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Infections with VIM-1 Metallo-β-Lactamase-Producing *Enterobacter cloacae* and Their Correlation with Clinical Outcome▽

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The aim of this study was to ascertain the incidence and clinical significance of metallo-β-lactamases among *Enterobacter* strains isolated from patients with nosocomial infections. We prospectively collected data on patients with *Enterobacter* infection during a 13-month period. All of the strains were investigated for antibiotic susceptibility, the presence and expression of metallo-β-lactamases, and clonality. Of 29 infections (11 involving the urinary tract, 7 pneumonias, 3 skin/soft tissue infections, 3 intra-abdominal infections, 3 bacteremias, and 2 other infections), 7 (24%) were caused by *Enterobacter cloacae* strains harboring a bla_{VIM-1} gene associated or not with a bla_{SHV12} gene. Infections caused by VIM-1-producing strains were more frequently associated with a recent prior hospitalization ($P = 0.006$), cirrhosis ($P = 0.03$), relapse of infection ($P < 0.001$), and more prolonged duration of antibiotic therapy ($P = 0.01$) than were other infections. All of the isolates were susceptible to imipenem and meropenem and had bla_{VIM-1} preceded by a weak P1 promoter and inactivated P2 promoters. Most VIM-1-producing *Enterobacter* isolates belonged to a main clone, but four different clones were found. Multiclonal VIM-1-producing *E. cloacae* infections are difficult to diagnose due to an apparent susceptibility to various beta-lactams, including carbapenems, and are associated with a high relapse rate and a more prolonged duration of antibiotic therapy.

The emergence of metallo-β-lactamases (MBLs) in the *Enterobacteriaceae* is a matter of major concern for clinicians worldwide (7, 28). Infection with VIM-1-producing *Klebsiella pneumoniae* has become endemic in some European nations (especially in the intensive care units of tertiary care hospitals in Greece [23]), and sporadic cases of infection due to multidrug-resistant *Enterobacteriaceae* carrying bla_{VIM-1} have also been reported (18, 23). In a recent Italian nationwide survey of acquired MBLs in gram-negative pathogens, among 14,812 consecutive nonreplicate clinical isolates (12,245 *Enterobacteriaceae* isolates and 2,567 gram-negative nonfermenters) screened for reduced carbapenem susceptibility during a 4-month period, 30 (0.2%) isolates (28 *Pseudomonas aeruginosa* isolates, 1 *Pseudomonas putida* isolate, and 1 *Enterobacter cloacae* isolate) carried acquired MBL determinants (20). Despite the presence of an MBL, the carbapenem MICs are frequently below the current resistance breakpoints (23), and no clinical information is available on the outcomes of patients infected with carbapenem-susceptible MBL-producing *Enterobacteriaceae*.

Enterobacter spp. are an important cause of nosocomial infection, but only a few cases of infection due to MBL-producing *E. cloacae* have been reported in the English literature worldwide (10, 16, 23, 29). In our hospital, we recently observed a fatal case of hospital-acquired pneumonia caused by a VIM-1-producing *E. cloacae* strain (18). This first episode prompted us to a careful monitoring of *Enterobacter* infections, to ascertain the incidence and the outcome of MBL production among cases of severe nosocomial infections caused by this species and to better define the biochemical characteristics of MBL-producing strains.

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MATERIALS AND METHODS

Setting and period of study. This study was conducted in the University Hospital Umberto I, Rome, Italy, during the period January 2007 to February 2008, in which *Enterobacter* infections were carefully monitored. Microorganisms primarily identified as *Enterobacter* were sent to the Department of Microbiology of the University of Catania and the Laboratory of Molecular Biology of the University of L'Aquila for identification confirmation, biochemical studies, and further molecular characterization.

