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Detection and Characterization of VIM-31, a New Variant of VIM-2 with Tyr224His and His252Arg Mutations, in a Clinical Isolate of *Enterobacter cloacae*

Pierre Bogaerts,^a Carine Bebrone,^b Te-Din Huang,^a Warda Bouchahrouf,^a Yves DeGheldre,^c Ariane Deplano,^d Kurt Hoffmann,^b and Youri Glupczynski^a

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We report the first description of the metallo- β -lactamase VIM-31, a new variant of VIM-2 with Tyr224His and His252Arg mutations, in *Enterobacter cloacae* 11236, which was isolated from blood specimens of a patient with colonic adenocarcinoma in Belgium. *bla*_{VIM-31} was found on a class 1 integron located on a self-transferable but not typeable 42-kb plasmid. Compared to values published elsewhere for VIM-2, the purified VIM-31 enzyme showed weaker catalytic efficiency against all the tested beta-lactam agents (except for ertapenem), resulting from lower k_{cat} (except for ertapenem) and higher K_m values for VIM-31.

The worldwide spread of metallo- β -lactamases (i.e., VIM, IMP, and NDM enzymes) in carbapenem-resistant Gram-negative bacteria presently constitutes a major public health issue.

So far, 33 distinct *bla*_{VIM} alleles have been described (www.lahey.org/Studies) for a variety of Gram-negative opportunistic pathogens, but only VIM-1, VIM-2, VIM-4, VIM-7, and VIM-27 enzymes (7, 12, 15, 22, 31) have been characterized biochemically and structurally. While VIM-1-derived enzymes have been reported largely for *Enterobacteriaceae* from around the world, especially from Greece but also recently from Belgium (4), VIM-2 has been associated mostly with *Pseudomonas aeruginosa* (32). The occurrence of VIM-2 has nevertheless also been reported for clinical isolates of *Enterobacteriaceae* from Asia (17, 24, 39), Mexico (28), Argentina (16), and Tunisia (10). Moreover, a recent report showed the presence of VIM-2 in *Enterobacter ludwigii* isolated from sewage water of a hospital, highlighting the involvement of environmental bacteria as potential reservoirs for the dissemination of clinically relevant metallo- β -lactamase resistance genes (37). In many subclass B1 (e.g., IMP and NDM) and B2 (e.g., CphA) metallo- β -lactamases, the amino acid at position 224 (a conserved Lys residue), close to the active site, plays an important role in substrate binding (2, 27). In the case of VIM enzymes, Lys224 is not present and is replaced by His224 in VIM-1 and by Tyr224 in VIM-2. The side chains of histidine and tyrosine residues are much shorter than that of a lysine residue, thereby preventing the interaction of these two amino acids with the carboxylate moiety (C₄ or C₃) of the substrate. For VIM enzymes, it is hypothesized that Arg228, with its long side chain, may replace Lys224 in interacting with the substrate (15, 22).

We report here the detection and characterization of VIM-31, a new variant of VIM-2 with Tyr224His and His252Arg mutations, in an *Enterobacter cloacae* clinical isolate recovered from an elderly patient in Belgium.

MATERIALS AND METHODS

Bacterial strains and antimicrobial susceptibility. *Enterobacter cloacae* 11236 was recovered in June 2011 from blood culture specimens from a patient hospitalized in Brussels, Belgium. The isolate was identified to the species level by matrix-assisted laser desorption/ionization–time of flight

mass spectrometry (MALDI-TOF MS) with a Microflex LT mass spectrometer (Bruker Daltonik GmbH, Leipzig, Germany). Susceptibility to antimicrobials was determined by the disc diffusion method, and MICs of antimicrobial agents were determined by Etest (bioMérieux, Marcy l'Etoile, France) or broth microdilution (Sensititre; Trek Diagnostic Systems, Cleveland, OH) according to CLSI guidelines (11). EDTA-disc synergy tests were performed for the screening of metallo- β -lactamase production (4).

