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Molecular Epidemiology of VIM-4 Metallo-β-Lactamase-Producing *Pseudomonas* sp. Isolates in Hungary[▽]

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VIM metallo-β-lactamase-producing serotype O11 or O12 *Pseudomonas aeruginosa* isolates infecting or colonizing 19 patients from seven hospitals in Hungary were characterized between October 2003 and November 2005. Macrorestriction analysis revealed the involvement of hospitals from three different towns in northwest Hungary in an outbreak caused by VIM-4-producing *P. aeruginosa*.

The worldwide spread of acquired metallo-β-lactamase (MBL)-producing gram-negative pathogens was observed in the past decade, with the bla_{VIM}-type acquired MBL genes currently being the most prevalent in Europe (16, 23). The first VIM-producing *Pseudomonas aeruginosa* clinical isolates in Hungary (isolates PA396 and PA450) were characterized at the National Center for Epidemiology in 2003 (7). We established routine screening of carbapenem-resistant *Pseudomonas* sp. isolates provided by collaborating regional laboratories for acquired MBL genes. In 2005, MBL-positive isolates from six towns in Hungary were detected (Fig. 1). Our aim was to characterize these isolates and to examine the clonal relationships between them and between the major European multiresistant serotype O12 *P. aeruginosa* clone, clone P12 (4, 8, 10).

The VIM-positive clinical and environmental isolates tested in this study are listed in Table 1, together with the previously published control isolates PA396 and PA450 (7). Isolates P12-Q and P12-E were from French patients "Q" and "E," respectively, who participated in a previous clone P12-related study (8). MICs were determined by the agar dilution method (1) for β-lactam antibiotics and by the Etest (AB Biodisk, Solna, Sweden) for other antibiotics. The MBL Etest and the imipenem-EDTA, ceftazidime-EDTA, and cefepime-EDTA double-disk methods were used for phenotypic screening (21, 22).

bla_{VIM} genes and class 1 integrons were detected by PCR (7). The variable regions of the integrons from isolates PA555 and MB197 were sequenced by using the following primers, together with those described previously (7): primer 197F (5′-AAT CGC TCA GTC GCC GAG-3′), primer 197R1 (5′-TAG TGC TTC TCC GTC GGG-3′), primer 197R2 (5′-AAT TCC GCA TTG CTG ATC G-3′), and primer 197R3 (5′-AGG TAT TGC TCC TGC ACT T-3′). Isolate PA555 was selected for full integron sequencing in 2003, as it was the first VIM-positive isolate from Pécs, Hungary, while isolate MB197 was selected as an invasive isolate from a cluster of clonally closely related VIM-positive isolates from northwest Hungary. For the other VIM-producing isolates, the integron structures were determined by PCR mapping and partial sequencing.

Pulsed-field gel electrophoresis (PFGE) was performed as described earlier (11), with modifications, and the patterns were interpreted by using Fingerprinting II Informatix software (Bio-Rad, Madrid, Spain). *Pseudomonas aeruginosa* antisera (Bio-Rad, Marnes-la-Coquette, France) were used for serotyping.

Conjugation experiments were carried out with strains *Escherichia coli* J5-3 Rif^r and *P. aeruginosa* PAO4089Rp (6, 7) as the recipients. Transconjugants were selected on Mueller-Hinton agar plates containing 300 μg/ml rifampin and 32 μg/ml cefotaxime, 128 μg/ml piperacillin-tazobactam, or 128 μg/ml ticarcillin.

A total of 758 carbapenem-resistant *Pseudomonas* sp. isolates collected on a voluntary basis from 85 epidemiological settings in 42 towns in Hungary were screened for MBL production between October 2003 and November 2005. The settings are distributed in every geographical region of Hungary. Fifty *P. aeruginosa* isolates and one *P. putida* isolate (isolate PP524) proved to be positive by the phenotypic tests. Seven of these positive isolates from a Budapest hospital were not included in this study. PCRs with VIM- and integron-specific primers (7) gave positive results for all these isolates. The bla_{VIM} genes of the isolates indicated by a superscript *g* in Table 1 were sequenced and were identified as bla_{VIM-4} in

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FIG. 1. Geographic distribution of the VIM-positive *P. aeruginosa* clinical isolates in Hungary characterized in the present study. Small black circles indicate the cities. Budapest (the capital) is underlined. Mosonm., Mosonmagyaróvár. The serotype and PFGE subtype of the VIM-positive *P. aeruginosa* isolates characterized are shown under or next to the names of the cities.

every case. P12-Q and P12-E were negative by the MBL phenotypic tests.