Patients. All hospitalized patients with a body fluid or tissue culture positive for *Enterobacter* spp. were initially included in the study. Patients with community-acquired infection (defined as an infection which occurred prior to admission or within 72 h of hospitalization) were excluded. All episodes of nosocomially acquired infection were defined according to the standard definitions of the Centers for Disease Control and Prevention (CDC) (8). Cases not fulfilling these definitions were excluded. Bloodstream infections, including sepsis, severe sepsis, and septic shock, were defined according to standard international criteria (4). Patients were followed up to discharge by the attending physician and, if requested, by an infectious disease specialist referring to the nosocomial infection unit of our hospital. We obtained written consent to use patient data.

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Measurements. We recorded the following data: age; sex; intrinsic and extrinsic risk factors, such as underlying diseases, previous antibiotic therapy, or previous surgery; clinical signs and symptoms; results from laboratory and microbiologic studies; antibiotic therapy; and outcome. We also recorded in-hospital complications and deaths. At the clinical end point of hospital discharge or death, we retrieved the variables of in-hospital death, length of hospital stay, and relapse rate.

The severity of clinical conditions was assessed at the time of presentation by using the sequential organ failure assessment (SOFA) score. Relapse was defined as recurrent *Enterobacter* infection during hospitalization, with at least 1 week of follow-up observation after antibiotic discontinuation.

Microbiological studies. All *Enterobacter* strains isolated from patients included in the study were collected and identified by using a Vitek 2 automatic system (bioMerieux Inc.). The strains were reconfirmed with an API 20E test (bioMerieux SA, Marcy l'Etoile, France) and stored at −80°C until their use for further molecular characterizations.

The preliminary antibiotic resistance profile determined by using the Vitek 2 automatic system was confirmed by the broth microdilution method, using Mueller-Hinton broth, according to CLSI guidelines. All experiments were performed in triplicate. For the imipenem-EDTA double-disk synergy test (DDST), an overnight culture of the test strain was suspended to the turbidity of a McFarland standard of 0.5 and used to swab inoculate a Mueller-Hinton agar plate. After drying of the plate, a 10-μg imipenem disk and a blank filter paper disk were placed 10 mm apart from edge to edge, and 10 μl of 0.5 M EDTA solution was then applied to the blank disk, which resulted in approximately 1.5 mg/disk. After overnight incubation, the presence of an enlarged zone of inhibition was considered EDTA synergy test positive (12). The presence of extended-spectrum β-lactamase (ESBL) activity was detected by placing disks containing clavulanate in combination with oxyimino-β-lactam antibiotics, such as ceftazidime, cefotaxime, ceftriaxone, and aztreonam. An increase of 5 mm in the zone of inhibition in a disk containing clavulanate compared to those for other β-lactams was considered positive for the presence of ESBLs.

(i) Molecular analysis. Plasmid DNA was extracted from *E. cloacae* by the procedure of Kado and Liu (11). Chromosomal DNA was extracted as previously described (21). DNA probes, specific for $bla_{\text{TEM-1}}$, $bla_{\text{SHV-1}}$, $bla_{\text{CTX-M-1}}$, $bla_{\text{PER-1}}$, $bla_{\text{VIM-1}}$, and $bla_{\text{IMP-1}}$, were labeled with dUTP-fluorescein by a random priming technique and were used for Southern blot experiments on genomic and plasmid DNAs, as previously described (11). Screening for resistance determinants (bla_{VIM}-type and bla_{SHV}-type genes) was performed by PCR analysis on genomic and plasmidic DNAs, using the primers VIM-F and VIM-R (18), TEM-F and TEM-R (17), and SHV/F (5′-GCCCGGGTTATTCTTATTTGT CGC) and SHV/R (5′-TCTTTCCGATGCCGCCGCCAGTCA). Detection and mapping of a class 1 integron were carried out using specific primers for the conserved segments, namely, 5′-CS (5′-GGCATCCAAGCAGCAAG-3′) and 3′-CS (5′-CTCTCAAGATTTTAATGCGGATG-3′), and then amplifying the variable region containing the resistance gene cassettes. The DNA fragments obtained by subsequent PCRs were sequenced on both strands with an ABI Prism 310 (Applied Biosystems, Monza, Italy) automatic sequencer. Sequence analysis was performed on three independent amplification products.

