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Infrequent Finding of Metallo-β-Lactamase VIM-2 in Carbapenem-Resistant *Pseudomonas aeruginosa* Strains from Croatia

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One hundred sixty-nine nonreplicate imipenem-resistant *Pseudomonas aeruginosa* strains isolated in a large hospital on the coastal region of Croatia were studied. The most active antibiotics were colistin and amikacin. Most of the isolates were multiresistant. The most prevalent serotype was O12, followed by O11. Six strains carried the bla_{VIM-2} gene located in a novel class 1 integron composed in its variable part of the bla_{VIM-2}-bla_{oxa-10}-Δ*qacF*-*aacA4* genes. Metallo-β-lactamase-producing strains belonged to sequence types ST235 and ST111.

Acquired metallo-β-lactamases (MBLs) are emerging worldwide as powerful resistance determinants in Gram-negative bacteria (3). So far, six MBL enzyme types have been described in clinical isolates of *Pseudomonas aeruginosa*—VIM type, IMP type, SPM type, GIM type, AIM type, and, most recently, the NDM type found among clinical isolates from Serbia, a Balkan country (3, 14).

VIM-type enzymes appeared in the Mediterranean area with the first report of VIM-1 in Italy from a *P. aeruginosa* strain isolated in 1997, and then the observation of an allelic variant, VIM-2, in France from a *P. aeruginosa* strain isolated in 1996 (3). To date, all Mediterranean countries on the European side reported the presence of MBL in *P. aeruginosa*, mainly of the VIM type (Spain, France, Italy, Croatia, Greece, and Turkey) but also of the IMP type (Italy and France). Some of the VIM-producing *P. aeruginosa* strains, particularly in Italy and Greece, were involved in nosocomial outbreaks (3, 29). Most acquired MBL genes are carried on mobile gene cassettes inserted in integrons, associated with mobile DNA elements (transposons and plasmids) (20). Those integrons often harbored additional gene cassettes carrying resistance determinants for other antibiotic classes (e.g., aminoglycosides), disinfectants, or other β-lactamase genes (7). Therefore, multidrug-resistant (MDR) strains with colistin-only-susceptible (COS) phenotypes are becoming highly prevalent (3, 8).

In Croatia, a Mediterranean country situated on the eastern coast of the Adriatic Sea, and in the west Balkans, two studies so far have reported the presence of VIM-2 MBL in *P. aeruginosa* (1, 28) without sufficient characterization or comparison with isolates from other countries. Here, a larger-scale analysis of the prevalence, type, and genetic support of MBL genes in a collection of imipenem-resistant *P. aeruginosa* isolates from Croatia is presented.

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A total of 169 consecutive nonredundant *P. aeruginosa* isolates resistant to imipenem (MIC, >8 μg/ml) were collected from various clinical specimens in Split University Hospital, the second largest tertiary-care hospital (1,400 beds) in Croatia, during the period 2001 to 2007. Isolates from 2001 and 2002 were periodically collected because of exceptional resistance, and from 2003 to 2007, most consecutively isolated imipenem-resistant strains were collected and conserved for further analyses. Testing of susceptibility to common antipseudomonal antibiotics was done using broth microdilution, and the results were interpreted according to CLSI standards (2). *P. aeruginosa* ATCC 27853 was used as a quality control strain (2). Multiresistance was defined as resistance to imipenem and to at least one antibiotic representative of two more classes (i.e., ureidopenicillins, cephalosporins, aminoglycosides, or fluoroquinolones), according to the last proposal for standard definitions (18).