Antibiotic susceptibility values for representative VIM-positive isolates and isolate P12-Q are shown in Table 2. All isolates were multidrug resistant, and some of them were only sensitive to aztreonam. Isolate MB397 was panresistant to all antimicrobials tested.

Two major groups could be identified among the VIM-positive *P. aeruginosa* isolates by serotyping and PFGE. Their geographical distribution is shown in Fig. 1. The first group comprised serotype O12 isolates from Budapest and Pécs (Table 1). These isolates were possibly related to each other, as determined by PFGE, and also to representative isolates of the major European P12 clone when a cutoff value of ≥80% was used (17–19). The O12 isolates from Budapest and Pécs carry an integron-borne $bla_{\mathrm{VIM\text{-}4}}$ gene with an identical 170-bp duplication in the last position, preceded by an *aacA4* gene (Fig. 2, integrons *a* and *b*). An additional bla_{OXA} cassette is present in the first position of integron *a* from Budapest (Table 1) (7).

The second group comprised all isolates from the Győr, Sopron, and Mosonmagyaróvár hospitals. These serotype O11 isolates were identical or closely related to each other by PFGE by use of a Dice coefficient of ≥95% (Table 1) and carried a $bla_{\mathrm{VIM\text{-}4}}$ gene without the 170-bp duplication on an integron that also harbored a $bla_{\mathrm{OXA\text{-}2}}$ cassette. Two variants

TABLE 1. Characteristics of VIM-producing *Pseudomonas* sp. isolates from Hungary[a]

Town	Hospital[b]	Ward	Patient[c]	No. of[d] isolates	Representative isolate[e]	Sample	Serotype	PFGE subtype	Integron[f]	Date of isolation (day.mo.yr)
Budapest	KHK	ICU	PA	1	PA396[g]	Urine	O12	A1	*a*	26.08.02
		ICU	PB	1	PA450[g]	Urine	O12	A1	*a*	24.09.02
		ICU	P1	4	MB242	Wound swab	O12	A2	*a*	04.07.05
		ICU	—	1	MB248[g]	Spillway	O12	A2	*a*	20.07.05
		ICU	—	1	PP524[g]	Sink			*a*	10.12.03
Pécs	BMK	Infectious disease	P2	1	PA555[g]	Urine	O12	A3	*b*	22.10.03
	PTE-BK	Hematology	P3	1	F69	Blood	O12	A3	*b*	03.08.04
	PTE-BK	Hematology	—	1	MB143[g]	Shower	O12	A3	*b*	20.04.05
	PTE-BK	Hematology	P4	1	MB397	Urine	O12	A3	*b*	19.08.05
Mosonmagyaróvár	KK	ICU	P5	3	MB93[g]	Tracheal aspirate	O11	B1	*d*	18.03.05
		ICU	P6	1	MB94[g]	Tracheal aspirate	O11	B2	*d*	18.03.05
		ICU	—	2	MB159	Sink	O11	B1	*d*	26.05.05
		ICU	P7	3	MB197[g]	Blood	O11	B1	*d*	06.06.05
		ICU	P8	6	MB240[g]	Tracheal aspirate	O11	B3	*d*	05.07.05
		ICU	P9	1	MB200	Tracheal aspirate	O11	B1	*d*	08.06.05
		ICU	P10	1	MB295[g]	Tracheal aspirate	O11	B2	*c*	04.07.05
		ICU	P11	1	MB296	Tracheal aspirate	O11	B1	*d*	28.07.05
		ICU	P12	1	MB449	Tracheal aspirate	O11	B2	*c*	30.09.05
Győr	PAK	Pediatrics	P13	1	MB219	Nasal swab	O11	B1	*d*	17.06.05
		ICU	P14	1	MB292	Feces	O11	B1	*d*	09.05.05
		ICU	P15	5	MB329[g]	Wound swab	O11	B2	*c*	13.08.05
		ICU	P16	1	MB330	Drain	O11	B2	*c*	12.08.05
		ICU	P17	3	MB447	Tracheal aspirate	O11	B2	*c*	14.10.05
Zalaegerszeg	ZMK	ICU	P18	1	MB346[g]	Urine	O11	C	ND	10.08.05
Sopron	EK	ICU	P19	2	MB387[g]	Urine	O11	B1	*c*	05.09.05

