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Short communication

Novel class 1 Integrons and sequence types in VIM-2 and VIM-11-producing clinical strains of *Enterobacter cloacae*

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ABSTRACT

All VIM-producing *Enterobacteriaceae* (six *Enterobacter cloacae*) submitted to the Argentinian Reference Laboratory in Antimicrobial Resistance in the period 2008–13 were characterized. The isolates were referred from 6 nosocomial institutions located in 5 different cities across the country. All isolates showed carbapenem disk diffusion inhibition zones ≤22 mm and synergism between a carbapenem disk and EDTA/SMA. The six isolates were PCR positive for bla_{VIM}. Imipenem MICs were ≤1 to 8 μg/ml. Typing by PFGE and MLST distinguished six pulsotypes and sequence types with bla_{VIM} located on novel class 1 integron arrays: ECL-1: ST182, In*883*; ECL-2, ST90, In*885;* ECL-3, ST88, In*346* with bla_{VIM-11}; ECL-4, ST184, In*900*; ECL-5, ST749-new, In*900*; ECL-6, ST*91* and uncharacterized In. Only ECL-2 was able to transfer bla_{VIM-2} to *E. coli* J53 by biparental conjugation. bla_{VIM} was located in plasmids of 53–82 Kb and in the chromosome (ECL-1 and ECL-5). The diversity of clones, class 1 integrons, plasmids and location of bla_{VIM}, reveals the plasticity of the genetic elements described and highlights the importance of surveillance programs as tools to identify the transmission of these highly resistant metallo-β-lactamase-producing *Enterobacteriaceae*.

1. Introduction

The prevalent metallo-β-lactamases (MBLs) reported to date are the IMP, VIM and NDM variants, which have been described from numerous geographic locations. Among the VIM enzymes, VIM-2 appears to be the dominant allelic variant, mostly reported in *Pseudomonas aeruginosa* but also frequent in *Enterobacteriaceae* with a broad geographical distribution (Cornaglia et al., 2011). VIM-11, in contrast, has been very infrequent worldwide and reported in *P. aeruginosa* from Argentina (2005) (Pasteran et al., 2005), India (2009) (Castanheira et al., 2009), Malaysia (2010) (Khosravi et al., 2010) and Mexico (2012) (Castillo-Vera et al., 2012) and in *Acinetobacter baumannii* from Taiwan (2010) (Huang et al., 2008). VIM-2 and VIM-11 are both able to hydrolyze penicillins, cephalosporins, carbapenems but not aztreonam, although VIM-11 has greater hydrolytic efficiency on imipenem (Marchiaro et al., 2008).

In Argentina, metallo enzymes of the VIM type are more frequently found in *Pseudomonas* than in *Enterobacteriaceae* (Pasteran et al., 2005, Marchiaro et al., 2010, Gomez et al., 2011). The first occurrence of MBL-producing enterobacteria in Argentina was in 2008 (Gomez et al., 2011). In this study we characterized six MBL-producing *Enterobacter cloacae* isolates submitted to the National Reference Laboratory in Antimicrobial Resistance in the period 2008–13.

2. Materials and methods

2.1. Clinical isolates and susceptibility testing

The epidemiological data of the patients was summarized in Table 1. MBL production was suspected in all isolates that exhibited: (i) imipenem inhibition zones ≤22 mm or a Vitek 2C MIC of imipenem ≥2 μg/ml plus a meropenem MIC ≥1 μg/ml, and (ii) a positive synergy test result between carbapenems and EDTA disks (Gomez et al., 2011). All isolates showed positive modified Hodge test (MHT) results (CLSI criteria). The antibiotic susceptibility profile of the isolates was determined by disk diffusion and MICs were obtained by VITEK-2 (bioMérieux, Marcy L'Étoile, France). Aztreonam, carbapenem and cephalosporin susceptibility was confirmed by the agar dilution method (CLSI criteria). Extended-spectrum β-lactamase (ESBL) production was screened by double-disk synergy testing with ceftazidime or cefepime

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Table 1
Epidemiological data of VIM-producing isolates.