Electroporation of the plasmid DNA preparation into *Escherichia coli* DH5α was carried out using a Bio-Rad Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, CA), with approximately 500 ng of plasmid, according to the manufacturer's recommendations. Pulsed-field gel electrophoresis was used for molecular typing of all isolates. XbaI restriction analysis was carried out in a CHEF-DR II apparatus (Bio-Rad, CA) under previously described running conditions (27). Isolates and their mutants were considered to be clonal when they had identical electrophoretic patterns and closely related when their patterns differed by three bands or more (25).

(ii) RNA extraction, retrotranscription, and quantitative real-time reverse transcription-PCR. Bacterial cells grown in brain heart infusion until the exponential growth phase, with and without 0.25 mg/liter and 8 mg/liter of meropenem, were harvested by centrifugation, and the bacterial pellet was stored and frozen at −80°C until use. The cultures were resuspended in 200 μl of diethyl pyrocarbonate-treated H_2O, 1 ml of Trizol reagent (GibcoBRL, Paisley, United Kingdom) was added, and incubation was continued for a further 5 min. Following incubation, 200 μl of chloroform was added and mixed by agitation; the mixture was then incubated for 5 min and centrifuged at 10,000 × *g* for 15 min. After centrifugation, 1 ml of ice-isopropanol was added, and the mix was incubated at −20°C for 30 min, followed by centrifugation at 12,000 × *g* for 15 min, resuspension of the pellet in 50 μl of diethyl pyrocarbonate-H_2O, and storage at −20°C. Genomic DNA was removed by treatment with RNase-free Dnase I (Ambion, Austin, TX), and the residual RNA concentration was determined spectrophotometrically. Each extracted RNA sample was also used as a template in the PCR assay to confirm the absence of DNA contamination. Retrotranscription was carried out by using an ImProm-II reverse transcriptase kit (Promega) according to the manufacturer's instructions. Real-time PCR was performed in an Mx 3000P instrument (Stratagene) with 2.5 μl of template, Brilliant SYBR green QPCR master mix (Stratagene), and 30 pmol of primers in a final volume of 25 μl. All PCRs were performed with 55°C as the annealing temperature. Primers for quantification were selected to amplify a fragment of less than 250 bp. Control primers were designed on the basis of the 16S rRNA gene sequence (CACGGTCCAGACTCCTACGG and TAACCACAATGCCTTCCT CC). Specific primers used for VIM-1 amplification were as follows: VIM 1-up, TCCGACTTTACCAGATTGCC; and VIM 1-down, CGAGAAGTGCCGCTG TGTTT. All PCRs were performed in triplicate. Relative gene expression was analyzed by the $2^{-\Delta\Delta CT}$ method. Primer efficiency was verified by using serial dilutions of cDNA ranging from 10^2 to 10^6 target copies per reaction (10^4 to 10^8 target copies per sample), and only oligonucleotides with comparable efficiency were chosen. Gene expression in cultures with added meropenem is represented as the increment/decrement relative to gene expression in culture without meropenem (used as a calibrator). For each analysis, three to five distinct biological replicates were done, and quantitative data are expressed as average values (± standard deviations). Values for changes in gene expression above 2-fold or below 0.5-fold, determined by the $2^{-\Delta\Delta CT}$ method, were considered significant.