[a] All isolates are *P. aeruginosa*, with the exception of isolate PP524, which is a *P. putida* strain.
[b] Hospitals are indicated by the abbreviation of their Hungarian name.
[c] Patients are indicated by codes PA and PB for the control isolates and P1 to P19 for the isolates characterized in this study. —, environmental isolates.
[d] The total number of characterized VIM-positive isolates from the patient. Except for P17, replicate isolates had the same serotype and the same PFGE type (A or B) and carried the same integron type (*a*, *b*, *c*, or *d*). P17 had two isolates carrying integron type *c* and one isolate carrying integron type *d*.
[e] Mating-out assays were performed with the underlined isolates.
[f] Integron codes correspond to those in Fig. 2. ND, not determined.
[g] Sequencing of the bla_{VIM} gene was performed, and the gene was identified as $bla_{\mathrm{VIM\text{-}4}}$ for these isolates, which represent the different PFGE subtypes and integron types.

TABLE 2. Antibiotic MICs determined for VIM-producing *Pseudomonas* sp. isolates and isolate P12-Q

Isolate	MIC (μg/ml)[a]									
	IPM	MEM	ATM	CAZ	FEP	TZP	GEN	AMK	CIP	PO
PA396[b]	64	>32	32	256	256	>256	8	32	>32	2
PA450[b]	256	>32	16	256	256	>256	8	32	>32	2
MB242	>256	>32	32	128	256	>256	32	64	>32	2
MB248	>256	>32	16	128	256	>256	>256	32	>32	2
PP524	256	>32	64	256	256	>256	8	32	1	2
PA555	>256	>32	8	256	256	>256	>256	>256	>32	4
F69	256	>32	4	>256	>256	>256	4	8	>32	4
MB143	>256	>32	16	>256	>256	>256	>256	>256	>32	2
MB397	>256	>32	16	256	>256	>256	>256	>256	>32	4
MB93	>256	>32	32	256	>256	256	64	>256	>32	2
MB94	>256	>32	32	256	>256	>256	64	>256	>32	2
MB159	>256	>32	8	>256	>256	>256	64	>256	>32	2
MB197	>256	>32	8	128	>256	>256	64	>256	>32	4
MB240	>256	>32	32	256	>256	>256	16	256	>32	1
MB200	256	>32	8	128	256	>256	64	>256	>32	2
MB295	>256	>32	8	>256	>256	>256	64	>256	>32	2
MB296	>256	>32	16	128	256	>256	64	>256	>32	2
MB449	>256	>32	8	>256	>256	>256	64	>256	>32	2
MB219	>256	>32	8	32	64	128	64	>256	>32	2
MB292	>256	>32	16	128	256	>256	32	>256	>32	2
MB329	256	>32	8	>256	>256	>256	128	>256	>32	2
MB330	256	>32	16	>256	>256	>256	64	>256	>32	2
MB447	>256	>32	8	>256	>256	>256	64	>256	>32	2
MB346	>256	>32	8	32	64	>256	16	>256	>32	4
MB387	>256	>32	4	64	128	>256	32	>256	>32	2
P12-Q	4	2	8	32	32	>256	128	16	32	4

[a] Abbreviations: IPM, imipenem; MEM, meropenem; ATM, aztreonam; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; TZP, piperacillin-tazobactam; GEN, gentamicin; AMK, amikacin; CIP, ciprofloxacin; PO, polymyxin B.
[b] Antibiotic MICs determined previously for control isolates PA396 and PA450 (7) are also shown for comparison.

of this integron were identified (Fig. 2, integrons *c* and *d*), with the only difference being that an additional *aacA7* cassette was present in the first position of integron *c*. We identified a carrier patient (patient P14) who was transferred between the intensive care units (ICUs) of the Győr and Mosonmagyaróvár hospitals, providing an epidemiological link between them. Another patient (patient P13) with no related clinical history was identified as a carrier on admission to the hospital, suggesting the presence of VIM-positive strains in the community. These results demonstrate the involvement of institutions from three towns in an outbreak of VIM-4-producing *P. aeruginosa*. Our observations and the available literature (2, 20, 23) underscore the role of human carriers and the hospital environment as potential reservoirs for MBL-producing *P. aeruginosa* strains.

