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Novel VIM Metallo-β-Lactamase Variant from Clinical Isolates of *Enterobacteriaceae* from Algeria▽

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Five different strains of bacteria belonging to the family *Enterobacteriaceae* were isolated from two patients hospitalized in the intensive care unit of the Central Military Hospital of Algiers, Algeria. All five strains, one *Providencia stuartii* strain, two *Escherichia coli* strains, and two *Klebsiella pneumoniae* strains, were intermediate or resistant to all β-lactams, including carbapenems. Synergy between imipenem and EDTA was observed for all five strains. The results of the PCR experiment confirmed the presence of a bla_{VIM} gene in all five strains. The bla_{VIM} genes were located as part of a class 1 integron on a 180-kb conjugative plasmid. They encoded a novel metallo-β-lactamase designated VIM-19, which differed from the parental enzyme VIM-1 by only two substitutions: Ser228Arg, previously observed in the closely related enzyme VIM-4, and Asn215Lys, not previously observed in other VIM-type carbapenemases. VIM-19 was further characterized after purification through determination of its kinetic constants. This enzyme was inhibited by EDTA and hydrolyzed penicillins, cephalosporins, and carbapenems, as observed for other VIM-type carbapenemases but with greater catalytic efficiency against penicillins than VIM-1. VIM-19 is the first carbapenemase enzyme identified from an isolate from Algeria. These results confirm the emergence of VIM-4-like enzymes in members of the family *Enterobacteriaceae* from Mediterranean countries.

Carbapenems are the most active molecule in β-lactams with 98% susceptibility worldwide among bacteria belonging to the family *Enterobacteriaceae* (24). However, a growing number of carbapenemase-producing strains have been identified since the beginning of the 1990s. They displayed variable *in vitro* levels of resistance to carbapenems, including susceptibility (24), but were clinically resistant to these molecules (35). The possible low level of resistance makes reliable detection of such strains difficult (35).

The geographical distribution of these strains differs: those producing class A KPC-type carbapenemases have been more frequently observed in the United States and in Israel, whereas those producing VIM- and IMP-type class B β-lactamases were generally encountered in Asia and in the northern part of the Mediterranean basin (24). Among these enzymes, metallocarbapenemases are especially worrying because they virtually hydrolyze all classes of β-lactams, except aztreonam (35). Initially observed in *Pseudomonas aeruginosa* and *Acinetobacter* spp., they spread to members of the family *Enterobacteriaceae* during the late 1990s and the 2000s (24). The most frequently acquired metallo-β-lactamases (MBLs) are the IMP and VIM types (24). Four other types of acquired MBLs in *P. aeruginosa* isolates from Brazil (SPM-1) (34) and Germany (GIM-1) (5), in *Acinetobacter baumannii* isolates from Korea (SIM-1) (17), and in a *Citrobacter freundii* isolate from Japan (KHM-1) (30) have recently been described.

VIM-producing members of the family *Enterobacteriaceae* have been involved in different outbreaks in particular in Italy and Greece during the 2000s (4, 23, 33). The southern part of the Mediterranean basin seemed to be spared by such strains until 2006, when VIM-4-producing strains of *Klebsiella pneumoniae* were identified during a nosocomial outbreak in a Tunisian hospital (14), suggesting a possible dissemination in northwestern Africa.

Between January and May 2008, five imipenem-resistant strains belonging to bacteria in the family *Enterobacteriaceae* were recovered from two patients in the intensive care unit of the Central Military Hospital of Algiers, Algeria. We identified a novel metallocarbapenemase, VIM-19, in these strains. This is the first report of VIM-producing strains in Algeria.