(iii) β-Lactamase assays. Fresh cultures of the seven $bla_{\text{VIM-1}}$-carrying *E. cloacae* isolates were grown overnight in 100 ml of TS broth without inducer and in the presence of 0.25 mg/liter and 8.0 mg/liter of meropenem. The β-lactamase extract obtained was suspended in 10 ml of 30 mM HEPES (pH 7.5) plus 50 μM $ZnCl_2$. The β-lactamase activity was determined spectrophotometrically by measuring the hydrolysis of 200 μM meropenem ($\lambda = 300$ nm; $\Delta\varepsilon^{300M} = -9{,}000\ M^{-1}\ cm^{-1}$) in 30 mM HEPES (pH 7.5) plus 50 μM $ZnCl_2$ and of 100 μM ceftazidime ($\lambda = 260$ nm; $\Delta\varepsilon^{260M} = -9{,}000\ M^{-1}\ cm^{-1}$) in 50 mM sodium phosphate buffer, pH 7.0. The protein content was measured using a Bio-Rad protein assay. Gel isoelectric focusing was performed in 5% polyacrylamide gels containing ampholines (pH range, 3.5 to 9.5) by using a Multiphor apparatus (Pharmacia LKB Biotechnology). The gel was focused at 4°C and 25 W for 180 min. Enzyme activity was revealed by overlaying the gel with a paper strip soaked in 250 μM nitrocefin in 30 mM HEPES (pH 7.5) plus 50 μM $ZnCl_2$.

Statistical analysis. The results were analyzed using a commercially available statistical software package (SPSS, version 12.0; SPSS Inc., Chicago, IL). To detect significant differences between groups, we used the chi-square test or Fisher exact test for categorical variables and the two-tailed *t* test or Mann-Whitney test for continuous variables, when appropriate. Statistical significance was established at *P* values of ≤0.05.

RESULTS

Overall, an *Enterobacter* sp. was isolated from 52 patients during the study period: 9 (17.3%) patients were excluded because they had a community-acquired infection, 14 (27%) had a positive body fluid or tissue culture without evidence of clinical infection (urine culture, 10 cases; skin colonization, 3 cases; single blood culture, 1 case), and 29 (55.7%) patients met the study definition criteria.

The infecting pathogen was identified as *E. cloacae* in 28 cases and as *Enterobacter aerogenes* in 1 case. Eleven patients (37.9%) had a complicated urinary tract infection, 7 (24.1%) a pneumonia, 3 (10.3%) a skin/soft tissue infection, 3 (10.3%) an intra-abdominal infection, 3 (10.3%) a bacteremia, and 2 (6.9%) another infection. Seven patients (24.1%) had an infection caused by an *Enterobacter* strain (*E. cloacae* in all cases) harboring the $bla_{\text{VIM-1}}$ gene. A comparison between patients with $bla_{\text{VIM-1}}$-positive and $bla_{\text{VIM-1}}$-negative *Enterobacter* infections is given in Table 1. The two study groups did not differ significantly in terms of median age, sex distribution, and severity of clinical conditions determined by mean SOFA scores. Compared with patients with VIM-1-negative *Enterobacter* infections, VIM-1-positive patients had a more frequent history of recent infection and prior hospitalization and a higher inci-

TABLE 1. Demographics and clinical characteristics of patients with bla_{VIM-1}-positive *Enterobacter* infection versus patients with bla_{VIM-1}-negative *Enterobacter* infection

Parameter	Value		*P* value[a]
	bla_{VIM-1}-positive infection ($n = 7$)	bla_{VIM-1}-negative infection ($n = 22$)	
Age (yr) (median [range])	68 (49–79)	61.5 (20–81)	1.0
Male sex (no. [%])	5 (71.4)	15 (68)	0.4
No. (%) of patients with underlying disease			
Cirrhosis	3 (42.8)	1 (4.5)	**0.03**
Neoplasm	3 (42.8)	8 (36.3)	1.0
Chronic obstructive pulmonary disease	0	2 (9)	1.0
Diabetes	2 (28.5)	5 (22.7)	1.0
Hemodialysis	1 (14.2)	0	0.2
Heart failure	0	7 (31.8)	0.1
Trauma	1 (14.2)	2 (9)	1.0
Dementia	0	2 (9)	1.0
No. (%) of patients with history of recent infection (3 mo)	6 (85.7)	9 (41)	**0.08**
No. (%) of patients with prior hospitalization (3 mo)	7 (100)	8 (36.3)	**0.006**
No. (%) of patients with prior antibiotic therapy (30 days)	7 (100)	15 (68)	**0.1**
SOFA score (mean)	4.4	3.9	**0.6**
No. (%) of patients with severe sepsis	5 (71.4)	9 (41)	**0.2**

[a] Values in bold indicate statistical significance.

dence of liver cirrhosis. Although not significant, severe sepsis was more frequent in patients with VIM-1-positive infection.