Strain no.	Age (gender)	Hospital (province)	Isolation site (date of isolation)	Underlying disease	LOS (days)	ATB treatment[a] (days of MBL-targeted treatment)	Diagnosis	Outcome
ECL-1	61 (f)	H1-CHACO	Foot abscess (June 3rd, 2011)	Diabetes type I	10	CIP (15 d), CLI[b]	Infection of diabetic foot	ND
ECL-2	80 (f)	H2-CABA	Rectal (colonization) (Jan 1st, 2012)	Hypertension, obesity	62	AMS, CLA[b], LVX, PTZ, VAN[b]	ND	Improved and discharged
ECL-3	84 (f)	H3-CABA	Abdominal drainage (Nov. 1st, 2011)	Colon cancer	ND	CRO, MET, PTZ, VAN[b], COL + IMP (3 d), COL + AMS (14 d)	Purulent peritonitis	Improved
ECL-4	62 (m)	H4-SALTA	Urine (May 5th, 2012)	Bladder stones, cholecystitis	>50	COL (10 d)	Intraabdominal infection	Reserved prognosis
ECL-5	17 days (f)	H5-JUJUY	Blood (Jan 1st, 2013)	Pre-term baby with renal malformation and bacteremia	375	AMP, GEN, PTZ (7 d)	Bacteremia	Dead
ECL-6	59 (f)	H6-NEUQUEN	Urine (June 6th, 2013)	Hemorrhagic Stroke	140	IMP + COL; TMS, VAN; PZT; TMS + PZT; VAN + IMP; PZT-COL-FLUCO-AKN (12 d)	Urinary tract infection	Dead

f, female; m, male; CABA, Capital district; CIP, ciprofloxacin; CLI, clindamycin; CRO, ceftriaxone; MET, metronidazole; PTZ piperacillin/tazobactam; VAN, vancomycin; COL, colistin, IMP, imipenem, AMS, ampicillin/sulbactam; AMP, ampicillin; GEN, gentamicin; LVX, levofloxacin; CLA, clarithromycin; FLUCO, fluconazole. ND, not-determined. LOS, length of stay; ATB, antibiotic.

[a] ATB treatment during hospital stay;

[b] Antibiotics used to treat Gram positive bacteria.

and amoxicillin-clavulanic acid (distance between disks, 20 mm, centre to centre). Disk diffusion and MIC results were interpreted according to the breakpoints established by the criteria set forth by CLSI 2014 (Table 2A, M100-S24).

2.2. Genotyping analysis

PFGE was done with total DNA digested with *Xba*I. PFGE was done in a Chef-DR® III System (Bio-Rad, Hercules CA, USA) with conditions and analysis criteria previously reported (Gomez et al., 2011). Multilocus sequences typing (MLST) was performed following the instructions described in the *E. cloacae* MLST website (http://pubmlst.org/ecloacae/). The allelic number and sequence types (STs) were assigned using the *E. cloacae* MLST website, available at http://pubmlst.org/ecloacae. The clustering of related STs (defined as clonal complexes, CCs) was analysed using eBURST software (http://eburst.mlst.net). A CC is defined as a set of similar ST(s) having six identical loci among seven, therefore a CC is formed by the founder ST and its single locus variants (SLV).

2.3. Detection of resistance genes and characterization of class 1 integrons

The detection of MBLs (bla_{VIM}, bla_{IMP} and bla_{NDM}), locally prevalent ESBLs (bla_{PER}, $bla_{CTX\text{-}M}$, bla_{SHV}) and plasmid mediated quinolone resistance (PMQR) genes was carried out by PCR using standard conditions (Gomez et al., 2011). Since most bla_{VIM} genes reported are located in class 1 integrons (Cornaglia et al., 2011), we performed PCRs combining 5′CS and 3′CS primers with VIM-R or VIM-F primers respectively (Gomez et al., 2011). PCR products were analysed by Sanger sequencing in an ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence analysis and alignments were performed using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and compared with sequences deposited in the GenBank database (www.ncbi.nlm.nih.gov). Integron numbers were assigned by INTEGRALL (Moura et al., 2009).