PCR detection of *bla* genes and sequencing. Detection of extended-spectrum β -lactamase (ESBL) genes (*bla*_{TEM,SHV}, *bla*_{CTX-M-1-like}, *bla*_{CTX-M-2-like}, *bla*_{CTX-M-9-like}, and *bla*_{CTX-M-8/25/26-like}) and of carbapenemase genes (*bla*_{VIM}, *bla*_{IMP}, *bla*_{NDM-1}, *bla*_{OXA-48}, and *bla*_{KPC}) was performed by DNA microarray analysis (Check-MDR CT102; Check-Points, Wageningen, The Netherlands) according to a previously described protocol (30). *bla*_{OXA-1-like}, *bla*_{OXA-2-like}, and *bla*_{OXA-10-like} genes, genes encoding class A ESBL enzymes (BEL, GES, VEB, and PER), and *qnr* genes, involved in quinolone resistance, were detected by PCR as previously described (3, 6). The genetic context of *bla*_{VIM-31} was assessed by PCR amplification and sequencing of the variable region of the integron by using primers designed on the basis of the 5' and 3' conserved segments of the class 1 integron (25), using an external sequencing service (Macrogen, Seoul, South Korea). Nucleotide sequences were analyzed with the BLASTN algorithm, available from the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Molecular typing. Pulsed-field gel electrophoresis (PFGE) analysis of XbaI-digested genomic DNA was used to examine the genetic relatedness of *E. cloacae* 11236 to other *E. cloacae* isolates known to be epidemic in several Belgian hospitals (A. Deplano, personal data). PFGE patterns were analyzed with BioNumerics software (Applied Maths, Kortrijk, Belgium), using the Dice similarity coefficient and the unweighted-pair group method using average linkages (UPGMA) as previously described (5). The PFGE classification criteria were described previously, and similar PFGE types included patterns showing differences of 0 to 6 DNA fragments (38).

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Plasmid analysis, mating assay, and electroporation experiments.

Plasmid DNA was extracted by the Kieser method (18) or by use of a QIAfilter Plasmid Midi kit (Qiagen, Venlo, The Netherlands). *Escherichia coli* NCTC50192, harboring four plasmids, of 154, 66, 48, and 7 kb, was used as a plasmid size marker. Plasmid DNA was analyzed by 0.7% agarose gel electrophoresis. Determination of the incompatibility groups of plasmids was done as described by Carattoli et al. (8). Transfer of the β -lactamase genes from *E. cloacae* 11236 to *Escherichia coli* J53 (azide resistant) and *Pseudomonas aeruginosa* PU21 (rifampin resistant) was attempted by solid and liquid mating assays at 37°C (13). Selection was performed on brain heart infusion (BHI) agar plates supplemented with ticarcillin at 100 μ g/ml and azide at 150 μ g/ml for *E. coli* and with ticarcillin at 100 μ g/ml and rifampin at 100 μ g/ml for *P. aeruginosa*. Moreover, plasmid DNA extracts from *E. cloacae* 11236 were electroporated into *E. coli* TOP10 cells (Invitrogen, Merelbeke, Belgium) by use of a Gene Pulser II apparatus (Bio-Rad, Marnes-la-Coquette, France) as previously described (1, 9), and transformants were selected on BHI agar plates containing 100 μ g/ml of ticarcillin. Electroporation of a plasmidic extract of *E. cloacae* 11236 into *Pseudomonas aeruginosa* PAO1 was also performed as previously described (13), and transformants were selected on BHI agar plates containing 100 μ g/ml of ticarcillin or 20 μ g/ml of tobramycin.

