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ARTÍCULO ORIGINAL

ß-lactamases produced by amoxicillin-clavulanate-resistant enterobacteria isolated in Buenos Aires, Argentina: A new *bla*$_{TEM}$ gene

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Abstract
Resistance to β-lactam/β-lactamase inhibitors in enterobacteria is a growing problem that has not been intensively studied in Argentina.
In the present work, 54/843 enterobacteria collected in a teaching hospital of Buenos Aires city were ampicillin-sulbactam-resistant isolates remaining susceptible to second- and third-generation cephalosporins. The enzymatic mechanisms present in the isolates, which were also amoxicillin-clavulanic acid (AMC)-resistant (18/54) were herein analyzed. Sequencing revealed two different variants of *bla*$_{TEM-1}$, being *bla*$_{TEM-1b}$ the most frequently detected allele (10 *Escherichia coli*, 3 *Klebsiella pneumoniae*, 2 *Proteus mirabilis* and 1 *Raoultella terrigena*) followed by *bla*$_{TEM-1a}$ (1 *K. pneumoniae*). Amoxicillin-clavulanate resistance seems to be mainly associated with TEM-1 overproduction (mostly in *E. coli*) or co-expressed with OXA-2-like and/or SHV β-lactamases (*K. pneumoniae* and *P. mirabilis*).
A new *bla*$_{TEM}$ variant (TEM-163) was described in an *E. coli* strain having an AMC MIC value of 16/8 μg/ml. TEM-163 contains Arg$_{275}$Gln and His$_{289}$Leu amino acid substitutions. On the basis of the high specific activity and low IC$_{50}$ for clavulanic acid observed, the resistance pattern seems to be due to overproduction of the new variant of broad spectrum β-lactamase rather than to an inhibitor-resistant TEM (IRT)-like behavior.

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PALABRAS CLAVE
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TEM-163;
Enterobacteria;
Hiperproducción de TEM-1

ß-lactamasas producidas por enterobacterias resistentes a amoxicilina-ácido clavulánico aisladas en Buenos Aires, Argentina: un nuevo gen bla_{TEM}

Resumen
La resistencia a la combinación de β-lactámico/inhibidor de β-lactamasa en enterobacterias es un problema creciente que no ha sido estudiado intensamente en Argentina.
En el presente trabajo, 54/843 enterobacterias recolectadas en un hospital universitario de la ciudad de Buenos Aires fueron resistentes a ampicilina-sulbactama, pero se mantuvieron sensibles a las cefalosporinas de segunda y tercera generación. Se analizaron los mecanismos enzimáticos presentes en los aislamientos que también fueron resistentes a amoxicilina-ácido clavulánico (AMC) (18/54).
La secuenciación reveló dos variantes diferentes de bla_{TEM-1}, donde bla_{TEM-1b} es el alelo más frecuentemente detectado (10 *Escherichia coli*, 3 *Klebsiella pneumoniae*, 2 *Proteus mirabilis* y 1 *Raoultella terrigena*), seguidos por bla_{TEM-1a} (1 *K. pneumoniae*). La resistencia a AMC parece estar asociada principalmente con la hiperproducción de TEM-1 (sobre todo en *E. coli*) o con la coexpresión con β-lactamasas tipo OXA-2 y/o SHV (*K. pneumoniae* y *P. mirabilis*).
Se describió una nueva variante de bla_{TEM} (TEM-163) en un aislamiento de *E. coli* que presentó una CIM frente a AMC de 16/8 μg/ml. La enzima TEM-163 contiene dos sustituciones de aminoácidos respecto de TEM-1, $Arg_{275}Gln$ y $His_{289}Leu$. Teniendo en cuenta la alta actividad específica observada y la baja IC_{50} para el ácido clavulánico, el patrón de resistencia de este aislamiento parece obedecer a la hiperproducción de la nueva variante de la β-lactamasa de amplio espectro, en lugar de vincularse con un comportamiento similar al de una TEM resistente a inhibidores (IRT).

