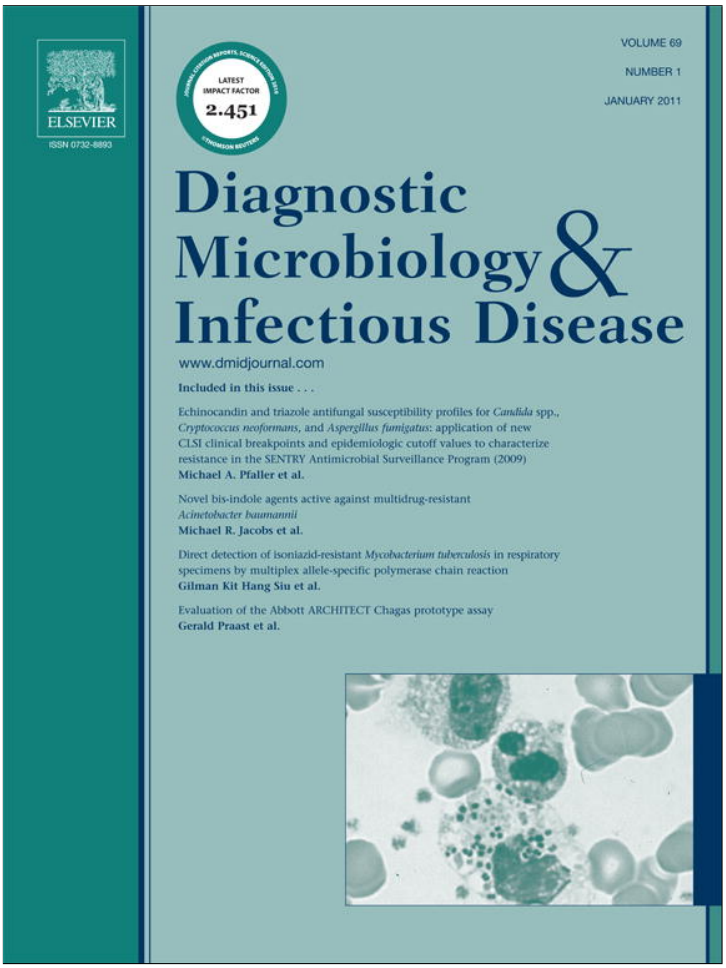

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Emergence of Metallo-β-lactamases in *Enterobacteriaceae* from Argentina☆

Sonia Gomez[a], Melina Rapoport[a], Ana Togneri[b], José Viegas-Caetano[c], Diego Faccone[a], Alejandra Corso[a], Alejandro Petroni[a], Fernando Pasteran[a,*]

[a]*Serv. Antimicrobianos, Instituto Nacional de Enfermedades Infecciosas (INEI)-ANLIS "Dr. Carlos G. Malbrán", CABA, Argentina*
[b]*Hospital Interzonal General de Agudos Evita, Lanús, Bs. As., Argentina*
[c]*Hospital Interzonal General de Agudos San Martín, La Plata, Bs. As., Argentina*

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Abstract

Carbapenem susceptibility in *Enterobacteriaceae* (M9921, M9959) revealed the presence of MBLs bla_{VIM-2} (M9959) and bla_{IMP-8} (M9921), both as first cassettes of class-1-integrons. ESBL bla_{PER-2} was detected in both strains and M9921 also harboured *qnrB10, aac(6′)-Ib* and *aac(6′)-Ib*-cr. This is the first report of MBLs in *Enterobacteriaceae* from Argentina.