2.4. Conjugative assays, location of bla_{VIM} genes and plasmid typing

The agar mating method was used to investigate transferability of resistance determinants from donor clinical isolates to the sodium azide-resistant *Escherichia coli* J53 recipient (Gomez et al., 2011). Donor and recipient strains were mixed in a 1:3 ratio and incubated overnight at 35 °C. Transconjugants were selected on Luria-Bertani agar plates containing ampicillin (50 μg/ml) plus sodium azide (200 μg/ml). The plasmid profile of all isolates and transconjugants was studied by S1 nuclease (Promega, Southampton, UK) digestion as described elsewhere (Barton et al., 1995). Briefly, agarose plugs containing total DNA were digested with S1 nuclease (0.5 U/plug, 45 min at 37 °C). Digested plugs were loaded and subjected to PFGE. Each band was considered a unit length linear plasmid. Plasmids were blotted onto a positively charged nylon membrane (Bio-Rad) and hybridized with a bla_{VIM} probe generated with PCR DIG DNA labelling and detection Kit (Roche Applied Sciences, Mannheim, Germany). A chromosomal location was investigated by using the endonuclease I-*Ceu*I (New England Biolabs, Beverly, MA) (Liu et al., 1993). I-Ceu-I-generated fragments were hybridized with a 16S rRNA probe and then with a bla_{VIM} probe. Plasmid replicons were identified using the PCR-based replicon typing method and were classified according to their incompatibility (Inc) group as described previously (Garcia-Fernandez and Carattoli, 2010).

2.5. Nucleotide accession numbers

The sequences of In883, In885 and In900 have been deposited in GenBank (accession numbers **KJ668593**, **KJ668592**, and **KJ668594**, respectively).

3. Results and discussion

3.1. Epidemiological and clinical features

In the present work, we studied six *E. cloacae* isolates that were referred as carbapenem resistant to the NRLAR between October 2008 and June 2013. The isolates were recovered from six patients: five adults with an age range of 59–84 years old and a newborn. Three patients cursed with a severe underlying disease or condition, three of them had prolonged hospital stay (>10 days) and multiple antibiotic treatments (Table 1). The isolates were detected in five provinces: three from the north of Argentina (Chaco, Salta and Jujuy), two from Buenos Aires City (CABA) and one from the south (Neuquén) (Table 1).

3.2. Susceptibility testing

Susceptibility to carbapenems and relevant antimicrobial agents were summarized in Table 2. Five isolates were non-susceptible to imipenem meanwhile all isolates were susceptible to meropenem. Most isolates were non-susceptible to piperacillin/tazobactam, ceftazidime and ciprofloxacin. Susceptibility to trimethoprim/sulfamethoxazole

Table 2
Antimicrobial susceptibility and molecular characterization of bla_{VIM}-harboring *E. cloacae isolates*.

Antibiotic	ECL-1		ECL-2		TC-ECL-2		ECL-3		ECL-4		ECL-5		ECL-6		Az^R *E. coli* J53	
	MIC(μg/ml)	Int.	MIC(μg/ml)	Int.	MIC (μg/ml)	Int.	MIC(μg/ml)	Int.	MIC(μg/ml)	Int.	MIC(μg/ml)	Int.	MIC(μg/ml)	Int.	MIC(μg/ml)	Int.
Piperacillin/tazobactam	≥128	R	32	I	16	S	≥128	R	≥128	R	≥128	R	≥128	R	ND	–
Cefepime	≤1	S[b]	≤1	S[b]	≤1	S[b]	8	S[b]	16	I[b]	≤1	S[b]	2	S[b]	0.015	S
Ceftazidime	16	R	16	R	≤1	I[b]	≥64	R	≥64	R	≥64	R	≥64	R	0.06	S
Imipenem	8	R	2	I	≤1	S	≤1	S	4	R	2	I	2	I	0.06	S
Meropenem	1	S[b]	≤0.25	S	≤0.25	S	1	S[b]	1	S[b]	0.5	S	1	S[b]	0.015	S
Gentamicin	4	S	2	S	≤1	S	≤1	S	≥16	R	≥16	R	≥16	R	1	S
Amikacin	≥64	R	≤2	S	≤2	S	≤2	S	≥64	R	16	S	≤2	S	1	S
Ciprofloxacin	≥4	R	≥4	R	≤0.25	S	≥4	R	≥4	R	≤0.25	S	≥4	R	0.015	S
Trimethoprim/sulfamethoxazole	≤1	S	≥16	R	≤1	S	≤1	S	≥16	R	≥16	R	≥16	R	ND	–
Aztreonam[a]	0.12	S	4	S	≤0.25	S	128	R	128	R	64	R	64	R	≤0.03	S
ST	182		90		–		88		184		749		91		–	
bla genes	bla_{VIM-2}		bla_{VIM-2}		bla_{VIM-2}		bla_{VIM-11}		bla_{VIM-2}		bla_{VIM-2}, bla_{PER-2}		bla_{VIM-2}		ND	
Location of bla_{VIM}	*ch*		*pl*		*pl*		*pl*		*pl*		*ch*, *pl*		*pl*		ND	
Plasmid size(s) Kb	<48; 100; 160		53; 73; 325		53		82; 154		61; 75; 367		50; 79; 325		55; 100		ND	
Rep content by PCR	NT		NT		NT		NT		HI2		HI2		A/C		ND	
In N°	In*883*		In*885*		In*885*		In*346*		In*900*		In*900*		ND		ND	
Class *1* Integron array	bla_{VIM-2}-*aacA4*-*aacA38*-bla_{OXA-2}-*gcuD3*		bla_{VIM-2}-*aacA4'*-*aadA5h*		bla_{VIM-2}-*aacA4'*-*aadA5h*		bla_{VIM-11}-*aacA35*		bla_{VIM-2}-*aadA7e*		bla_{VIM-2}-*aadA7e*		bla_{VIM-2}-*aacA53Δ*		ND	