Introduction

Amoxicillin-clavulanate (AMC) is one of the most frequently prescribed antibiotic combinations in many countries, especially in ambulatory patients. For this reason, even if it is still a low occurrence event, resistance to β-lactam/β-lactamase inhibitors among enterobacteria clinical isolates is an emerging worldwide problem. Different enzymatic mechanisms are associated with AMC resistance: hyperproduction of chromosome-encoded class C β-lactamases, acquired plasmid-encoded cephalosporinases (AmpC type), hyperproduction of plasmid-mediated class A β-lactamases (such as TEM-1 and SHV-1 enzymes), production of class D oxacillinases and inhibitor-resistant TEM (IRTs or CMTs) and SHV mutants[6,14,19,23] (whose β-lactamase activities are poorly inhibited by clavulanate). In addition to enzymatic mechanisms, a decrease in the expression or absence of outer membrane proteins might also be involved, i.e., a deficiency in the OmpF and/or OmpC production has been associated to this phenotype in *Escherichia coli*[4].

IRTs were frequently described as plasmid-encoded enzymes derived from TEM-1 or TEM-2 β-lactamases and detected mainly from European isolates. Substitutions of one or more amino-acid residues at positions 69, 244, 275 and 276 render structural changes in β-lactamases that affect affinity for inhibitors, but produce only slight modifications in the isoelectric point (pI) or activity on other β-lactam compounds. Other substitutions also found in IRT β-lactamases do not seem to be involved in the IRT phenotype[9].

Overproduction of both TEM-1 and SHV-1, or production of an IRT may increase resistance to amoxycillin, ticarcillin, amoxicillin-clavulanic acid and, frequently to piperacillin and ticarcillin-clavulanic acid, while only slightly affecting susceptibility to narrow spectrum cephalosporins, cephamycins, extended-spectrum cephalosporins, and, in most cases, to piperacillin-tazobactam[3].

Over the last years, an increase in the rate of resistance to AMC has been noted among *E. coli* isolates in our country. According to the "Sistema Informático de Resistencia (SIR)" susceptibility to ampicillin-sulbactam in *E. coli* has been decreasing from 62%[22] (years 2004-2005) to 50% (years 2006-2008; M. Radice, personal communication). In spite of the high use of this association, there is little information available about the prevalence of AMC resistance mechanisms in *Enterobacteriaceae*.

The aim of this study was to investigate the enzymatic mechanisms of amoxicillin-clavulanate-resistant enterobacteria lacking inducible chromosomal *ampC* genes isolated from a teaching hospital of Buenos Aires city.

Materials and methods

Microorganisms

Isolates recovered from different clinical specimens that fulfilled a screening criteria (*Enterobacteriaceae* resistant to ampicillin-sulbactam but remaining susceptible to cefoxitin, ceftazidime and cefotaxime by a disk diffusion method) were studied. Isolates were collected within a 5-month period (May to September 2008) from patients attending the Hospital de Clínicas that belongs to UBA (University of

Buenos Aires) "José de San Martín", Buenos Aires, Argentina.

E. coli ATCC 35218, TEM-1 basal level producer, was used as reference.

Susceptibility testing

Antimicrobial susceptibility testing was performed using the disk diffusion method and the agar dilution method as described by the Clinical Laboratory Standard Institute (CLSI). Results were interpreted according to the CLSI guidelines[2]. Tested antibiotics were ampicillin (AMP), ampicillin-sulbactam (AMS), Amoxicillin-clavulanic acid (AMC), piperacillin (PIP), piperacillin/tazobactam (PTZ), cephalothin (CTN), cefoxitin (FOX), ceftriaxone (CRO), ceftazidime (CAZ), cefepime (FEP), imipenem (IMP), meropenem (MER), ertapenem (ERT), amikacin (AMI), gentamicin (GEN), polymyxin B (POL), trimethoprim-sulfamethoxazole (TMS), ciprofloxacin (CIP), nitrofurantoin (NIT). AMS and AMC MICs were tested at a 2:1 fixed ratio. Antimicrobial agents tested and reported were obtained from Britania S.A. (disks) and Klonal laboratorios (drugs), Argentina.