The most effective antipseudomonal antibiotics were colistin (85% susceptible strains) and amikacin (83% susceptible strains), followed by ceftazidime, piperacillin-tazobactam, and ciprofloxacin (with 31%, 28%, and 27% of susceptible strains). One hundred thirty-eight *P. aeruginosa* isolates (82%) were multiresistant. Eight strains (5%) exhibited a colistin-only susceptible (COS) phenotype. To assess the possible role of derepressed AmpC or upregulated efflux in resistance, MICs of ceftazidime of COS isolates were determined in the presence of 300 μg/ml of phenylboronic acid (Sigma) (32), while MICs of meropenem were determined in the presence of 12.5 μM carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) (Sigma) (24). The presence of OprD in COS isolates was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4% stacking and 11% separating gels, as previously described (4). The carbapenemase activity of crude cell extracts of COS strains was assessed spectrophotometrically, as previously described (20). In seven out of eight COS

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TABLE 1 MICs of antipseudomonal antibiotics and effects of phenylboronic acid and CCCP, OprD presence, and serotypes of colistin-only-susceptible *Pseudomonas aeruginosa* isolates

Strain no.	MIC (μg/ml) of[a]:								OprD presence	Serotype
	CAZ	CAZ + BA	TZP	IPM	MEM	MEM + CCCP	AMK	CIP		
31	32	4	128	16	32	8	32	64	No	O12
146	32	4	>128	16	>128	64	32	32	No	O11
149	128	16	>128	16	64	16	32	32	No	O11
150	64	8	>128	16	32	8	64	32	No	O3
153	64	16	>128	16	64	8	32	32	No	O11
154	64	16	>128	16	32	32	32	32	No	O11
156	64	32	>128	16	128	32	32	32	No	O11
175	32	32	>128	16	64	16	32	32	Yes	O12

[a] CAZ, ceftazidime; BA, phenylboronic acid; TZP, piperacillin-tazobactam; IPM, imipenem; MEM, meropenem; CCCP, carbonyl cyanide-*m*-chlorophenylhydrazone; AMK, amikacin; CIP, ciprofloxacin.

strains, phenylboronic acid significantly (4 times or more) decreased MICs of ceftazidime, while in six out of eight COS isolates, CCCP significantly decreased MICs of meropenem, as shown in Table 1. The majority of COS isolates were found to lack an OprD outer membrane protein (data not shown). No imipenem hydrolysis was detected in the spectrophotometric assay. The multiresistance of COS *P. aeruginosa* strains appeared to be due to an interplay of hyperexpressed AmpC, upregulation of efflux pumps, and the lack of the outer membrane D1 pathway, as previously described (6). MICs of imipenem were also determined in the presence of EDTA and phenanthroline using the EPI (EDTA-phenanthroline-imipenem) test, with *P. aeruginosa* VA-182 as a control strain (19, 20). MBL genes were screened by multiplex PCR using pairs of VIM and IMP primers amplifying specifically bla_{VIM} and bla_{IMP} genes, using bla_{VIM}- and bla_{IMP}-harboring strains (VA-182, and AC-54/97, respectively) as positive controls (20, 25). Additional PCRs, with primers specific for bla_{SPM}, bla_{GIM}, and bla_{NDM} genes were performed with COS isolates (14, 33). Class 1 integrons were amplified by PCR using INT primers which targeted conserved regions, while variable arrays of integrons were characterized by a "primer-walking" strategy with specific primers (20).

Six isolates (3%) showed a significant decrease of imipenem MICs in the presence of EDTA and phenanthroline ranging from 16 to >32-fold and carried the bla_{VIM-2} MBL gene. MBL-producing strains were susceptible to colistin, and all except one (susceptible to aztreonam) were susceptible to piperacillin-tazobactam. None of the COS strains revealed the presence of an MBL gene, indicating other mechanisms of resistance besides carbapenemase were involved, as discussed previously. According to PCR mapping and sequence analysis, the bla_{VIM-2} gene was located within the first gene cassette of a class 1 integron cassette array, which also included a bla_{OXA-10}, a truncated *qacF*, and an *aacA4* cassette, followed by *qacEΔ1* typical of 3′-conserved segments (Fig. 1). All VIM-producing isolates carried the same integron. According to the sequences, the Pc promoter of this integron was a variant designated PcW_{TGM-10}, providing strong expression of the gene cassette array, but associated with weak integrase IntI activity (15). This could possibly explain the stable integron content observed during a 6-year period with a likely repetitive independent acquisition of the integron from environmental sources (7, 21). The cassette array of this class 1 integron was unusual in two aspects: first, although the coexistence of different β-lactamases is frequent in MBL-carrying integrons, in this one, two neighboring gene cassettes encoded two different β-lactamases (VIM-2 and OXA-10), a situation similar to the one described for *P. aeruginosa* isolates harboring VIM-6 and OXA-10 in Asia (16); second, a remnant of the *qacF* gene, seldomly reported in integrons harboring MBL genes, could have a residual function in promoting transcription of adjacent genes, as already described for other *qac* genes (*qacEΔ1* and *qacH*) (7, 9, 13). The truncated *qacF* cassette possibly originated through an anomalous recombination event at its 3′ end (Fig. 1). The fourth gene cassette, *aacA4*, encoding a 6′-*N*-aminoglycoside acetyltransferase, is frequently found in integrons containing MBL-encoding genes (20, 29).