Among patients with VIM-1-positive *E. cloacae* infection, five were treated with a carbapenem therapy (alone or in combination), one patient received ciprofloxacin plus amikacin, and one received cefepime plus gentamicin. The drugs were used at standard dosages or adjusted for the creatinine clearance of the patient. The mean duration of antibiotic therapy (23.6 days [95% confidence interval {CI}, 10.3 to 36.8 days] versus 13.3 days [CI, 10.7 to 15.8 days]; $P = 0.01$) and the relapse rate (71.4% [CI, 54.3 to 88.5%] versus 0%; $P < 0.001$) were both significantly higher for patients with VIM-1-positive infection than for those with VIM-1-negative *E. cloacae* infection. A relapse of infection was observed in four of five patients treated with carbapenems and in one treated with cefepime plus amikacin. After the recurrence of infection, one patient was treated with higher doses of meropenem (2 g every 8 h), while among the remaining three patients the antibiotic therapy was modified according to in vitro susceptibility tests, as described in Table 2. The length of hospital stay (41.6 days [CI, 22.4 to 60.7 days] versus 29.7 days [CI, 21.5 to 37.8 days]) and the mortality rate (42.8% [CI, 24.1 to 61.5%] versus 27.3% [CI, 17.8 to 36.8%]), although not statistically significantly different, were higher in patients with VIM-1-positive *E. cloacae* infection.

Susceptibility to the most commonly used antibiotics with gram-negative activity, genetic backgrounds, and resistance gene cassette contents of the seven VIM-1-producing *E. cloacae* strains are shown in Table 3. All strains showed MICs for imipenem and meropenem that were still in the range of susceptibility, while four strains were shown to be resistant to ertapenem following the EUCAST breakpoint but defined with reduced susceptibility to this drug following CLSI guidelines, with reduced susceptibility or a low-level resistance to ertapenem (MICs, 2 to 8 mg/liter). All strains were resistant to ceftazidime, three strains were resistant to piperacillin-tazobactam, aztreonam, and levofloxacin, two were resistant to colistin, and one was resistant to amikacin. All strains were susceptible to tigecycline. All of the isolates showed a negative imipenem-EDTA DDST. In agar diffusion tests, small-colony mutants were selected in ertapenem halos in four cases (3442, 63016, 67141, and 68722), and the mutants displayed a meropenem MIC of 16 mg/liter and were isogenic to the wild-type

TABLE 2. Relationship between site of infection, mutant selection, antimicrobial therapy, and outcome among seven patients with bla_{VIM-1}-positive *Enterobacter* infection

Case no.	Type of infection	Mutant selection	Antibiotic regimen	Outcome
2971	Bacteremia	No	Meropenem plus amikacin	Death, septic shock
2270	Skin/soft tissue infection	No	Ciprofloxacin plus amikacin	Cure
3442	Intra-abdominal abscess	Yes[a]	Cefepime plus gentamicin	Relapse, therapy shifted to ciprofloxacin plus amikacin, cure
63016	Bacteremia	Yes[a]	Ertapenem	Breakthrough bacteremia, death
68722	Complicated urinary tract infection	Yes[a]	Imipenem	Relapse, therapy shifted to high doses of meropenem (2 g every 8 h), cure
67110	Complicated urinary tract infection	No	Meropenem	Relapsing infection, therapy shifted to cefepime, death
67141	Complicated urinary tract infection	Yes[a]	Imipenem plus levofloxacin	Relapse, therapy not changed, cure

[a] Isogenic mutants had meropenem MICs of 8 mg/liter.