Keywords: Metallo-β-lactamases; Extended-spectrum-β-lactamases; Plasmid-mediated-quinolone-resistance; *Enterobacteriaceae*; Multi-resistance

1. Introduction

Carbapenems are the drug of last resort in the treatment of infections caused by multidrug-resistant *Enterobacteriaceae* clinical isolates. Carbapenem resistance in the hospital setting is mainly attributed to the production of Ambler class B acquired metallo-β-lactamases (MBLs) of the IMP and VIM types. These enzymes posses great clinical significance because they hydrolyze all β-lactams except monobactams (Carattoli, 2009). MBL genes can be chromosome or plasmid borne, and are often located in integrons as gene cassettes. Transmissible MBLs were first described in *Pseudomonas aeruginosa* in Asia in the 1980s, but they soon appeared in *Enterobacteriaceae* causing nosocomial infections and outbreaks (Walsh et al., 2005).

MBLs history in Argentina involved only *Pseudomonas* species mainly with VIM-producing strains (Pagniez et al., 2006; Pasteran et al., 2005). Since 2005, gram-negative bacilli are routinely being screened for MBL production (synergism between EDTA/SMA and imipenem and meropenem disks) by WHONET-Argentina Network members (69 hospitals) (Lee et al., 2003). From 184.222 enterobacterial isolates tested to date, 6 were suspected of MBL production, but only 2 were confirmed by the National Reference Laboratory (INEI). This is the first report in Argentina of MBLs found in *Enterobacteriaceae;* furthermore, these strains harbor extended spectrum β-lactamases (ESBL) and plasmid mediated quinolone resistance mechanisms.

In 2008, *Providencia rettgeri* M9959 was identified in Hospital San Martín, La Plata, Buenos Aires province, recovered from the urine of a 81-year-old woman. The patient had been hospitalized with lumbar fracture, deep venous thrombosis, pulmonary embolism and shock due to uro-sepsis, and was being treated successfully with piperacillin/tazobactam (no more data was available). The same year, *Enterobacter cloacae* M9921 was identified in Hospital Evita, Lanus, Buenos Aires province. This pathogen was isolated from the blood sample of a 81-year-old woman with hypertension, mitral valve prolapse and Chagas disease but no hospitalization in the previous six months. She was admitted to the hospital on August 11th with hip-bone fracture. Therefore she remained hospitalized waiting for hip-bone replacement, and received medication and hydration protocol with parenteral catheter. On August

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* Corresponding author. Tel.: +54-11-4301-9346; fax: +54-11-4301-9346.
E-mail address: fpasteran@anlis.gov.ar (F. Pasteran).

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16th the patient developed fever, hypotension and sepsis, receiving ceftazidime as an empiric treatment (presumptive pneumonia). Blood samples were positive for *Enterobacter cloacae* M9921 phenotypically resistant to expanded spectrum cephalosporins (ESC) and possible carbapenemase production. Upon phenotypic confirmation of MBL, the treatment was rotated to intravenous piperacillin/tazobactam considering the lack of therapeutic options for M9921. The hospital implemented contact isolation protocols and surveillance swabs were taken but resulted negative. After completion of the antibiotic treatment and negative blood samples the patient underwent hip-bone surgery and was discharged from the hospital in October 4th, 2008.

In both M9959 and M9921, microbiological assays confirmed carbapenemase activity (Masuda et al., 1976). The minimal inhibitory concentration (MIC) was determined by agar dilution according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2009). EDTA reduced the carbapenems MICs by at least three dilutions in both strains, suggesting the presence of MBLs (Table 1) (CLSI, 2009). *E. cloacae* M9921 was resistant to expanded spectrum cefalosporins with and without inhibitors such as clavulanate, possibly due to the hyperproduction of chromosome-encoded AmpC besides the MBL. Moreover, this strain was susceptible to ertapenem, imipenem and meropenem by the CLSI old breakpoints (susceptible: ertapenem ≤2 μg/ml, imipenem and meropenem ≤4 μg/ml) (CLSI, 2009). However, the use of the new breakpoins for carbapenems released in 2010 by CLSI changes the interpretation for M9921 as ertapenem resistant (susceptible: ertapenem ≤0.25 μg/ml) although it continued to be characterized as susceptible to imipenem and meropenem (≤1 μg/ml) (CLSI, 2010). Additionally, *E. cloacae* M9921 showed intermediate susceptibility to ciprofloxacin and reduced susceptibility to nalidixic acid and levofloxacin. *P. rettgeri* M9959 was also resistant to ESC and clavulanate reduced ESC MICs by more than three dilutions (Table 1).