MATERIALS AND METHODS

Bacterial isolates and plasmids. The bacterial strains used in this study were *Escherichia coli* 138, *E. coli* 2603, *K. pneumoniae* 2878, *K. pneumoniae* 6828, and *Providencia stuartii* 6858 isolated from two different patients hospitalized in the intensive care unit of the Central Military Hospital of Algiers, Algeria. *E. coli* DH5α (Novagen, Darmstadt, Germany) and *E. coli* BL21(DE3) (Novagen) were used for cloning experiments, and *E. coli* C600 was used for mating-out assays. Plasmid pBK-CMV (CMV stands for cytomegalovirus) (Stratagene, Amsterdam, The Netherlands) was used for the initial cloning experiments, and a modified pET9a plasmid harboring a NotI restriction site (18) was used for overexpression of the β-lactamase-encoding genes.

Phenotypic detection of metallo-β-lactamases. EDTA-combined disk method was performed with imipenem-containing disks by the method of Picão et al. (20). Two imipenem-containing disks were placed on a Mueller-Hinton agar plate (bioMérieux, Marcy l'Etoile, France), and 10 μl of a 100 mM EDTA solution were added to one of the disks. After overnight incubation at 37°C, an

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inhibition diameter extension of at least 5 mm for EDTA-containing disk indicated synergy.

Genomic typing. The clinical isolates *Escherichia coli* 138, *E. coli* 2603, *K. pneumoniae* 2878, and *K. pneumoniae* 6828 were compared by enterobacterial repetitive intergenic consensus sequence PCR (ERIC2-PCR) as previously described (8).

Susceptibility to β-lactams. Antibiotic-containing disks were used for antibiotic susceptibility testing by the disk diffusion assay (MAST Diagnostic, Amiens, France). Results of susceptibility testing were interpreted according to the recommendations of the Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM) (7). MICs were determined by a microdilution method on Mueller-Hinton agar (Bio-Rad, Richmond, CA) with an inoculum of 10^4 CFU per spot and were interpreted according to the guidelines of the CA-SFM (7). The antibiotics were provided as powders by GlaxoSmithKline (amoxicillin, ticarcillin, cefuroxime, ceftazidime, and clavulanic acid), Wyeth Laboratories (piperacillin and tazobactam), Eli Lilly (cephalothin), Roussel-Uclaf (cefotaxime and cefpirome), Bristol-Myers-Squibb (aztreonam and cefepime), Merck Sharp and Dohme-Chibret (cefoxitin, imipenem and ertapenem) and AstraZeneca (meropenem).

Isoelectric focusing. Isoelectric focusing of β-lactamases was performed with polyacrylamide gels containing Ampholines with a pH range of 3.5 to 10.0, as previously described (2), with TEM-39 (pI 5.2), TEM-1 (pI 5.4), TEM-2 (pI 5.6), CTX-M-15 (pI 8.6), and CMY-4 (pI 9) as standards.

Plasmid analysis. Mating experiments were performed with *in vitro*-obtained rifampin-resistant mutants of *E. coli* C600 as previously described (27). Transconjugants were selected on agar containing rifampin (300 μg/ml) and cefotaxime (0.5 μg/ml). Plasmid DNA extraction of the different clinical strains was performed by using the Qiagen plasmid DNA maxikit (Qiagen, Courtaboeuf, France). The extracted plasmids were electroporated onto *E. coli* DH5α with selection on agar containing cefotaxime (0.5 μg/ml). Plasmid DNAs were extracted from the clinical strains, transconjuguants, and transformants by the method of Kado and Liu (13). Plasmid size was determined by comparison with that of plasmids Rsa (39 kb), TP114 (61 kb), pCFF04 (85 kb), and pCFF14 (180 kb) as previously described (19). The plasmids of the transformants corresponding to the five clinical strains were extracted by the alkaline lysis method (1) and were digested with EcoRI, HindIII, and PstI restriction endonucleases according to the manufacturer's recommendations (Boehringer Mannheim, Meylan, France).