ß-lactamase assays

Cells were harvested by centrifugation from overnight LB broth cultures and resuspended in 0.5 ml of sodium phosphate buffer (0.1 M, pH 7). β-lactamases were released by sonication (ten 1-min high-frequency discontinuous bursts on ice at output 4-5 and duty cycle: 50%) using SonicVibra Cell ultrasonic processor (Sonics & Materials Inc., USA).

Isoelectric focusing was performed with crude extracts applied to polyacrylamide gels (pH gradient 3-10) in an LKB 2117-Multiphor II isoelectric focusing tank (LKB Produkter AB, Sweden), as previously described[15]. β-Lactamase activity was revealed by the agar iodometric system using AMP (500 mg/l) as substrate, as previously described[25]. TEM-1 (pI 5.4), SHV-2 (pI 7.6), OXA-2 (pI 7.7) and CTX-M-2 (pI 8.2) producing strains were used as standards and were run simultaneously with the samples.

Semi-quantification of β-lactamase production was determined as described below: total protein concentration in crude enzyme preparations was determined by the Bradford method (Bio-Rad Laboratories, USA), adjusted at the same protein concentration and diluted with 100 mM sodium phosphate buffer (pH 7.0). β-Lactamase activity of each enzymatic extract and decimal dilutions (20 μl) were evaluated using the iodometric method employing AMP as substrate. Clinical isolates that showed β-lactamase activity in dilutions higher than the reference strain were considered enzyme overproducers.

Specific activity was determined in those isolates harboring β-lactamases that focused at pI 5.4. This activity was measured three times per strain by the spectrophotometric assay using nitrocefin (482 nm) as substrate at a final concentration of 100 μM. TEM-1 overproduction was arbitrarily defined when specific activity was at least two-fold higher than *E. coli* ATCC 35218.

Inhibition studies were performed by measuring the residual β-lactamase activity of crude extracts previously preincubated with clavulanic acid (0.1, 1 and 10 μM) for 15 min at room temperature. IC_{50} was defined as the clavulanic acid concentration required to inhibit 50% of the β-lactamase activity.

Detection of bla_{TEM}, bla_{SHV} and bla_{OXA} genes

Molecular detection of bla_{TEM}, bla_{SHV} and bla_{OXA-1}, bla_{OXA-2} and bla_{OXA-10} group was carried out by PCR amplification using specific primers (Table 1). The reaction mixture contained 2 μl of total heat extracted DNA as template, and 1.25 U of T-free DNA polymerase (Inbio Highway, Argentina) was added to a total volume of 50 μl containing 0.2 mM deoxynucleoside triphosphate, 20 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, 50 mM KCl, and 0.5 μM primers. The reaction protocol required an initial step of 5 min at 95°C, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 20 min. *E. coli* ATCC 35218, *K. pneumoniae* ATCC 700603, *Salmonella* Infantis S21[5] and *Enterobacter cloacae* 153[21] strains were used as positive controls.

Sequencing of the bla_{TEM} genes

PCR products were purified using either the GFX™ PCR DNA or Gel Band Purification Kit (Amersham Biosciences, Pisca-

Table 1 PCR primers used to detect β-lactamase genes and the expected amplicon sizes