Cloning of *bla*_{VIM-31}. The *bla*_{VIM} gene was amplified with HotStarTaq DNA polymerase (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions, using primers 5'-CATGCCATGGTCAAAC TTTTGAGTAAGTTATTG-3' and 5'-CTGAATTCCTACTCAACGACT GAGCGATTGTG-3', designed to add NcoI and EcoRI restriction sites (underlined in the sequences) upstream and downstream of the VIM-31 gene, respectively. The PCR product was then cloned into pET-28a (Novagen, Madison, WI), and the expression vector construct, named pET-28a/VIM-31, was introduced into *E. coli* BL21(DE3) (New England Biolabs, Ipswich, MA) by electroporation. The construct was verified by PCR and sequencing with primers targeting the T7 RNA polymerase promoter and terminator.

Production and purification. pET-28a/VIM-31-expressing *E. coli* was grown overnight at 37°C with shaking in 50 ml LB medium supplemented with 50 μ g/ml kanamycin. The bacterial suspension was diluted 100-fold in a total of 2 liters of Terrific Broth supplemented with kanamycin (50 μ g/ml), and the expression of VIM-31 was induced with isopropyl- β -D-thiogalactopyranoside (final concentration, 1 mM) when the culture reached an A_{600} of 0.6. The induced culture was incubated overnight at 28°C with shaking.

VIM-31 was purified by ion-exchange chromatography and gel filtration. Briefly, the bacterial suspension was pelleted, resuspended in 60 ml of buffer A (50 mM HEPES, pH 7.5, 50 μ M ZnCl₂), disrupted by sonication, and cleared by ultracentrifugation. The supernatant was then dialyzed against buffer A and loaded onto a Q-Sepharose FF column equilibrated with the same buffer. The proteins were eluted with a linear NaCl gradient (0 to 1 M). Fractions were analyzed by SDS-PAGE and by the ability to hydrolyze nitrocefin. Those fractions containing β -lactamase activity were pooled and dialyzed against buffer A overnight at 4°C. This partially purified enzyme was loaded onto a MonoQ column (GE Healthcare, Diegem, Belgium) equilibrated with the same buffer. The proteins were eluted with a linear gradient of NaCl (0 to 0.5 M). The fractions containing β -lactamase activity were pooled and concentrated to a volume of 2 ml. Size-exclusion chromatography was performed on a Hiload 16/60 Superdex 75 preparation-grade column (GE Healthcare, Diegem, Belgium) equilibrated with buffer A supplemented with 250 mM NaCl. Finally, fractions containing the highest β -lactamase activities were pooled and subsequently dialyzed overnight at 4°C against buffer A. The VIM-31 enzyme was >95% pure as judged by SDS-PAGE (data not shown).

Determination of kinetic parameters. All experiments were performed at 30°C in buffer A in the presence of 50 μ M Zn²⁺ (22). Imipenem and ertapenem were obtained from Merck Sharp and Dohme Research Laboratories (Rahway, NJ). Ampicillin, benzylpenicillin, piperacillin,

cephalothin, cefoxitin, cefotaxime, ceftazidime, cefepime, meropenem, and aztreonam were obtained from Sigma-Aldrich (Steinheim, Germany). Nitrocefin was a gift from ProGenosis (Liège, Belgium). Hydrolysis of the antibiotics was monitored by following the absorbance variation resulting from the opening of the β -lactam ring, using a Uvikon 943 spectrophotometer equipped with thermostatically controlled cells. The wavelengths and extinction coefficients used were those previously reported (21, 33) for all drugs tested except for ertapenem (36). The k_{cat} and K_m parameters were determined under initial rate conditions, using the Hanes linearization of the Henri-Michaelis-Menten equation.

Homology model. A homology model of VIM-31 was constructed using the EsysPred3D server (www.fundp.ac.be/urbm/bioinfo/essypred), based on the structure of reduced VIM-2 (Protein Data Bank [PDB] accession number 1KO3).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been assigned to the EMBL/GenBank nucleotide sequence database under accession number JN982330. VIM numbering was assigned according to the method of the Lahey clinic.

