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Emergence of VIM-12 in *Enterobacter cloacae*[▽]

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Detection of the *bla*$_{\text{VIM-12}}$ gene within the originally described Inh12 integron in a clinical isolate of *Enterobacter cloacae* is reported for the first time worldwide. Integron Inh12 was carried on a conjugative plasmid of approximately 85 kb which also conferred resistance to aztreonam, likely due to AmpC production.

In recent years, metallo-β-lactamases (MBLs) have emerged among several Gram-negative pathogens, namely, species of the *Enterobacteriaceae* family, as well as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Proteus mirabilis* (13). VIM types are the most frequently acquired MBLs and in some regions are widespread (2). To date, 23 VIM variants have been described (www.lahey.org/studies/other.asp#table 1). In Greek hospitals, the *bla*$_{\text{VIM-1}}$ allele prevails and is frequently located in a known class 1 integron structure, designated In-e541 (8). Furthermore, alleles *bla*$_{\text{VIM-4}}$ and *bla*$_{\text{VIM-12}}$ were originally described in Greek clinical isolates (10, 11). Since their original isolation, these alleles, along with others (namely, *bla*$_{\text{VIM-1}}$ and *bla*$_{\text{VIM-2}}$), have been reported from various hospitals in our country, which documents the endemic trait of VIM-type MBLs in Greece.

Enterobacter cloacae is an important nosocomial pathogen in which carbapenem resistance is still unusual, and reports of MBL-producing *E. cloacae* clinical isolates remain limited. Here we report on the first detection of VIM-12 in a clinical isolate of *E. cloacae*, which was recovered at the University Hospital of Alexandroupolis, Thrace, Greece. The hospital is located at the northern east border of Greece, about 300 km from the city of Thessaloniki, and has approximately 700 beds serving a population of about 200,000 citizens. The onset and gradual increment in occurrence of multiresistant *E. cloacae* in our hospital prompted us to monitor isolates for MBL-encoding genes due to their wide dissemination in our country.

From June 2007 to October 2009, 27 multiresistant *E. cloacae* clinical isolates exhibiting resistance to at least three classes of antimicrobial agents (mainly β-lactams, aminoglycosides, and trimethoprim-sulfamethoxazole) were routinely classified by the Vitek 2 automated system (bioMérieux, Marcy l'Etoile, France). The MICs of various antimicrobials were determined by Etest (AB Biodisk, Solna, Sweden). Phenotypic detection of MBLs was performed by the MBL Etest (AB Biodisk), imipenem-EDTA double-disk synergy test (DDST), as well as combined-disk test (CDT) using imipenem (4). Phenotypic detection of AmpC type β-lactamases and tests for KPC production were performed using boronic acid (1, 12). PCR detection of various *bla* gene types (including *bla*$_{\text{IMP}}$, *bla*$_{\text{VIM}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{CTX-M}}$, and *bla*$_{\text{KPC}}$) as well as PCR mapping of integrons related to the MBL-encoding genes detected was performed using the primers and amplification conditions described previously (9, 10). Mating experiments using *Escherichia coli* 26R793 (Lac$^-$ Rif^r) as the recipient strain were also performed as described previously (5). Plasmid extraction was performed using a CompactPrep plasmid midi-core kit (Qiagen, Hilden, Germany) and *E. coli* 39R861 as the standard plasmid control strain.

Three of the isolates tested were MBL positive by phenotypic methods, and PCR screening showed that these isolates were positive for the *bla*$_{\text{VIM}}$ gene and negative for the other *bla* genes for which the isolates were tested. All of the rest of the isolates were also PCR negative for any of the MBL-encoding genes for which the isolates were tested. Integron mapping and nucleotide sequence analysis revealed the presence of integron Inh12, carrying allele *bla*$_{\text{VIM-12}}$, in one isolate (Ec119) and integron In-e541, carrying allele *bla*$_{\text{VIM-1}}$, in two isolates (Ec120 and Ec123). Mating experiments yielded transconjugant cells at a median frequency of 3.3×10^{-1} per donor cell of Ec119 as well as 4.3×10^{-2} and 1.5×10^{-2} per donor cell of Ec120 and Ec123, respectively. Transconjugant cells of Ec119 had elevated MICs of cephalosporins (but not cefepime), amikacin, and aztreonam, as well as tetracycline (Table 1). Transconjugant cells of Ec120 and Ec123 had elevated MICs of gentamicin and amikacin. Plasmid analysis of both clinical and transconjugant colonies showed a plasmid of approximately 85 kb for isolate Ec119 and one of approximately 60 kb for isolates Ec120 and Ec123. Transconjugant cells were also PCR positive for the *bla*$_{\text{VIM}}$ gene, indicating its plasmid location in the respective clinical isolates of the study.