ECL, *Enterobacter cloacae;* Int., interpretation; R: resistant; S, susceptible; I, intermediate.
ND, not determined. In, integron; Rep., Replicon; ST, sequence type. TC-ECL-2, transconjugant of ECL-2; ch, chromosome; pl, plasmid. Plasmid size numbers in bold indicate Southern hybridization with the bla_{VIM} probe.
[a] MIC of aztreonam was determined by the agar dilution method.
[b] Categorized as I by the Advanced Expert System of VITEK 2; MICs were interpreted according to CLSI 2013 guidelines.

and aminoglycosides was variable, while most isolates were susceptible to cefepime. Four isolates were resistant to aztreonam but only ECL-5 showed positive synergy between aztreonam and amoxicillin/clavulanic acid disk, suggesting the presence of an ESBL (see below Section 3.5).

3.3. Genes detected

All isolates were PCR positive for bla_{VIM}-type genes and negative for the other β-lactamases tested with the exception of the aztreonam resistant ECL-5, which was positive also for bla_{PER-2}, as well as for *qnrB* and *aac*(6′)*Ib*-cr (Table 2). The other aztreonam-resistant strains were negative for the ESBL genes tested but showed negative synergy between 3rd generation cephalosporins and clavulanic acid, probably owing this profile to the chromosomal cephalosporinase of the *Enterobacter* species.

3.4. PFGE and MLST analysis of VIM-producing E. cloacae isolates

PFGE was performed on all *E. cloacae* isolates studied and the band pattern observed revealed no clonal relatedness between isolates (Supplementary Fig. 1). These results are in agreement with the absence of data about VIM-2-producing *Enterobacteriaceae* causing outbreaks. Indeed, all reported outbreaks of bla_{VIM-2} have been caused by *P. aeruginosa* (Jeannot et al., 2013) or other alleles like VIM-16 in *Serratia marscecens* (Nastro et al., 2013). Six STs were identified: ST88, ST749, ST184, ST182, ST90 and ST91 (Fig. 1). Based on eBURST analysis, there were 2 singletons, ST88 previously reported in China (Liu et al., n.d.) and ST749, which is a new sequence type not previously reported. As observed in Fig. 1, ST90 and ST91 are SLV between them and part of a cluster of 6 members. ST182 belongs to a cluster of 5 members and consequently clonal complex 182 (CC182) (Fig. 1). ST184 is part of the CC234 (Fig. 1). Initially, CC234 was defined by Izdebski and collaborators (Izdebski et al., 2015) but the entry of new STs to the database broadened this group.