RESULTS AND DISCUSSION

Clinical isolate, susceptibility profile, and patient outcome. In June 2011, an 87-year-old female underwent a left hemicolectomy for intestinal adenocarcinoma. Her immediate postoperative status was complicated by the occurrence of a deep surgical site infection, for which she received a 2-week course of antimicrobial treatment, first comprising piperacillin-tazobactam and then comprising meropenem. Six days after the end therapy, she developed a peritonitis caused by a carbapenem-resistant *Enterobacter cloacae* isolate (*E. cloacae* 11236) as suspected by Vitek2 (meropenem MIC of >16 mg/liter and imipenem MIC of 4 mg/liter [data not shown]).

Etest MIC determination (Table 1) confirmed that *E. cloacae* 11236 was intermediately susceptible to imipenem (MIC = 3 μ g/ml) and resistant to ertapenem (MIC = 6 μ g/ml), but it was susceptible to meropenem (MIC = 0.5 μ g/ml) and also to aztreonam, tigecycline, amikacin, and ciprofloxacin. The presence of a metallo- β -lactamase in this isolate was suspected by a positive double-disc synergy test with imipenem and EDTA.

The patient gradually improved following a 9-day course of treatment with ciprofloxacin (400 mg twice daily [BID] intravenously for 3 days, followed by 500 mg BID orally for 6 days), but she remained persistently colonized with *E. cloacae* 11236 in the urine for several weeks and returned home.

β -Lactamase genes, genetic support transfer, and typing. Analysis of β -lactamase-encoding genes detected the presence of *bla*_{VIM} in addition to *bla*_{CTX-M} of group 9. PCR amplification and sequencing of the *bla*_{VIM}-bearing integron (In669 [GenBank accession no. JN982330]; the integron number was assigned by INTEGRALL, the integron database [29]) revealed the presence of a new *bla*_{VIM-31} allele located upstream of the *aacA4* allele. The *bla*_{VIM}/*aacA4* structure of In669 was found to match almost perfectly with a VIM-2-harboring integron described for an *Acinetobacter* sp. isolate in Korea (GenBank accession no. EF125009), except for three single nucleotide polymorphisms (one upstream and two within *bla*_{VIM-2}), as well as with the first 2 cassettes of In71 in *P. aeruginosa* TS-832347 (GenBank accession no. AM180753) (20), recovered in Italy. *bla*_{VIM-31} differs from *bla*_{VIM-2} by two single nucleotide polymorphisms, resulting in Tyr224His and His252Arg mutations according to the class B standard numbering of Garau et al. (14). Additional PCR amplification and sequencing revealed the presence of *bla*_{CTX-M-9} and *qnrA1* in *E. clo-*

TABLE 1 Etest or microdilution MICs of *E. cloacae* 11236, *E. coli* J53 (ECJ53) and *E. coli* BL21 (ECBL21) acceptor strains, a transconjugant, and VIM-2- and VIM-31-expressing clones

	MIC ^a (μg/ml)	<i>E. coli</i>				
Antibiotic	<i>Enterobacter cloacae</i> 11236 ^b	J53	J53/p11236 ^c	BL21 ^d	BL21/pET-28a/VIM-31	BL21/pET-28a/VIM-2
Ticarcillin	>256	1.5	>256	1.5	>256	>256
Ticarcillin-clavulanate	>256	1.5	>256	1.5	>256	>256
Temocillin	192	4	256	3	32	64
Piperacillin	96	0.75	32	0.75	12	12
Piperacillin-tazobactam	24	1.5	16	0.75	8	16
Ceftazidime	4	0.125	1.5	0.064	0.5	2
Cefepime	2	0.064	0.5	0.064	0.19	0.19
Aztreonam	1	0.032	0.032	0.0016	0.016	0.016
Imipenem	3	0.19	1	0.25	0.75	1
Meropenem	0.5	0.032	0.125	0.023	0.064	0.125
Ertapenem	6	0.032	0.38	0.064	0.125	0.19
Doripenem	0.5	0.016	0.094	0.032	0.094	0.125
Amikacin	≤4	≤4	≤4	NA	NA	NA
Gentamicin	4	≤1	2	NA	NA	NA
Tobramycin	8	≤1	4	NA	NA	NA
Ciprofloxacin	0.5	≤0.25	≤0.25	NA	NA	NA
Tigecycline	≤0.25	≤0.25	≤0.25	NA	NA	NA
Colistin	≤0.25	≤0.25	≤0.25	NA	NA	NA