Southern blot hybridization was performed on whole genomic DNA of two representatives of MBL-carrying strains using a PCR-generated fragment of the entire bla_{VIM-2} gene (*P. aeruginosa* strain VA-182) labeled with ^{32}P by the random priming technique (Rediprime II DNA labeling system; Amersham Biosciences), as previously described (20). For both isolates, Southern blot hybrid-

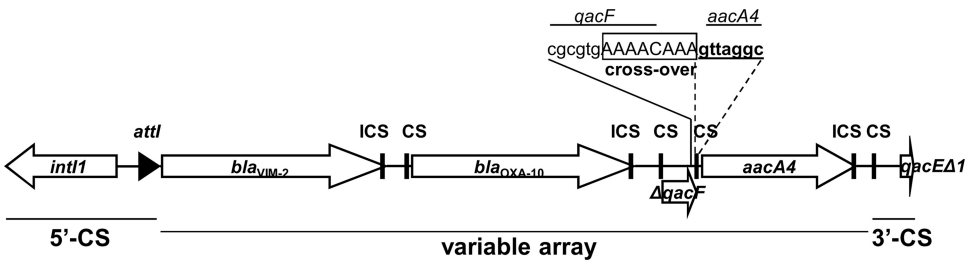
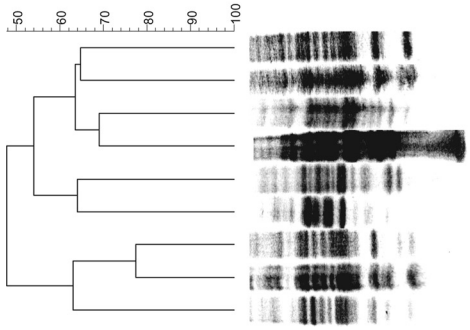


FIG 1 Schematic presentation of the class 1 integron carrying bla_{VIM-2} gene cassette (In493; no. AM392427). CS, core site; ICS, inverse core site; 5′- and 3′-CS, 5′- and 3′-conserved segments.



Strain No	PFGE clonal lineage	Department	Specimen	Serotype		Year of isolation	MLST
43	A	SURG.ICU	URINE	O12	mbl+	2004	ST 111
41	D	ICU	URINE	O12		2004	ST 111
52	F	MED.	URINE	O12		2004	ST 257
66	G	PED.	TRACH.ASP.	O1		2005	ST 794
3	B	SURG.	W.SW.	O11	mbl+	2001	ST 235
46	H	PED	TRACH.ASP.	UND.		2004	ST 1074
104	C	ICU	BLOOD	O12		2005	ST 111
161	E	ICU	BRONCH.ASP.	O12		2006	ST 1075
44	I	PED.	TRACH.ASP.	O12		2004	ST 111

FIG 2 Dendrogram showing PFGE profiles and corresponding STs of *Pseudomonas aeruginosa* strains representative of the clonal lineages. SURG.ICU, Surgical Intensive Care Unit; MED., Department of Medicine; PED., Department of Pediatrics; TRACH.ASP., tracheal aspirate; W.SW., wound swab; BRONCH.ASP., bronchial aspirate; UND., undetermined.

ization yielded a positive signal indicative of the presence of the bla_{VIM-2} determinant on the chromosomal DNA (data not shown).