Oligonucleotide name	Sequence 5´→3´	Expected size (bp)	Reference
TEM-F	TAACCCTGGTAAATGCTTCA	940	This study
TEM-R	CAATCTAAAGTATATATGAG		This study
SHV-GF	ATGCGTTWTDTTCGCCKGTG	861	This study
SHV-GR	TTAGCGTTGCCAGTGCTCGAT		This study
OXA-1F	CCATTATTTGAAGGAACTGA	720	This study
OXA-1R	TAGTGTGTTTAGAATGGTGA		This study
OXA-2A	CCTGCATCGACATTCAAGATA	460	[5]
OXA-2B	CTCAACCCATCCTACCCACCA		[5]
OXA-10F	GCCATGAAAACATTTGCCGC	801	[21]
OXA-10R	GCCACCAATGATGCCCTCAC		[21]

taway, NJ, USA). bla_{TEM} sequences were performed on an ABI 377 DNA sequencer (Perkin Elmer, Applied Biosystems). The nucleotide sequences and their derived amino acid sequences were compared to previously described sequences obtained from the GenBank database and β-lactamase classification in Lahey website (http://www.lahey.org/Studies/), respectively. Sequences were aligned using Vector NTI Suite 9 program (InforMax, Inc.).

Molecular epidemiology

The genetic relationship between the-AMC-resistant *E. coli* isolates was determined by REP-PCR.

Nucleotide accession numbers

The nucleotide sequence of a novel variant of bla_{TEM} gene ($bla_{TEM-163}$) was deposited in the GenBank database under accession number EU815939.

Results

Susceptibility testing

Fifty-four (6.4%) out of a total of 843 documented infections were caused by different *Enterobacteriaceae* that fulfilled the proposed selection criterion. These bacteria, lacking inducible chromosomal AmpC genes, were: 38 *E. coli*, 8 *K. pneumonia*, 5 *P. mirabilis*, 2 *K. oxytoca* and 1 *Raoultella terrigena*, and displayed MIC values of AMS higher than 16/8 μg/ml.

As not all AMS-resistant isolates are also AMC-resistant[16,18]; therefore, AMC susceptibility was first determined by the agar disk diffusion method. Taking this feature into account, 18/54 (33.3%) isolates were finally selected to characterize the β-lactamase/s involved: 7 of them were clearly AMC-resistant with inhibition zone diameters <14 mm (2 *E. coli*, 1 *R. terrigena*, 2 *K. pneumoniae* and 2 *P. mirabilis*), 8 had intermediate susceptibility (all *E. coli*, with inhibition zone diameters between 14-17 mm) and 3 had border line susceptibility (1 *E. coli* and 2 *K. pneumoniae*, inhibition zone = 18 mm).

Resistance profiles were further explored by MIC determination to a large set of antibiotics. Relevant results are shown in Table 2. All of them were sensitive to FOX but PIP and CTN susceptibility was variable. All isolates remained susceptible to PTZ, extended-spectrum cephalosporins (CRO, CAZ, FEP) and carbapenems (IMP, MER, ERT) tested (data not shown). Thus, chromosomal or plasmid AmpC hyper-producing and CMT-producing isolates were ruled out.

Regarding susceptibility to non β-lactam antibiotics, 9/11 *E. coli* were resistant to TMS, 4/11 to CIP and 3/11 to NIT; 4/4 *K. pneumoniae* were resistant to TMS and NIT, 2/4 were resistant or had intermediate susceptibility to GEN and AMK, and 1/4 was resistant to CIP; and finally 2/2 *P. mirabilis* were resistant to GEN and NIT, and 1/2 was resistant to CIP. Except for natural resistance in *P. mirabilis*, all isolates were susceptible to POL.