Table 1
Antimicrobial drug susceptibility [MIC (μg/ml)], isoelectric focusing of β-lactamases, PCR and sequencing of antimicrobial resistance determinants, transcojugants (TC) and recipient strain *(E. coli* J53 resistant to sodium azide)

	Original Isolates		Acceptor	*E. cloacae* transconjugants (M9921)				
	P. rettgeri E.cloacae		*E. coli*	*E. coli*				
Strain number	M9959	M9921	J53	TC1	TC2	TC3	TC4	TC5
Antibiotics				MICs (μg/ml)				
Imipenem	256	1	0.06	1	1	4	0.125	0.125
Imipenem/EDTA[a]	4	0.125	ND[b]	0.06	0.06	0.125	0.06	0.06
Meropenem	256	1	ND	0.25	1	2	0.008	0.008
Meropenem/EDTA[a]	16	0.125	ND	0.008	0.008	0.008	0.008	0.008
Ertapenem	256	2	ND	0.25	0.5	2	0.015	0.015
Cefotaxime	256	128	0.06	64	32	128	16	64
Cefotaxime/CLA[c]	64	128	ND	32	32	128	0.015	0.015
Ceftazidime	>1024	1024	0.06	512	512	512	256	512
Ceftazidime/CLA[c]	32	1024	0.06	512	512	256	0.03	0.03
Cefepime	256	64	0.01	8	16	64	4	16
Cefepime/CLA[c]	8	32	ND	4	16	32	0.008	0.008
Aztreonam	1024	128	0.06	256	0.125	0.25	128	128
Nalidixic Acid	>128	16	4	16	16	2	16	16
Ciprofloxacin	8	2	0.01	2	0.25	0.03	1	0.25
Levofloxacin	2	0.5	ND	0.25	0.25	0.03	0.25	0.5
Chloramphenicol	>128	>128	4	>128	4	4	>128	4
	Isoelectric focusing results							
pI Band[d]	5.0+5.4+9.0	5.4+7.5+8.5		8.5+5.4	8.5+5.4	8.5	5.4	5.4
PCR results Gene detected								
bla_{VIM-2}	+	-	ND	ND	ND	ND	ND	ND
bla_{IMP}	-	+	ND	+	+	+	-	-
bla_{PER}	+	+	ND	+	+	-	+	+
qnrB10	ND	+	ND	+	+	-	+	+
aac(6′)-Ib	ND	+	ND	+	+	-	+	+
aac(6′)-Ib-cr	ND	+	ND	+	-	-	+	-

[a] EDTA 0.4 mmol/L.
[b] ND not determined.
[c] CLA: clavulanic acid (0.4 μg/ml).
[d] pI isoelectric point.

Isoelectric focusing was performed as already described (Melano et al., 2003). *P. rettgeri* M9959 and *E. cloacae* M9921 produced β-lactamases bands that correlated with VIM (pI = 5.0) or IMP (pI = 8.5) respectively, in addition to PER (pI = 5.4) (Table 1). The isoelectric focusing bands at pI 5.0 (M9959) and 8.5 (M9921) showed MBL activity by previous EDTA (5 mM) in situ-inhibition (Melano et al., 2003). The remnant bands suggest the presence of AmpC type enzymes.

PCR studies were performed using specific primers and DNA sequencing was done on both strands by the method of Sanger *et al.* (Sanger et al., 1977) using the BigDye terminators methodology (Applied Biosystems/Perkin Elmer, Foster City, CA). The sequences were analyzed in an ABI 3500 Genetic Analyzer (Applied Biosystems). Sequencing results confirmed the presence of $bla_{\text{IMP-8}}$ and $bla_{\text{VIM-2}}$ in *E. cloacae* M9921 and *P. rettgeri* M9959 respectively and $bla_{\text{PER-2}}$ was detected in both strains. In addition, PCR and DNA sequencing of amplicons confirmed the presence of *qnrB10, aac(6′)-Ib,* and *aac(6′)-Ib-cr* in M9921 (Table 1).