TABLE 3. Microbiological and molecular characteristics of seven VIM-1-producing *E. cloacae* strains

Strain	Pulsed-field gel electrophoresis clone	Genetic element	Mechanism of resistance	MIC (mg/liter)[a]											
				IPM	MEM	ERT	TIGE	CEF	CAZ	ATM	PIP/T	AMP	LEV	AK	COL
3442	A	Plasmid int1 bla_{VIM-1}; *Int1-aadA2*	VIM-1 + SHV-12	2	2	8	0.5	>64	>128	>128	>128	>32	32	0.5	0.5
63016	A	Plasmid int1 bla_{VIM-1}; *Int1-aadA2*	VIM-1 + SHV-12	1	2	4	0.5	>64	>128	128	>128	>32	32	0.5	0.25
67141	A	Plasmid int1 bla_{VIM-1}; *Int1-aadA2*	VIM-1 + SHV-12	2	2	2	1	64	>128	128	>128	>32	>32	1	0.5
67110	B	Plasmid int1 bla_{VIM-1}; *Int1-aadA2*	VIM-1	<0.12	0.5	0.5	0.5	8	>128	16	32	>32	<0.03	4	>256
68722	B	Plasmid int1 bla_{VIM-1}	VIM-1 + TEM-1	<0.12	<0.12	0.25	0.5	1	128	64	64	>32	<0.03	1	256
2270	C	int1 bla_{VIM-1}	VIM-1	0.25	0.06	0.25	0.12	0.12	>128	4	32	>32	<0.03	32	2
2971	D	int1 bla_{VIM-1}	VIM-1	1	0.12	1	0.5	2	128	4	64	>32	<0.03	1	0.5

[a] IPM, imipenem; MEM, meropenem; ERTA, ertapenem; TIGE, tigecycline; CEFE, cefepime; CAZ, ceftazidime; ATM, aztreonam; PIP/T, piperacillin-tazobactam; AMP, ampicillin; LEV, levofloxacin; AK, amikacin; COL, colistin.

strains, as demonstrated by identical pulsed-field gel electrophoresis profiles (data not shown).

The seven VIM-1-producing strains belonged to four main clones, namely, A, B, C, and D; in two cases (clones A and B), a nosocomial transmission was documented. Among these strains, three had a contemporary presence of bla_{VIM} and bla_{SHV} and one had the contemporary presence of bla_{VIM} and bla_{TEM} alleles. The bla_{VIM} allele showed an open reading frame of 837 bp that encodes a preprotein of 278 amino acids which shares 100% identity with the VIM-1 MBL. Where present, the bla_{SHV} and bla_{TEM} genes were identified as the genes for the SHV-12 ESBL and a TEM-1 enzyme. In a Southern blot assay, genomic DNA showed a positive hybridization signal with the bla_{VIM-1} probe, whereas the plasmid DNA showed a positive signal only with the bla_{SHV-1} (isolates 3442, 63016, and 67141) and bla_{TEM-1} (isolates 68722) probes. The presence of class 1 integrons was detected in all *E. cloacae* isolates by PCR amplification with primers designed on 5′-3′-conserved segments directly on genomic and plasmid DNAs. An amplification product of 1,060 bp was obtained from genomic DNA, and direct sequencing revealed the presence of one gene cassette carrying bla_{VIM-1}. The 5′-conserved segment carried an integrase gene (*Int1*), a promoter region, and an *attI1* site. The P1 promoter had features of an intermediate promoter (its −35 [TGGACA] and −10 [TAAACT] regions were spaced by 17 bp), while the P2 promoter (−35 [TTGTTA] and −10 [TACAGT] regions 90 bp downstream of P1) was in the inactive form. This finding indicated that the promoter driving the expression of the gene cassette inserted in the integron was only the P1 promoter. A second amplification fragment of 1,010 bp was obtained from plasmid DNA, and it showed a gene cassette carrying an *aadA2* gene encoding an aminoglycoside adenylyltransferase.