Sequencing and analysis of *bla* genes. Molecular screening for bla_{VIM}, bla_{TEM}, $bla_{CTX\text{-}M}$, and bla_{CMY} was performed using PCR with primers VIM-F, VIM-B, CitMF, CF2, CTX-M A, and CTX-M B (3, 9, 21, 26). VIM-1-specific primers VIM-1A NdeI (5′-GGAATTCCATATGTTAAAAGTTATTAGTAGT-3′) and VIM-1B NotI (5′-ATAGTTTAGCGGCCGCCCTACTCGGCGACTGAGCG ATT-3′) were used to amplify and sequence the bla_{VIM} gene. Direct sequencing was performed on PCR products, which were obtained from the five clinical strains, the transconjugant *E. coli* C600, and the different recombinant *E. coli*. These PCR products were sequenced by dideoxy chain termination on both strands with an Applied Biosystems sequencer (ABI 377) (29). Primer INT/5CS targeting the 5′ conserved sequence of class 1 integron previously described by Riccio et al. (25) was used with VIM-1B NotI to amplify and sequence a part of the bla_{VIM} genetic environment. The nucleotide and deduced protein sequences were analyzed using software available at the website of the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov). The ClustalW program (http://ebi.ac.uk/Tools/clustalW2/index.html) was used for the alignment of amino acid sequences (32).

Cloning experiments. Recombinant DNA manipulation and transformations were performed by the methods of Sambrook et al. (27). The PCR products obtained with primers VIM-1A NdeI and VIM-1B NotI were cloned into SmaI (Roche Diagnostics, Meylan, France) restriction site of the pBK-CMV plasmid. Recombinant *E. coli* DH5α bacteria were selected on Mueller-Hinton agar plates supplemented with 30 μg/ml kanamycin and 0.5 μg/ml cefotaxime. The plasmid was then digested by NdeI and NotI (Roche Diagnostics, Meylan, France) and ligated into the corresponding restriction sites of a modified pET-9a plasmid. Recombinant *E. coli* BL21 bacteria were selected on Mueller-Hinton agar plates supplemented with 30 μg/ml kanamycin and 0.5 μg/ml cefotaxime. All the cloned fragments were subjected to confirmatory sequencing.

Overproduction and purification of β-lactamases. The VIM-19-producing *E. coli* BL21(DE3) clone was used to overproduce the VIM-19 β-lactamase as previously described (6). Bacteria were disrupted by sonication. The supernatant was loaded onto a Q Sepharose column (10 ml; Amersham Pharmacia Biotech., Orsay, France) equilibrated with 20 mM Tris-HCl (pH 7.4). The bound proteins were eluted with a linear NaCl gradient (0 to 500 mM). The β-lactamase-containing elution peak was loaded onto a Superose 12 column (Amersham Pharmacia Biotech) and eluted with buffer consisting of 5 mM Tris-HCl and 100 mM NaCl (pH 7.4). The β-lactamase-containing elution peak was dialyzed against 100 mM NaCl, concentrated, and stored at −20°C until use. The total protein concentration was estimated by the Bio-Rad protein assay (Bio-Rad, Richmond, CA), with bovine serum albumin (Sigma Chemical Co., St. Louis, MO) used as a standard.

Determination of β-lactamase kinetic parameters k_{cat} and K_m. The Michaelis constant (K_m) and catalytic activity (k_{cat}) were determined with purified extracts using a computerized microacidimetric method (15) at pH 7 and 37°C in 0.1 M NaCl solution.

Nucleotide sequence accession number. The nucleotide sequence has been assigned accession number FJ499397 in the GenBank database.

RESULTS

Clinical strains and resistance phenotype. *E. coli* 138 was isolated in January 2008 from the urinary tract of a patient hospitalized for acute renal failure in the intensive care unit of the Central Military Hospital of Algiers, Algeria. He was treated with imipenem and amikacin and died from septic shock 1 day later. *E. coli* 2603 was isolated in February 2008 from the urinary tract of a second patient hospitalized in the same ward. *K. pneumoniae* 2878 and 6828 and *P. stuartii* 6858 were isolated in May 2008 from two different urinary samples of this second patient. *P. stuartii* 6858 was isolated from two samples, whereas *K. pneumoniae* 2878 and 6828 were only isolated from the first and second sample, respectively. She was treated with cefotaxime and died from a pulmonary embolism.