Biparental conjugation was performed as described (Melano et al., 2003) with *E. coli* J53 (sodium azide resistant) as the recipient strain, and selecting transconjugants with sodium azide (200 μg/ml) and ampicillin (50 μg/ml). *P. rettgeri* M9959 did not render transconjugant strains using different experimental conditions. In order to see if $bla_{\text{VIM-2}}$ could be located on a non-transferable plasmid, DNA plasmid extraction was performed three times and using two commercial kits (Concert™, Rapid Plasmid Miniprep System, GibcoBRL, UK, and Wizard Plus SV Minipreps DNA Purification System, Promega, WI). DNA bands were not observed. Moreover, plasmid extractions were used to electroporate *E. coli* JM109 competent cells but electrotransformants were not obtained under ceftazidime (5 μg/ml) selection. These results strongly suggest that $bla_{\text{VIM-2}}$ is chromosomally located in *P. rettgeri* M9959. Interestingly, *E. cloacae* M9921 rendered five phenotypes of transconjugants when transferred to *E. coli* J53 (Table 1). Phenotypic (disc diffusion and MIC) and PCR results demonstrated that $bla_{\text{IMP-8}}$ was not associated to $bla_{\text{PER-2}}$, *qnrB10, aac(6′)-Ib* or *aac(6′)-Ib-cr* (Table 1). Notably, $bla_{\text{PER-2}}$ was co-transferred with *qnrB10* and *aac(6′)-Ib* in all cases, which suggests a possible linkage between them (Table 1). Additionally, the genetic determinants involved in resistance to ciprofloxacin in *E. cloacae* M9921 were completely transferred to *E. coli* TC1 and TC4, and partially to TC2 and TC5 (ciprofloxacin MICs of 1-2 μg/ml versus 0.25 μg/ml respectively), corroborating the presence of at least two plasmid mediated quinolone resistance mechanisms in the clinical isolate *E. cloacae* M9921 (Table 1).

The genetic contexts of MBL genes were studied by PCR cartography and DNA sequencing. *P. rettgeri* M9959 harboured $bla_{\text{VIM-2}}$-*aadA*1 cassette array in a class-1-integron identical to that previously described in *P. aeruginosa* (GenBank DQ522235, unpublished). *E. cloacae* M9921 harboured the $bla_{\text{IMP-8}}$ gene as the first cassette of a class-1-integron. However, we could not characterize the 3′ flanking region of $bla_{\text{IMP-8}}$. Diverse PCR strategies were used to characterize this region (48th Interscience Conference on Antimicrobial Agents and Chemotherapy, abstract C1-1062) but neither the 3′CS of a typical class-1-integron nor the *tni* module of *Tn402* were found associated with $bla_{\text{IMP-8}}$.

This is the first report of MBLs in *Enterobacteriaceae* from Argentina. In recent years, our laboratory reported the emergence and dissemination of *P. aeruginosa* carrying VIM, IMP and SPM (Pasteran et al., 2005), (IV Congreso SADEBAC 2006, Sociedad Argentina de Bacteriología Clínica, abstract 15531). Soon after, we reported the appearance of MBLs in *P. putida* (49th Interscience Conference on Antimicrobial Agents and Chemotherapy, abstract C2636/96). Only two additional reports of MBLs in *Enterobacteriaceae* from South America appeared in 2005 in Brazil (Sader et al., 2005) and 2008 in Venezuela (Marcano et al., 2008). Our work further documents the increasing frequency of detection of MBLs in gram-negative strains in this region.