^a MICs were evaluated by Etest for ticarcillin to doripenem and by microdilution for amikacin to colistin. NA, not analyzed.^b Strain isolated from blood culture and harboring *bla*_{VIM-31}, *bla*_{CTX-M-9}, *aacA4'*, and *qnrA1*.^c The plasmid p11236 (42 kb) harbored *bla*_{VIM-31} and *aacA4'* but not *bla*_{CTX-M-9} and *qnrA1*.^d The MICs observed for *E. coli* BL21 were identical to those for the strain transformed with the original (without VIM) pET28a vector. The selection marker for pET28a was kanamycin.

aca 11236. Plasmid extracts showed the presence of three plasmids, of ca. 290, 136, and 42 kb, with the latter (p11236) being a *bla*_{VIM-31}-harboring nontypeable plasmid (8) which could be transferred by electroporation into *E. coli* TOP10 (not shown) and also by mating experiments into *E. coli* J53 (Table 1). Despite several attempts, p11236 could not be transferred to *P. aeruginosa* PAO1 or PU21, suggesting that it was not self-replicative in *P. aeruginosa*. Molecular detection of the resistance genes in the *E. coli* transformant (*E. coli* TOP10/p11236) and transconjugant (*E. coli* J53/p11236) confirmed that *bla*_{VIM-31} was not located on the

same plasmid as the *qnrA1* and *bla*_{CTX-M-9} genes (data not shown). The VIM-31-expressing *E. coli* J53 transconjugant displayed lower MICs, especially for cephalosporins, monobactam, and carbapenems (Table 1). Regarding carbapenems, this suggests that reduced outer membrane permeability could also contribute to decreased susceptibility or to resistance to carbapenems in this clinical isolate, as already demonstrated (23). According to PFGE analysis, *E. cloacae* 11236 corresponds to a sporadic isolate and does not cluster with any of the major CTX-M-9-producing *E. cloacae* epidemic clones identified previously in Belgian hospitals (35; data not

TABLE 2 Kinetic parameters of VIM-31 and VIM-2 enzymes

Antibiotic	VIM-31 parameters ^a			VIM-2 parameters ^b			Ratio of k_{cat}/K_m for VIM-2 to k_{cat}/K_m for VIM-31
	k_{cat} (s ⁻¹)	K_m (μM)	k_{cat}/K_m (mM ⁻¹ s ⁻¹)	k_{cat} (s ⁻¹)	K_m (μM)	k_{cat}/K_m (mM ⁻¹ s ⁻¹)	
Benzylpenicillin	108	270	400	280	70	4,000	10
Ampicillin	36	40	900	125	90	1,400	1.6
Piperacillin	66	72	920	300	125	2,400	2.6
Cefotaxime	62	32	1,900	70	12	5,800	3
Ceftazidime	2.2	490	4.5	3.6	72	50	11
Cefepime	8.5	470	18	>40	>400	100	5.6
Cefoxitin	1.6	74	22	15	13	1,200	54
Cephalothin	88	20	4,400	130	11	12,000	2.7
Nitrocefin	152	35	4,350	770	18	43,000	9.9
Imipenem	12	5	2,400	34	9	3,800	1.6
Meropenem	0.9	3	300	5	2	2,500	8.3
Ertapenem	0.6	9	67	0.2	9	22	0.33
Aztreonam	<0.01	>1,000	<0.01	<0.01	>1,000	<0.01	