Both patients had been previously treated with different broad-spectrum β-lactams, the first with cefotaxime and the second with cefotaxime, ceftazidime, and imipenem. These five strains were the first strains of bacteria belonging to the family *Enterobacteriaceae* intermediate or resistant to carbapanems isolated in this hospital.

All five strains were intermediate or resistant to all β-lactams including carbapenems (Table 1). They were resistant to sulfonamides, trimethoprim, tetracycline, and chloramphenicol. They were intermediate or resistant to kanamycin, tobramycin, netilmicin, amikacin, and gentamicin. They were also susceptible to all quinolones and to fosfomycin.

The two *E. coli* strains and the two *K. pneumoniae* strains presented different ERIC2-PCR patterns, confirming that they were not clonally related (data not shown). The results of the EDTA-combined disk method suggested that the five strains produce a metallo-β-lactamase.

Molecular β-lactamase identification. Isoelectric focusing experiments showed that the five clinical strains produced different β-lactamases of pI 5.4 and 9 in addition to their natural respective β-lactamases. The PCR-based analysis of β-lactamase-encoding genes revealed that they all harbored a *bla* gene encoding a VIM-type carbapenemase and a *bla* gene encoding a CMY-type cephalosporinase corresponding to the enzyme with pI 9. Partial sequencing of the bla_{CMY} genes showed that the gene coded for a CMY-4-like enzyme. The two *E. coli* strains 138 and 2603 also presented a bla_{TEM} gene, which was identified as $bla_{TEM\text{-}1}$. No CTX-M-type enzyme was detected in any of these strains, unlike in the Tunisian VIM-4-producing strains (14).

The five clinical strains harbored the same novel bla_{VIM} gene, designated $bla_{VIM\text{-}19}$. This gene differed from $bla_{VIM\text{-}4}$ by only one substitution at position 1034 from A to C (numbering according to the sequence deposited in EMBL under accession

TABLE 1. MICs of β-lactam antibiotics for the five clinical strains, the transconjugant, the transformant, and the clone which produce VIM-19

β-Lactam[a]	MIC (μg/ml) for strain:								
	E. coli 138	*E. coli* 2603	*K. pneumoniae* 2878	*K. pneumoniae* 6828	*P. stuartii* 6858	TC *E. coli* C600 2878	TR *E. coli* DH5α 2878	*E. coli* DH5α (pBK-VIM-19)	*E. coli* (pBK-CMV)
Amoxicillin	>2,048	>2,048	>2,048	>2,048	>2,048	>2,048	>2,048	>2,048	4
Amoxicillin + CLA	>2,048	>2,048	>2,048	>2,048	>2,048	>2,048	>2,048	>2,048	4
Ticarcillin	>2,048	>2,048	>2,048	>2,048	>2,048	>2,048	>2,048	>2,048	2
Ticarcillin + CLA	>2,048	>2,048	>2,048	>2,048	>2,048	>2,048	>2,048	>2,048	2
Piperacillin	>2,048	>2,048	2,048	1,024	256	512	1,024	512	2
Piperacillin + TZB	2,048	1,024	2,048	1,024	256	512	1,024	512	2
Cephalothin	>2,048	2,048	>2,048	2,048	>2,048	1,024	2,048	1,024	4
Cefuroxime	>1,024	>1,024	1,024	1,024	1,024	512	1,024	1,024	4
Cefoxitin	512	512	512	128	128	128	256	128	4
Cefotaxime	512	256	256	128	128	64	256	128	0.06
Cefotaxime + EDTA	32	4	4	4	2	1	1	0.06	0.06
Ceftazidime	512	128	128	64	64	32	128	32	0.12
Ceftazidime + EDTA	128	16	8	8	4	1	8	0.12	0.12
Aztreonam	128	64	16	8	2	4	16	0.12	0.12
Aztreonam + EDTA	128	64	16	8	2	4	16	0.12	0.12
Cefepime	64	4	64	4	2	2	4	2	<0.12
Imipenem	32	16	64	4	16	4	8	16	<0.12
Imipenem + EDTA	0.5	0.25	0.5	<0.12	0.5	<0.12	<0.12	<0.12	<0.12
Meropenem	16	8	32	4	8	1	8	8	<0.12
Meropenem + EDTA	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12
Ertapenem	32	16	64	16	32	1	8	4	<0.12
Ertapenem + EDTA	0.25	<0.12	0.25	<0.12	<0.12	<0.12	<0.12	<0.12	<0.12