Mating-out assays were performed with the isolates underlined in Table 1. These experiments did not result in transconjugants under the experimental conditions applied, suggesting that the integrons are located either on the chromosome or on nonconjugative plasmids (12, 15). While integrons *a*, *c*, and *d* have so far been reported only from Hungary, class 1 integrons carrying the same gene cassettes in their variable regions as integron *b* were also identified in Poland and Belgium (3, 7, 9; P. Bogaerts, H. Rodriguez, C. Bauraing, A. Deplano, Y. Glupczynski, and M. J. Struelens, Abstr. 15th Eur. Congr. Clin. Microbiol. Infect. Dis., abstr. P929, 2006).

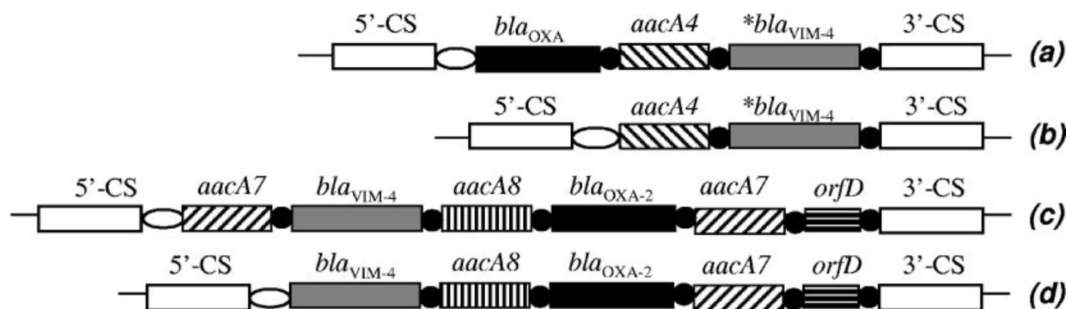

FIG. 2. Comparison of the schematic structures of the bla_{VIM-4}-carrying class 1 integrons from Hungary. Open ellipses, *attI1* site; black circles, 59-base elements; asterisks, bla_{VIM-4} cassettes with the 170-bp duplication; 5′-CS and 3′-CS, 5′ and 3′ conserved sequences, respectively. GenBank accession numbers and isolates harboring the integrons are as follows: (*a*) AY509609 and isolate PA396, (*b*) AY702100 and isolate PA555, (*c*) isolate MB387, (*d*) DQ357197 and isolate MB197.

The rates of imipenem resistance among *P. aeruginosa* clinical isolates in Hungary rose from 5.4% in 1996 to 13% in 2005 (5). The prevalence of VIM-positive *P. aeruginosa* isolates was estimated in the epidemiological settings from which the isolates were collected. On the basis of our experimental results and data from the National Bacteriological Surveillance database, we estimate that in 2004, VIM producers constituted about 0.4% and 0.05% of the imipenem-resistant isolates and all *P. aeruginosa* clinical isolates, respectively. In 2005, these values rose to about 6.5% and 0.8%, respectively. The observed increase could mainly be attributed to the clonal spread of the serotype O11 VIM-4-positive *P. aeruginosa* isolates in northwest Hungary.

This is the first report of an outbreak caused by acquired MBL-producing pathogens in Hungary. In hospitals in Greece, Italy, Korea, and Colombia, the dissemination of integrons carrying the $bla_{\text{VIM-1}}$, $bla_{\text{VIM-2}}$, or $bla_{\text{VIM-8}}$ gene mostly occurred in serotype O11 and O12 clones of *P. aeruginosa* (2, 6, 14, 20). These studies, as well as ours, indicate that serotype O11 and O12 multiresistant clones of *P. aeruginosa* (13) play an important role in the dissemination of bla_{VIM} through clonal spread but that other mechanisms, such as horizontal transfer, are also involved (3, 16, 23).

Nucleotide sequence accession numbers. The sequences of bla_{VIM} harboring integrons were deposited in GenBank under accession numbers AY702100 and DQ357197.

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