrons. Studies on the structure of these elements are under way.

There were no phenotypic characteristics suggestive of bla_{VIM} carriage. The majority of bla_{VIM}-positive and bla_{VIM}-negative isolates were resistant to all tested antibiotics. Additionally, no consistent quantitative differences in the levels of resistance to β-lactams, including carbapenems, were observed between the two groups. The MAA synergy test exhibited low sensitivity. Its specificity, however, appeared to be adequate. This test may be a useful adjunct to trace bla_{VIM}-containing *P. aeruginosa* in this setting.

The relatively small number of isolates examined did not allow a reliable estimation of the prevalence of the bla_{VIM}-containing *P. aeruginosa*. Also, there were sampling differences between hospitals. Finally, the possibility that bla_{VIM} genes may be present among IPM-susceptible strains cannot be excluded. These resistance determinants are carried by integrons, and thus, their expression and subsequently their levels of resistance to carbapenems may vary significantly. Nevertheless, this preliminary study shows that bla_{VIM} genes have spread not only in the large tertiary-care hospitals of Athens and Thessaloniki but also in district hospitals throughout the country.

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