O serotypes were determined by the slide agglutination method using commercial antisera O1 to O16 (Bio-Rad, France). The most common serotype was O12 (99 isolates; 59% of all isolates), followed by O11 (29 isolates; 17%) and O1 (7 isolates; 4%), respectively. Eighteen strains (11%) were nontypeable. Most of the O12 (85%) and O11 (69%) isolates were multiresistant and also represented the majority of COS isolates, which is in accordance with previous studies suggesting a possible role of these serotypes in dissemination of multiresistance (11, 12, 23).

Clonal diversity was assessed by pulsed-field gel electrophoresis (PFGE) using XbaI restriction enzyme and a CHEF-DRIII apparatus (Bio-Rad) as previously described (27). Clonal relatedness was determined using GELCompar software (Applied Maths, Kortrijk, Belgium) with 2% position tolerance and Dice coefficient. A dendrogram representing the relatedness between the isolates was built by the unweighted-pair group method using arithmetic averages (UPGMA) based on the similarity values calculated with Dice coefficient. A cutoff of ≥80%, equivalent to the criterion of "possibly related" by Tenover et al., was applied (31). Among the 169 isolates, PFGE analysis allowed us to define nine major clonal lineages. Seventy-seven isolates (45%) belonged to lineage A, which was found to be the most prevalent, while 61 isolates (36%) belonged to clonal lineage B. The dominant clone A consisted mainly of O12 isolates (86%), whereas clone B was more heterogeneous, consisting mostly of O11 (40%) and O12 (21%) isolates and suggesting that serotyping can lead to a misleading classification of the isolates. Variable serotypes among a clonal lineage could involve recent genetic changes and recombination events (12, 22). All COS strains belonged to the same clone (clone B). Five MBL-positive strains were of the O11 serotype and belonged to clone B, while the remaining MBL-producing strain was of the O12 serotype and belonged to clone A. Further analysis of *P. aeruginosa* infraspecific diversity was performed by multilocus sequence typing (MLST) according to the published protocols (5, 10). MLST was applied to two VIM-carrying *P. aeruginosa* isolates on representative strains of the remaining clonal lineages C to I (Fig. 2). Nucleotide sequences were determined on both strands and searched against the MLST database (http://pubmlst.org/paeruginosa/) for the assignment of allelic profiles and sequence types (STs). MLST analysis identified six STs among nine strains with distinct PFGE profiles and two newly described STs—ST1074 (closest to ST212) and 1075 (closest to ST111). The MBL-producing O12 strain belonging to PFGE clone A was typed as ST111, while the MBL-producing O11 strain of clone B belonged to ST235. Representatives of clones A, C, D, and I were part of ST111, while other clones' representatives displayed distinct STs, as shown in Fig. 2. The MLST findings were in support of previous analysis proving the presence of two international clonal complexes—CC235, represented by the predicted founder, ST235, and CC111, represented by ST111—in dissemination of bla_{VIM}-harboring *P. aeruginosa* (34). VIM-producing *P. aeruginosa* strains belonging to these two international clones had already been reported from countries bordering Croatia, like Serbia (ST235), Hungary (ST235 and ST230), and Italy (ST111, ST235, and ST227) (10, 17, 34). The prevalence of MBL-producing *P. aeruginosa* isolates in this part of Europe ranged from 0.1% in Spain and 0.8% in Greece to 1.3% in Italy (3, 26, 29). Taking into account the 10% of imipenem-resistant isolates reported during the last decade in Croatia (30), the estimated prevalence of MBL-producing *P. aeruginosa* strains would be 0.3% in Croatia. In spite of these numbers, *P. aeruginosa* can evolve rapidly and can select a beneficial genetic configuration that can lead to dramatic hospital outbreaks, as already reported in the Mediterranean basin and throughout the world (3, 4, 8, 34).

In conclusion, a low prevalence of metallo-β-lactamase-producing *P. aeruginosa* isolates in Croatia was observed, but the reported strains belonged to epidemic clones that spread over the whole of Mediterranean Europe.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper was assigned GenBank accession no. AM392427. The integron sequence reported in this paper was submitted to the INTEGRALL database (http://integrall.bio.ua.pt/) and was assigned no. In493.

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