[a] CLA, clavulanic acid at 2 μg/ml; TZB, tazobactam at 4 μg/ml; EDTA at 0.4 mM.

number AM181293 [14]) which led to the substitution Asn215Lys according to the MBL scheme (11). VIM-19 was therefore a new VIM enzyme which differed from VIM-1 by two amino acid substitutions, Asn215Lys and Ser228Arg (previously observed in VIM-2 and VIM-4).

Genetic support. Different transconjugants and transformants were obtained from the five clinical strains by mating out and electroporation. Specific PCR experiments confirmed the presence of the bla_{VIM} and bla_{CMY} genes in all five strains. No bla_{TEM} was detected in any of the transconjugants or transformants. Plasmid content analysis of the clinical strains and their respective transformants and transconjugants revealed the transfer of a 180-kb plasmid that encoded both VIM-type and CMY-type β-lactamases, as previously observed in the Tunisian VIM-4-producing strains (14). The restriction profiles of the transformants were identical, suggesting the dissemination of a single plasmid among the different isolates (data not shown).

PCR experiments and sequencing of bla_{VIM-19} genetic environment showed that this gene was the first gene cassette of a class 1 integron as previously observed for other bla_{VIM} genes (14).

The transconjugant (TC) *E. coli* C600 2878 and the transformant (TR) *E. coli* DH5α 2878 of the clinical strain *K. pneumoniae* 2878 were chosen for further characterization.

β-Lactam MICs. All the clinical strains, TC *E. coli* C600 2878, TR *E. coli* DH5α 2878, and the clone *E. coli* DH5α(pBK-VIM-19) were highly resistant to all penicillins (alone or in association with clavulanic acid or tazobactam), cephalothin, and cefuroxime (MICs, 1,024 to >2,048 μg/ml) (Table 1). The MICs of cefoxitin, cefotaxime, and ceftazidime were slightly lower, ranging from 32 to 512 μg/ml. Carbapenems and cefepime were more active with MICs ranging from 1 to 64 μg/ml. Aztreonam was only active against *E. coli* DH5α(pBK-VIM-19), which produced only VIM-19. The other strains, which also produced a CMY-4-like enzyme, had MICs of aztreonam between 2 and 128 μg/ml. In the presence of EDTA, the activity of the different carbapenems was restored (MICs, <0.12 to 0.5 μg/ml). The MICs of ceftazidime and cefotaxime decreased 2-fold to 11-fold,but aztreonam MICs were not significantly modified.

Kinetic constants. The two steps of purification yielded approximately 1.6 mg of pure enzyme per liter of culture. The specific activity of the extract against benzylpenicillin was 419.6 μmol/min/mg. The level of purity was estimated >97% by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (2, 16).

Table 2 lists the steady-state kinetic parameters k_{cat}, K_m, and k_{cat}/K_m of VIM-19 and compares these data with previously published values of VIM-1 and VIM-2 (10, 22, 29). These data have been measured under different experimental conditions, so the data should be compared with caution.

Like other VIM enzymes, VIM-19 has a broad hydrolysis profile. However, it was more active against penicillins than against cephalosporins and carbapenems, whereas the K_m values remained lower than 100 μM for all of the tested substrates. The greatest catalytic efficiency was observed for benzylpenicillin, cloxacillin, amoxicillin, piperacillin, and cephalothin. Among the oxyimino cephalosporins, VIM-19 was especially active and efficient against cefotaxime. Finally, VIM-19 was 6-fold to 14-fold more active against imipenem than against ertapenem and meropenem. As previously observed for other metallo-β-lactamases, VIM-19 had no detectable activity against aztreonam.