3.5. Plasmid analysis and conjugation results

To determine whether bla_{VIM} containing plasmids can disseminate horizontally, the isolates were subjected to conjugation studies. Only ECL-2 rendered transconjugants that tested positive for bla_{VIM-2} in a non-typeable 53 Kb plasmid (Table 2). This results suggest that the plasmids present in the rest of the isolates might not be conjugative or mobile as reported by others (Porres-Osante et al., 2014), at least under the experimental conditions tested here. S1 nuclease digestion showed that all isolates harboured multiple plasmids of sizes between 50 and 370 Kb (Table 2, Supplementary Fig. 2). Specific hybridization bands were seen in four isolates and ECL-2 transconjugant confirming that the bla_{VIM} gene was located in plasmids of 53–82 Kb. In addition, we investigated the chromosomal location of bla_{VIM} in all the strains (Supplementary Fig. 3). Co-hybridization bands with 16S rDNA and bla_{VIM} probes were observed in ECL-1 and ECL-5 demonstrating the chromosomal location of bla_{VIM} in ECL-1 and the double location, plasmid and chromosome of bla_{VIM} in ECL-5 (Table 2, Supplementary Fig. 3). Plasmid analysis showed that three of the isolates were non-typeable (ECL-1, ECL-2 and ECL-3); (Table 2) but we detected HI2 replicon in ECL-4 and ECL-5 and A/C replicon in ECL-6. However, it was not possible to associate those plasmids to bla_{VIM}.

3.6. Genetic environment of bla_{VIM}

PCR mapping and sequencing analysis showed that all isolates harboured bla_{VIM} as the first cassette of class 1 integrons (Table 2). Five isolates harboured bla_{VIM-2} and one bla_{VIM-11}. VIM-2 is the common allele found in Argentina, generally detected in *P. aeruginosa,* but VIM-11 has also been detected previously (Pasteran et al., 2005). The second