^a Data are the means for three independent experiments. Standard deviations were within 10% of the means.^b VIM-2 values were reported by Docquier et al. (12), except for those for ertapenem (36). Experimental conditions are identical to those used in the study of Docquier et al. Regarding ertapenem, the conditions used by Samuelsen et al. were slightly different (50 mM sodium cacodylate [pH 7.0], 100 μM ZnCl₂ for VIM-2) but did not involve major discrepancies in hydrolysis efficiency.

shown), suggesting the independent acquisition of VIM-31 by *E. cloacae* 11236.

VIM-expressing clones and kinetics. MICs of VIM-31- and VIM-2/pET-28a-expressing *E. coli* BL21 (Table 1) for various antibiotics were not significantly different from each other, with the single exception of ceftazidime (MIC = 2 µg/ml for *E. coli* BL21/pET-28a/VIM-2 and MIC = 0.5 µg/ml for *E. coli* BL21/pET-28a/VIM-31).

Under the experimental conditions employed, purified VIM-31 (calculated pI = 5.00; molecular mass = 25,069 Da) hydrolyzed all compounds tested except for aztreonam. The individual kinetic parameters (k_{cat} and K_m) of VIM-31 with several β -lactam substrates and a comparison with those of VIM-2 are reported in Table 2.

The highest catalytic efficiencies for the VIM-31 enzyme were observed for cefotaxime, cephalothin, nitrocefin, and imipenem, while ceftazidime, cefepime, and ceftioxitin were rather poor substrates. With carbapenems, the k_{cat}/K_m ratios ranged from 67 mM⁻¹ s⁻¹ (ertapenem) to 2,400 mM⁻¹ s⁻¹ (imipenem), resulting from a combination of low K_m values and very low turnover rates, a characteristic behavior of the VIM-type enzymes. Of the three carbapenems tested, VIM-31 showed greater hydrolytic efficiency against imipenem (Table 2), a result also reported for VIM-2 by Docquier et al. (12) and Samuelsen et al. (36).

VIM-2 had a higher catalytic efficiency than its His224-possessing VIM-31 variant for all substrates tested, with the exception of ertapenem, with the ratio of k_{cat}/K_m for VIM-2 to k_{cat}/K_m for VIM-31 ranging from 1.6 (ampicillin and imipenem) to 54 (ceftioxitin) (Table 2). For each individual substrate, the k_{cat} values determined for VIM-31 were lower than those for VIM-2 (only 1.1-fold lower for cefotaxime but up to 9-fold lower for ceftioxitin). On the other hand, the K_m values for VIM-31 were increased compared to those for VIM-2, with the exception of those for ampicillin, imipenem, and meropenem (12). VIM-31 was slightly more efficient than VIM-2 for the hydrolysis of ertapenem, with the k_{cat} value determined for VIM-31 being 3-fold higher than that obtained for VIM-2 by Samuelsen et al. (36) and with their K_m values remaining similar.

Positions of the mutations. A homology model of VIM-31 (Fig. 1) was constructed based on the structure of VIM-2 (PDB accession no. 1KO3). The two single mutations (Tyr224His and His252Arg) observed between VIM-31 and VIM-2 are both situated in the C-terminal part of the protein. The Tyr224His substitution, also present in VIM-1, VIM-4 (22), VIM-7 (7, 36), and VIM-12 (19), is located close to the active site and is situated on the L3 loop (Fig. 1). Tyr224 in VIM-2 was previously suggested to enhance the binding affinity of certain substrates, such as ceftazidime (12, 15), for which VIM-2 exhibits a lower K_m value than that for VIM-1 (His224) (the K_m values were 800 and 72 µM for VIM-1 and VIM-2, respectively). In line with these findings, VIM-2 also exhibits higher affinity than its His224-possessing variant for this substrate (the K_m value of VIM-31 is increased 7-fold compared to that of VIM-2). The His252Arg substitution is situated on the α 4 helix. This residue is distant from the active site of the VIM enzyme (more than 22 Å away), and its side chain points outside the protein surface, toward the external solvent. This renders unlikely any interaction of Arg252 with catalytically important residues of the protein or with the substrates of the enzyme. Nevertheless, we cannot totally exclude that the His252Arg mutation may also contribute to tuning the