Although the combinations of *qnrB*-type, *aac(6′)-Ib-cr*, ESBL and MBL have been described (Ode et al., 2009; Wu et al., 2007), the combination of these four resistance mechanisms in the same clinical strain, as we found in *E. cloacae* M9921, has never been described in Latin America.

References

Carattoli A (2009) Resistance plasmid families in *Enterobacteriaceae*. *Antimicrob Agents Chemother* 53:2227–2238.

Clinical and Laboratory Standards Institute (CLSI) (2009). Performance Standards for Antimicrobial Susceptibility Testing: 19th informational supplement. Wayne, PA. M100-S19.

Clinical and Laboratory Standards Institute (CLSI) (2010). Performance Standards for Antimicrobial Susceptibility Testing; 20th Informational Supplement (update). Wayne, PA. M100-S20-U Vol.30 No. 15.

Lee K, Lim YS, Yong D, Yum JH, Chong Y (2003) Evaluation of the Hodge test and the imipenem-EDTA double-disk synergy test for differentiating metallo-beta-lactamase-producing isolates of *Pseudomonas* spp. and *Acinetobacter* spp. *J Clin Microbiol* 41:4623–4629.

Marcano D, Pasteran F, Rapoport M, Faccone D, Ugarte C, Salgado N, Payares D, Spadola E, Lopez Y, Maggi G, Galas M, Sanchez D (2008) First isolation of a VIM-producing *Klebsiella pneumoniae* from a seven-year-old child in Venezuela. *J Infect Dev Ctries* 2:241–244.

Masuda G, Tomioka S, Hasegawa M (1976) Detection of β-lactamase production in gram-negative bacteria. *J Antibiot (Tokio)* 29:662–664.

Melano R, Corso A, Petroni A, Centron D, Orman B, Pereyra A, Moreno N, Galas M (2003) Multiple antibiotic-resistance mechanisms including a novel combination of extended-spectrum beta-lactamases in a *Klebsiella pneumoniae* clinical strain isolated in Argentina. *J Antimicrob Chemother* 52:36–42.

Ode T, Saito R, Kumita W, Sato K, Okugawa S, Moriya K, Koike K, Okamura N (2009) Analysis of plasmid-mediated multidrug resistance in *Escherichia coli* and *Klebsiella oxytoca* isolates from clinical specimens in Japan. *Int J Antimicrob Agents* 34:347–350.

Pagniez G, Radice M, Cuirolo A, Rodriguez O, Rodriguez H, Vay C, Famiglietti A, Gutkind G (2006) [Prevalence of metallo-beta-lactamase in carbapenem resistant *Pseudomonas aeruginosa* at a university hospital of Buenos Aires City]. *Rev Argent Microbiol* 38:33–37.

Pasteran F, Faccone D, Petroni A, Rapoport M, Galas M, Vazquez M, Procopio A (2005) Novel variant bla_{VIM-11} of the metallo-{beta}-lactamase bla_{VIM} family in a GES-1 extended-spectrum-{beta}-lactamase-producing *Pseudomonas aeruginosa* clinical isolate in Argentina. *Antimicrob Agents Chemother* 49:474–475.

Sader HS, Castanheira M, Mendes RE, Toleman M, Walsh TR, Jones RN (2005) Dissemination and diversity of metallo-beta-lactamases in Latin America: report from the SENTRY Antimicrobial Surveillance Program. *Int J Antimicrob Agents* 25:57–61.

Sanger S, Micklen S, Coulson A (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 74:5463–5467.

Walsh TR, Toleman MA, Poirel L, Nordmann P (2005) Metallo-beta-lactamases: the quiet before the storm? *Clin Microbiol Rev* 18:306–325.

Wu JJ, Ko WC, Tsai SH, Yan JJ (2007) Prevalence of plasmid-mediated quinolone resistance determinants QnrA, QnrB, and QnrS among clinical isolates of *Enterobacter cloacae* in a Taiwanese hospital. *Antimicrob Agents Chemother* 51:1223–1227.