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Emergence in *Klebsiella pneumoniae* and *Enterobacter cloacae* Clinical Isolates of the VIM-4 Metallo-β-Lactamase Encoded by a Conjugative Plasmid

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Resistance to carbapenems is an emerging problem among gram-negative hospital pathogens. A transferable plasmid encoding the VIM-4 metallo-β-lactamase was detected in isolates of *Klebsiella pneumoniae* and *Enterobacter cloacae* obtained from a single patient under carbapenem therapy. Thus, enterobacteria appear to increasingly contribute to the spread of VIM-type enzymes.

The spreading of new resistance determinants among nosocomial pathogens represents a worldwide problem. Two major classes of broad-spectrum β-lactamases have been emerging among gram-negative pathogens over the last decade: the extended-spectrum β-lactamases (ESBLs), most commonly observed in members of the family *Enterobacteriaceae*; and the metallo-β-lactamases (MBLs), which are most prevalent in nonfermenting gram-negative bacteria (1).

The acquired MBLs belong to two major types (IMP and VIM), each comprising multiple allelic variants (12). Recently, a third type of acquired MBL (named SPM-1) was reported from South America (17). Enzymes of the IMP and VIM types are clinically relevant, since they are able to degrade virtually all β-lactams except monobactams; bacteria expressing these traits can thus cause difficult-to-treat infections. MBLs are commonly encoded by genes carried on mobile elements (integron-borne gene cassettes) that can spread horizontally among different replicons and strains (7, 12).

The VIM-type MBLs were originally detected in Europe (5, 14), where these enzymes apparently prevail over IMP-type enzymes (12). Subsequently, VIM-type enzymes were also reported in Asia (6, 12, 18–20) and, more recently, in the Americas (M. A. Toleman, K. Rolston, R. N. Jones, and T. R. Walsh, Abstr. 42nd Intersci. Conf. Antimicrob. Agents Chemother., abstr. C1-1851, 2002; N. Woodford, A. Sinclair, M. E. Kaufmann, J. D. Velez, C. R. Castañeda, M. Recalde, D. M. Livermore, and M. P. Crespo, Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. C2-2017, 2003; A. C. Gales, A. O. Reis, A. P. Penteado, S. Silbert, M. C. Tognim, and H. S. Sader, Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. C2-2018, 2003). Unlike IMP-type enzymes, which are frequently reported in enterobacteria (12, 18), VIM-type enzymes are commonly found in *Pseudomonas aeruginosa* and other nonfermenting gram-negative bacteria (e.g., *Acinetobacter baumannii*, *Pseudomonas putida*, and *Achromobacter xylosoxidans*) (6, 12). There are only a few reports on the production of VIM-type enzymes in members of the family *Enterobacteriaceae*, including the production of VIM-2 in *Serratia marcescens*, *Citrobacter freundii*, and *Enterobacter cloacae* isolates from far east Asia (12, 18, 20) and production of VIM-1 in *Escherichia coli* and *Klebsiella pneumoniae* isolates from Greece (3, 10).

Since 1999, VIM-positive isolates of nonfermenting gram-negative bacteria have been occasionally recovered at our institution (2, 8, 9). Here we report for the first time the isolation of *K. pneumoniae* and *E. cloacae* strains producing the VIM-4 MBL.

On 18 May 2002, a 72-year-old female patient was admitted to the General Surgery Department of the Varese University Hospital (northern Italy) with the diagnosis of obstructive jaundice. On 2 June, due to the development of hemorrhagic necrotizing pancreatitis, the pancreas tail and body were resected. The patient was then transferred to the Intensive Care Unit, where after 40 days, she died of multiorgan failure. During hospitalization, the patient had been treated with multiple courses of antimicrobials. After an initial therapy with piperacillin for 14 days (2 g twice a day), treatment was switched to imipenem (0.5 g twice a day) because of the recovery from pancreatic drainages of a multidrug-resistant isolate of *E. cloacae* (isolate VA-341/02). This isolate was found to produce an ESBL activity, as shown by the synergistic effect between clavulanate and aztreonam, ceftriaxone, cefotaxime, and ceftazidime in the double-disk assay (4). After 4 weeks of imipenem therapy, two multidrug-resistant isolates of *K. pneumoniae* (isolate VA-416/02) and *E. cloacae* (isolate VA-417/02) were simultaneously recovered from pancreatic drainages.