The plasmid DNA preparations of all *E. cloacae* strains revealed the presence of plasmid DNA sized larger than 100 kb (data not shown) in only five of seven strains. Electroporation of these plasmids into *E. coli* DH5α was carried out using approximately 500 ng of each plasmid. Selected transformants were obtained on media containing ceftazidime at 16 mg/liter (isolates 3442, 63016, and 67141) and ampicillin at 100 mg/liter (isolate 68722), suggesting a plasmidic location of the bla_{SHV-12} and bla_{TEM-1} genes. Southern blot analysis on plasmid extracts from *E. coli* transformant strains showed no positive signal after hybridization with the bla_{VIM-1} probe, suggesting a chromosomal location of the bla_{VIM-1} gene.

VIM-1 expression experiments with strain 67141 and its isogenic mutant, cultured at a subinhibitory (0.25 mg/liter) or higher (8 mg/liter) meropenem concentration, demonstrated a low increase in VIM-1 expression only in the wild-type strain at 0.25 mg/liter of meropenem (Table 4). No induction was observed with meropenem at 8 mg/liter in the wild type or at either concentration in the mutant, confirming a low level of VIM-1 induction under subinhibitory meropenem pressure and a lack of induction at higher concentrations for both strains.

DISCUSSION

Enterobacter spp. are significant causes of nosocomial infections and are intrinsically resistant to aminopenicillins, cefazolin, and cefoxitin due to the production of constitutive chromosomal AmpC β-lactamases. Moreover, ESBL-producing *Enterobacter* spp., particularly *E. cloacae*, have been identified in the United States (13) and Europe (27), and carbapenems are considered the drug of choice in these cases. To our knowledge, this is the first study that compares the epidemiology and outcomes of VIM-1-positive and VIM-1-negative *Enterobacter* nosocomial infections.

A major finding of our study was that the 24.1% of clinically significant *Enterobacter* infections observed in our hospital during a 1-year period were caused by VIM-1-producing strains. The presence of this resistance determinant is probably underreported because it is not related to a phenotype of resistance by standard susceptibility tests. In some cases, only ertapenem demonstrated a reduced susceptibility phenotype. For four strains of seven, the MIC of ertapenem was >1 mg/liter, i.e.,

TABLE 4. Change to VIM-1 expression in *E. cloacae* 67141 and its isogenic mutant in presence of meropenem

Strain	Meropenem MIC (mg/liter)	Log fold change in VIM-1 expression with meropenem	
		0.25 mg/liter	8 mg/liter
67141	2	2.01 ± 0.05	−0.20 ± 0.05
Isogenic mutant	16	−0.25 ± 0.03	−0.30 ± 0.04

they were considered resistant following EUCAST guidelines. This finding is concordant with previous reports which analyzed the microbiological features of VIM-1-producing *Enterobacteriaceae*, in which ertapenem was demonstrated to be a better indicator for phenotypic carbapenemase detection. A study conducted in Spain (24) found that most MBL-producing isolates (including *K. pneumoniae* and *E. cloacae*) had imipenem and meropenem MICs within the range of susceptibility, as stated by the CLSI guidelines, and similar results were obtained by analyzing 17 patients with serious infections caused by VIM-1-producing *Enterobacteriaceae* (only two cases involving *Enterobacter* spp.) in Greece (23). Susceptibility to carbapenems was also documented for most strains isolated in two case series of VIM-1-positive *Klebsiella pneumoniae* bloodstream infections (1, 19) and among *Proteus mirabilis* strains carrying the VIM-1 gene (26). We recently published the first case of VIM-1-positive *P. mirabilis* infection in Italy and also found a full susceptibility to all carbapenems and various β-lactams (5).