It is noteworthy that isolate Ec119 was resistant to aztreonam (MIC = 128 mg/liter), and its transconjugant cells also showed an elevated MIC to that agent (16 mg/liter). PCR for the *bla*$_{\text{KPC}}$ gene was negative, but both clinical and transconjugant cells were phenotypically positive for AmpC production. Accordingly, resistance to aztreonam might be attributed to AmpC production (7), with the *bla*$_{\text{AmpC}}$ gene being carried in a conjugative plasmid along with the *bla*$_{\text{VIM-12}}$ gene.

VIM-type MBL-encoding genes associated with class 1 integron structures have become endemic in Greece, and phenotypic screening for MBLs in Gram-negative pathogens is routinely performed in laboratories of clinical microbiology in our hospitals. In particular, In-e541 is an integron widely dis-

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TABLE 1. Susceptibility status of VIM-producing *E. cloacae* clinical isolates and the respective transconjugants

Isolate	Etest MIC (mg/liter)[a]									
	IPM	MEM	CTX	CAZ	FEP	ATM	TZP	GEN	AMK	TET
Ec119	1	1.5	>32	>256	6	128	48	0.75	32	>256
E. coli 26R793(pEc119)	1	0.064	>32	64	0.5	16	6	0.75	16	128
Ec120	0.5	0.125	4	64	2	0.5	16	16	24	1
E. coli 26R793(pEc120)	0.5	0.032	1.5	6	0.25	0.25	3	12	16	1
Ec123	0.5	0.125	16	64	4	0.5	16	16	24	1
E. coli 26R793(pEc123)	0.5	0.032	1.5	6	0.25	0.25	2	12	16	1
E. coli 26R793	0.5	0.032	0.094	0.75	0.094	0.094	0.75	0.75	2	1

[a] IPM, imipenem; MEM, meropenem; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam; TZP, piperacillin-tazobactam; GEN, gentamicin; AMK, amikacin; TET, tetracycline.

tributed among several species in our hospitals (8). Its variable region consists of $bla_{\text{VIM-1}}$, *aacA7*, *dhfrI*, and *aadA1* genes in succession; and these are under the control of a strong P1 promoter and an inactivated P2 promoter. Integron Inh12 was originally described in a *Klebsiella pneumoniae* clinical isolate from Greece and carries the $bla_{\text{VIM-12}}$ allele flanked by two *aacA7* alleles, while it also has a strong P1 promoter and an inactivated P2 promoter. The respective promoting genetic constitution has previously been associated with phenotypically undetectable MBLs not only in *E. cloacae* but also in other species (3, 4). However, our isolates were typically positive by all MBL-related phenotypic assays. A plausible explanation could be that specific molecular mechanisms not present in our isolates might mask the phenotypic manifestation of MBL-encoding genes. Therefore, phenotypic screening for MBLs may not be adequate in all cases, while the use of PCR screening of phenotypically negative isolates has previously been suggested in the literature (4).

The University Hospital of Alexandroupolis is a health care setting relatively remote in respect to the other Greek hospitals where VIM-producing Gram-negative isolates have been isolated. Furthermore, patients from whom the isolates used for the present study were derived were native citizens of the city of Alexandroupolis; thus, horizontal transfer among different Greek hospitals could not explain the appearance of VIM-type MBLs in our hospital. Also, this is the first report worldwide of a VIM-12-producing *E. cloacae* clinical isolate. It is noteworthy that no VIM-producing Gram-negative isolates were previously detected in our hospital. Thus, it is more likely that the $bla_{\text{VIM-12}}$ gene is independent and is widely distributed in bacteria rather than being the spontaneous result of homologous recombination between the $bla_{\text{VIM-1}}$ and $bla_{\text{VIM-2}}$ genes, which already existed and which are disseminated within the respective health care settings, as commented on previously (6).

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