In contrast with VIM-1, VIM-19 was slightly more efficient against penicillins than against cephalosporins, as observed for

TABLE 2. Kinetic parameters of β-lactamases VIM-1, VIM-2, and VIM-19[a]

β-Lactam	Kinetic parameter of β-lactamase								
	VIM-19			VIM-1[b]			VIM-2[b]		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)
Benzylpenicillin	206	57	3.6	29	841	0.034	55.8	49	1.14
Cloxacillin	79.2	14	5.7	ND	ND		350	250	1.40
Amoxicillin	112	34	3.3	ND	ND		29.7	54	0.55
Ticarcillin	45.8	68	0.7	ND	ND		31.7	46	0.69
Piperacillin	160	37	4.3	1,860	3,500	0.53	32.7	72	0.45
Cephalothin	21.2	15	1.4	281	53	5.3	56.2	44	1.28
Cefoxitin	4.0	53	0.08	26	131	0.2	2.8	24	0.12
Cefuroxime	5.3	13	0.4	324	42	7.7	12.1	22	0.55
Aztreonam	<0.1			<0.01	>1,000		<0.5		
Ceftazidime	6.3	88	0.07	60	794	0.076	88.7	98	0.90
Cefotaxime	34.3	36	0.95	169	247	0.68	27.5	32	0.86
Cefepime	12.5	55	0.23	549	145	3.8	4.7	184	0.03
Imipenem	8.2	22	0.37	2	1.5	1.3	9.9	10	0.99
Meropenem	1.3	9	0.14	13	48	0.27	1.4	5	0.28
Ertapenem	0.6	21	0.03	ND	ND		0.2	9	0.022

[a] The standard deviation for analysis was ≤10%.
[b] The kinetic parameters for VIM-1 are from Franceschini et al. (10) and those for VIM-2 are from Poirel et al. (21), except for cloxacillin and ertapenem, which are from Samuelsen et al. (28). ND, not determined.

VIM-2, because of lower K_m values. Both VIM-2 and VIM-19 harbored an arginine residue at position 228 instead of a serine in VIM-1. The crystallographic structure of VIM-2 revealed that the side chain of this residue was close to one of the two Zn atoms (Zn 2) and interacted, in the oxidized form of VIM-2, with the residues of the active site of VIM-2 located at positions 224 and 263 (12). In addition, in the reduced form of VIM-2, Arg228 contributed to the creation of a positively charged environment for substrate binding (12). These structural features, which are shared by VIM-2 and VIM-19, could be involved in the differences in the hydrolysis profiles observed for VIM-1 and VIM-19. The role of the substitution Asn215Lys of VIM-19 remained unclear, but this residue seemed to have less impact because its side chain was oriented toward the solvent in the structure of VIM-2.

Concluding remarks. Infections caused by metallo-β-lactamase-producing bacteria of the family *Enterobacteriaceae* are currently a worrying threat because the antibiotic options available are extremely limited and the infections are associated with a high risk of mortality (31). We report here the first documented cases of infections caused by VIM-producing strains belonging to the family *Enterobacteriaceae* in Algeria. The five strains produced a novel VIM-type metallo-β-lactamase, VIM-19. This enzyme is closely related to VIM-4 and VIM-1, which are so far the most frequently observed VIM-type enzymes in strains of the *Enterobacteriaceae* (14, 22, 24). As in a recent outbreak of VIM-4-producing *Klebsiella pneumoniae* in Tunisia (14), the bla_{VIM-19} gene was located among a class 1 integron on a large plasmid and was associated with a bla_{CMY} gene. Our study confirms the emergence of VIM-producing bacteria of the *Enterobacteriaceae* in northwest Africa and their dissemination all around the Mediterranean basin. A large multicentric study would be necessary to assess the dissemination of such strains in the different countries of the region.

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