Another matter of concern is that all VIM-1-carrying isolates showed a negative imipenem-EDTA DDST; this phenomenon has already been observed for VIM-1-positive *Acinetobacter baumannii* (14) and MBL-producing *Pseudomonas aeruginosa* (22) strains. Combined disk tests using various amounts of EDTA/disk have previously been evaluated for detection of MBLs (22), but they showed that disks containing 930, 744, 518, or 292 mg of EDTA/disk did not perform well in separating the MBL-positive and -negative isolates, a fact suggesting that the optimal amount of EDTA may depend on the strain collection studied. All of these findings confirm a poor efficacy of conventional screening tests for MBLs.

Only a few studies have tried to elucidate the mechanisms underlying the diversity in β-lactam resistance among VIM-1-producing strains. Loli and coworkers found a correlation between increases of carbapenem MICs, alterations of outer membrane protein profiles, and rearrangements in the $bla_{\text{VIM-1}}$-carrying plasmid (15). In our strains, the low expression level of the $bla_{\text{VIM-1}}$ gene was related to an inactivated P2 promoter and to a weak activity of a promoter (P1) which drives the expression of the gene cassette inserted into the integron. The presence of a weak P1 promoter has previously been shown to result in a 20-fold decrease in the level of resistance conferred by the downstream gene cassettes (3) relative to the levels seen with promoters like that of the control strain. This mechanism was recently identified as the cause of hidden MBL phenotypes among 87 *Acinetobacter baumannii* clinical isolates (9). This is the first description of this phenomenon in *Enterobacter cloacae*. So far, the identification of MBL-carrying gram-negative isolates may represent a new challenge for clinical microbiologists, because they may be dismissed as carbapenem susceptible in daily laboratory practice.

Our data suggest that patients with VIM-1-producing *Enterobacter* infection are more likely to develop antibiotic therapy failure and usually need a more prolonged course of antibiotic therapy. The correlation between the presence of the VIM-1 gene and clinical outcome has not been studied extensively. Souli et al. studied the clinical features of 17 patients with serious infections caused by members of the *Enterobacteriaceae* (including two *Enterobacter* strains) carrying $bla_{\text{VIM-1}}$ genes and found a high incidence of antibiotic failure among patients treated with carbapenem therapy (23); however, a comparison with VIM-1-negative strains was not available. In our series, the comparison between VIM-1-negative and VIM-1-positive *Enterobacter* infections demonstrated a higher relapse rate and a more complicated outcome in the latter group. Interestingly, most patients who had a complicated outcome developed isogenic mutants with higher meropenem MICs under antibiotic pressure, a fact suggesting a potential development of resistance under certain circumstances.

From a clinical point of view, the spread of carbapenem-susceptible MBL-carrying *Enterobacteriaceae* is extremely worrisome, because carbapenems are frequently used in patients with gram-negative nosocomial infections, especially if the strain exhibits an ESBL phenotype. Thus, a hidden resistance to these antibiotics may represent a major problem in the treatment of serious nosocomial infections. Alternative options are limited and are represented by tigecycline and colistin, alone or in combination (2). However, as demonstrated in our series, some VIM-1-producing *Enterobacter* strains were resistant to colistin, and adverse toxicity effects and pharmacokinetic properties limit its use. Colistin resistance is becoming a sporadic but increasingly reported phenomenon, not only in nonfermentative bacteria but also in many species of *Enterobacteriaceae* (6). Furthermore, the clinical use of tigecycline for the treatment of infections with these organisms has scarcely been reported.

In conclusion, we observed the spread of VIM-1 beta-lactamase among *E. cloacae* strains isolated in our hospital. In the present study, $bla_{\text{VIM-1}}$-producing *E. cloacae* clinical isolates showed a negative MBL phenotype. This observation raises the issue of whether standard susceptibility tests are sufficient in terms of sensitivity, since patients with nosocomial infections caused by VIM-1-positive *E. cloacae* infections have a high risk of therapeutic failure. Our findings highlight the need for rapid identification methods for MBL-producing *Enterobacteriaceae*.

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