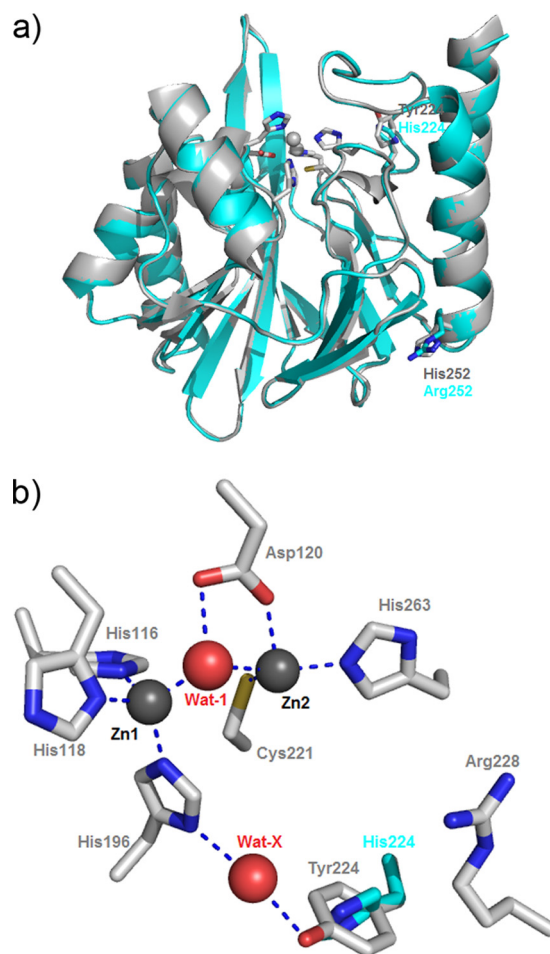


FIG 1 (a) Superposition of VIM-2 and the homology model constructed for VIM-31. Positions showing variation between VIM-2 and VIM-31 (224 and 252) are labeled in gray for VIM-2 and in cyan for VIM-31. (b) VIM-2 active site. Zinc ions and water molecules are shown as gray and red spheres, respectively. Residues are numbered according to the BBL standard numbering scheme (14). Metal-ligand and hydrogen bonds are shown as dashed blue lines. The side chain of His224 in the VIM-31 homology model is shown with carbon atoms rendered in cyan. This figure was rendered using Pymol.

metallo- β -lactamase activity. Indeed, observations made previously for VIM-11 and VIM-19 indicate that amino acid substitutions located outside the active site (Asn165Ser compared to the VIM-2 sequence and Asn215Lys compared to the VIM-1 sequence, respectively) may also modulate the hydrolytic properties of the enzyme (26, 34).

Conclusions. We have characterized VIM-31, a new VIM variant closely related to VIM-2 that was detected in a clinical isolate of *E. cloacae* in Belgium and located on a new integron, In669.

Comparison of the kinetic parameters of VIM-31 to those previously obtained by other groups for VIM-2 (12, 36) revealed that VIM-31 exhibits globally lower catalytic efficiencies than those of VIM-2 (because of lower k_{cat} and higher K_m values). This could be explained mostly by the single Tyr224His point mutation.

From an epidemiological point of view, the low level of resistance of *E. cloacae* 11236 to carbapenem drugs underscores the difficulties that may be associated with the detection of such resistance mechanisms in clinical practice.

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