In vitro susceptibility tests showed that both of these isolates were characterized by reduced susceptibility to carbapenems, although MICs of imipenem and meropenem, as measured by the Etest method (AB Biodisk, Solna, Sweden), were still in the range of susceptibility according to National Committee for Clinical Laboratory Standards guidelines (11). Compared to the MICs for *E. cloacae* VA-341/02 (which was clonally

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TABLE 1. Antimicrobial susceptibility of *E. cloacae* and *K. pneumoniae* clinical isolates producing SHV-12 and/or VIM-4 enzymes

Antimicrobial agent	MIC (μg/ml)		
	E. cloacae VA-341/02 (SHV-12 positive)	*K. pneumoniae* VA-416/02 (VIM-4 positive)	*E. cloacae* VA-417/02 (SHV-12 and VIM-4 positive)
Amoxicillin	>128	>128	>128
Piperacillin	>128	>128	>128
Cephalothin	>128	>128	>128
Cefoxitin	>128	32	>128
Cefotaxime	>128	32	>128
Ceftriaxone	>128	32	>128
Ceftazidime	>128	32	>128
Cefepime	2	2	8
Aztreonam	>128	4	>128
Imipenem	0.25	2	4
Meropenem	0.125	0.5	1
Amikacin	1	8	8
Gentamicin	64	16	64
Tobramycin	4	16	16
Ciprofloxacin	0.25	0.032	0.25
Levofloxacin	0.5	0.064	0.5

related, as demonstrated by pulsed-field gel electrophoresis analysis [data not shown]), the MICs for isolate VA-417/02 were increased: not only those of carbapenems but also those of cefepime, amikacin, and tobramycin. These isolates' susceptibilities to other antimicrobial agents were identical (Table 1). ESBL production was also detected in *E. cloacae* VA-417/02, although the synergistic activity was seen only between clavulanate and aztreonam in the double-disk assay. Based on the susceptibility profile of these multidrug-resistant pathogens, ciprofloxacin (0.4 g twice a day) was added to imipenem therapy. *K. pneumoniae* and *E. cloacae* isolates, however, were not cleared from pancreatic drainages, as shown by subsequent cultures. Low antimicrobial dosages were used because the patient was in renal failure.

Multiplex PCR analysis of *K. pneumoniae* VA-416/02 and *E. cloacae* VA-417/02, carried out with crude DNA extracts together with VIM/DIA primers (2), yielded a 0.5-kb amplification product that suggested the presence of a bla_{VIM} allele. Analysis of the *Rsa*I restriction pattern of the amplicon yielded two 0.25-kb fragments, revealing a pattern compatible with the presence of a bla_{VIM-1}-like allele (5). The entire bla_{VIM} determinants from VA-416/02 and VA-417/02 were amplified by PCR using external primers designed on bla_{VIM-1} flanking sequences (5). Both strands of the amplification products were sequenced as described previously (13). The presence of a bla_{VIM} allele identical to bla_{VIM-4} (15) was evident in both cases. In the case of *E. cloacae* VA-341/02, PCR analysis with the VIM/DIA primers yielded negative results. PCR analysis for bla_{TEM} and bla_{SHV} genes, carried out as described previously (13), revealed the presence of a bla_{SHV} allele that encoded a mature protein identical to SHV-12 (http://www.lahey.org/studies/webt.htm) in both VA-341/02 and VA-417/02.