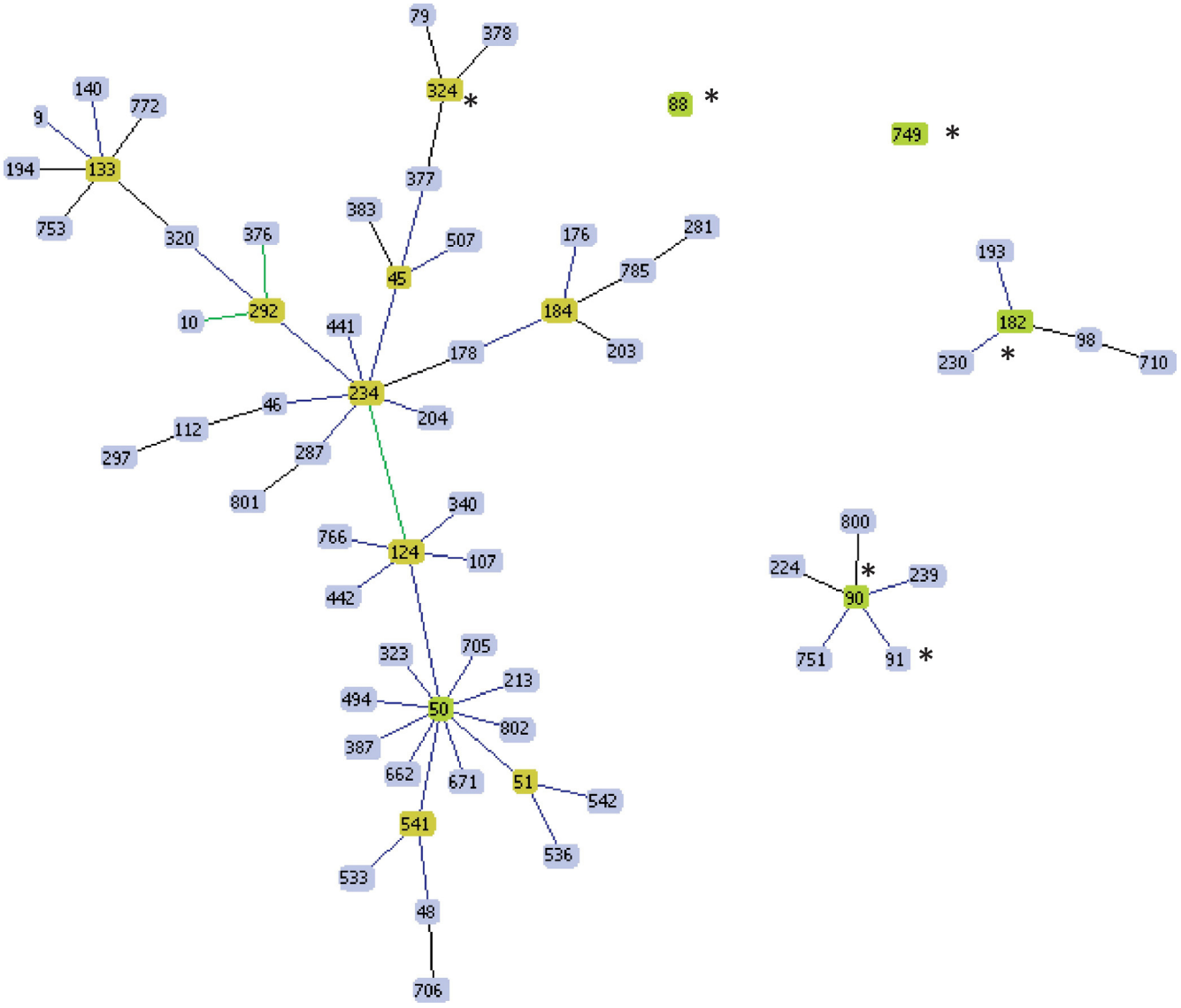

Fig. 1. Population structure of the six VIM-producing *E. cloacae* isolates, shown in the context of the STs present in the global MLST database (http://pubmlst.org/ecloacae/, as of 7th Dec. 2016). The scheme was constructed using eBURST analysis as a close-in view of all the STs present in the database that are part of the clonal complex (cc) or single locus variant (SLV) of the STs found in this study (marked with asterisk). SLVs are linked by continuous lines.

and third cassettes found were genes encoding aminoglycoside modifying enzymes. Interestingly, we found new integron cassette arrays and novel gene cassettes (Table 2). The isolate from Chaco, ECL-1, harboured bla_{VIM-2} in In*883* which has two novel gene cassettes: *aacA38* and *gcuDD3*, a third truncated version of the *gcuD* gene of unknown function. *aacA38* gene has never been detected in a functional gene cassette because the one reported previously (GenBank **EF382672**) has a truncated attC site. The isolate ECL-3 from Buenos Aires City, harboured bla_{VIM-11} in In*346* (GenBank **AJ628983**). To the best of our knowledge, this is the first report of bla_{VIM-11} in *Enterobacteriaceae*. The second isolate form Buenos Aires City, ECL-2, harboured the integron In*885* with *aac(6′)-IId* (GenBank **AF453998**) with the novel cassette *aadA5h* (GenBank **AY139600**), which is an allele of *aadA5* (GenBank **AF170088**). ECL-4 from Salta and ECL-5 from Jujuy, harboured bla_{VIM-2} in In*900*, followed by *aadA7e* (Table 2). Finally, ECL-6 from Neuquén harboured bla_{VIM-2} followed by the truncated gene *aacA53*Δ (95% identity with aacA44, GenBank **JQ407409.1**). The mapping studies performed on this isolate gave a unique band when amplifying with Class I integron conserved segment primers (5′-CS and 3′-CS) alone or in combination with VIM primers. Alternative strategies, e.g. amplifying with *sul1*, *qacE* and *tni* were negative, suggesting the presence of alterations in the 3´end sequence of this integron. For these reasons, this isolate is still under study.

4. Conclusions

In summary, our work showed that VIM-2 and VIM-11 enzymes were detected in genetically unrelated *E. cloacae* clinical isolates from Argentina. These resistance determinants were found in novel class 1 integrons with distinct gene cassette composition and in a variety of plasmid sizes and location (chromosome and/or plasmid). The epidemiological data reported here suggest that the dissemination of VIM in Argentina is not linked to a particular epidemic clone and that the occurrence is still low. In this context, the burden of carbapenem-resistant *Enterobacteriaceae* due to the broad spread of KPC-producing strains is significant. Therefore, the emergence of VIM and other metallo enzymes has the potential to deepen the already existent problem. The implementation of recommended infection control policies to prevent the transmission of these organisms should be a priority for institutions where these organisms are detected.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meegid.2017.07.019.

Transparency declarations

None to declare.

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