Sonic extracts of isolates VA-416/02 and VA-417/02, analyzed by a spectrophotometric assay (5), showed the presence of carbapenemase activity (imipenem-hydrolyzing specific activities of 14.8 ± 0.2 and 23.6 ± 0.2 nmol/min/mg of protein, respectively), which was inhibited by over 90% following exposure to 5 mM EDTA for 20 min at 20°C. No comparable activity was detected in crude extracts from *E. cloacae* VA-341/02. (The imipenem-hydrolyzing specific activity was lower than 0.5 nmol/min/mg of protein). In an agar dilution test using 5×10^8 CFU per spot as an inoculum, the imipenem and meropenem MICs for both MBL-producing isolates were >64 μg/ml. Southern blot analysis of genomic DNAs of VA-416/02 and VA-417/02 was carried out with a ^{32}P-labeled bla_{VIM} probe as described previously (5). Plasmid DNA was recognized by the probe in both isolates (data not shown).

Conjugation experiments were performed with liquid medium (16) by mating *K. pneumoniae* (isolate VA-416/02) or *E. cloacae* (isolate VA-417/02) with the rifampin-resistant *E. coli* strain J53 as a recipient. The initial donor/recipient ratio was 0.1. Transconjugants were selected on MacConkey agar containing ceftazidime (1 μg/ml) plus rifampin (100 μg/ml). Transfer of ceftazidime resistance was observed at a frequency of approximately 10^{-5} transconjugants per recipient from both *K. pneumoniae* and *E. cloacae*. The transconjugants obtained from either donor harbored an apparently identical plasmid that was recognized by the bla_{VIM} probe in Southern blot hybridization. Compared to the recipient *E. coli* J53 strain, the transconjugants exhibited decreased susceptibility to several β-lactams (including carbapenems) and aminoglycosides. Transconjugants also produced an EDTA-inhibitable carbapenemase activity comparable to that of the parent *K. pneumoniae* and *E. cloacae* strains (data not shown).

This is the first report of clinical isolates of *K. pneumoniae* and *E. cloacae* producing the VIM-4 MBL, as well as the first report from Europe of *E. cloacae* producing a VIM-type enzyme.

In the two isolates investigated (*K. pneumoniae* VA-416/02, and *E. cloacae* VA-417/02), the VIM-4 gene was carried on an apparently identical plasmid that could be transferred to *E. coli* by conjugation. Since the two isolates had been obtained at the same time from the same clinical specimen—while an isolate of *E. cloacae* clonally related to VA-417/02 (but not containing the VIM-4 determinant) had been obtained from the same body site 4 weeks earlier—*K. pneumoniae* most likely represented the original vehicle of the bla_{VIM-4}-coding plasmid. Transfer of the plasmid to *E. cloacae* had probably occurred in vivo.

Overall, these findings are alarming, considering the clinical relevance of enterobacteria and of *K. pneumoniae* in particular. This species, in fact, represents an important nosocomial pathogen known for its ability to act as a reservoir for a variety of resistance plasmids, although the current spread of CTX-M-type ESBL genes in *E. coli*, *Salmonella enterica*, and *Proteus mirabilis* indicates that other enterobacterial species are also becoming important as a reservoir of resistance determinants.

The emergence of carbapenemases in *K. pneumoniae* and *E. cloacae* poses relevant clinical problems. It could be argued that carbapenems maintain clinical efficacy on MBL-positive enterobacteria, since these drugs appear to allow a low-level expression of MBL enzymes, while carbapenem MICs remain below the susceptibility breakpoint (10, 18, 20). However, in our isolates, a significant increase of carbapenem MICs was observed at higher inoculum sizes. This finding strongly suggests the possibility of a clinical failure of carbapenem therapy.

This report demonstrates that the problem of MBL-producing pathogens no longer entails gram-negative nonfermenters alone but also involves enterobacteria. The need for continuous surveillance of the spread of these resistance determinants is underscored. In enterobacteria, however, MBL detection on the basis of resistance phenotypes may be particularly difficult due to the relatively low-level carbapenem MICs in standard susceptibility assays. Therefore, adequate screening methods should be adopted when an even modest increase in carbapenem MICs is observed. Surveillance programs of this kind may be of value in the difficult battle against life-threatening bacterial infections.

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