Table 2 Species, specimens and MIC determinations for eighteen selected isolates

			MIC (μg/ml)				
Strain	Species	Specimen	AMP	AMC	PIP	CTN	FOX
2772	*E. coli*	urine	1024	16/8	32	16	1
3184	*E. coli*	urine	≥1024	32/16	512	64	4
3637	*E. coli*	urine	≥1024	16/8	512	64	4
3313	*E. coli*	urine	≥1024	16/8	256	32	2
41002	*E. coli*	blood	≥1024	32/16	1024	≥64	2
2754	*E. coli*	urine	≥1024	32/16	256	64	2
3317	*E. coli*	urine	≥1024	16/8	128	64	2
40952	*E. coli*	blood	≥1024	16/8	256	8	1
3343	*E. coli*	urine	≥1024	16/8	128	32	2
40479	*E. coli*	blood	≥1024	16/8	128	32	4
2617	*E. coli*	urine	1024	16/8	64	32	4
2574	*R. terrigena*	urine	≥1024	16/8	1024	≥64	4
2875	*K. pneumoniae*	urine	≥1024	16/8	1024	≥64	1
3400	*K. pneumoniae*	urine	1024	16/8	16	8	4
41362	*K. pneumoniae*	blood	512	32/16	16	2	2
41318	*K. pneumoniae*	blood	≥1024	32/16	512	64	2
1130	*P. mirabilis*	bone	≥1024	64/32	32	32	8
1132	*P. mirabilis*	wound	≥1024	64/32	32	64	8

AMP, ampicillin; AMC, amoxicillin-clavulanic acid; PIP, piperacillin; CTN, cephalothin; FOX, cefoxitin.

Resistance mechanisms

All crude extracts showed β-lactamase activity when analyzed by the iodometric assay employing AMP as substrate. Characterization of the β-lactamases revealed that all extracts possessed, at least, an enzyme that focused at pI 5.4 and 7/18 co-produced other enzymes that focused at different pI (Table 3). Ten of eleven *E. coli* isolates only displayed the pI 5.4 enzyme.

All isolates were positive for bla_{TEM} and PCR products were sequenced subsequently. Variants of the bla_{TEM-1} gene were detected, being bla_{TEM-1b} described in 16 isolates. The bla_{TEM-1a} allele was only noted in one *K. pneumoniae* strain (Table 3) and no other variants of bla_{TEM-1} gene were detected. An *E. coli* isolate (strain 3343) produced a new variant of the bla_{TEM} gene (named $bla_{TEM-163}$), which differed from bla_{TEM-1b} in 3 bp (see below for more details).

When tested for other β-lactamase genes by PCR, only one *E. coli*, three *K. pneumoniae* and two *P. mirabilis* isolates were positive for bla_{OXA-2}-group genes (Table 3). The bla_{SHV} gene was only detected in all *K. pneumoniae* isolates. Not a single isolate rendered a positive amplification for bla_{OXA-1} or bla_{OXA-10} group. These results agreed with pI values previously obtained.

Most isolates that only harbored the bla_{TEM-1} determinant (7 *E. coli* and 1 *R. terrigena)* showed a higher level of TEM-1 production, and could be considered as probable TEM-1 overproducers since they showed an enzyme production 2-12-fold higher than *E. coli* ATCC 35218 (Table 3). The remaining two *E. coli* strains, with intermediate susceptibility to AMC, displayed a basal or slightly increased level of TEM-1, suggesting that other mechanisms would be involved in the susceptibility decrease observed. Results achieved by the spectrophotometer method were coincident with those obtained by the iodometric semi-quantitative assay.

E. coli 3343, in which the new TEM-163 was present, also showed a profile compatible with TEM overproduction (with a specific activity that was 6-fold higher than *E. coli* ATCC 35218). In this case, IC_{50} values for clavulanic acid were similar to the IC_{50}value measured on the reference TEM-1-producing strain (IC_{50} around of 1μM). Because of these characteristics, rather than thinking of TEM-163 as a probable IRT β-lactamase, the AMC-resistance phenotype is likely due to overproduction of this new β-lactamase.

As expected from isolates that were mostly obtained from urine samples with no strong epidemiological link, even those producing only a TEM β-lactamase, the different *E. coli* isolates are assumed as the different clones displayed a dissimilar banding profile by REP-PCR (data not shown).

TEM-163 versus TEM-122

TEM-163-producing *E. coli* was resistant to AMP, AMS (MIC≥32/16 μg/ml), PIP and CTN, of intermediate susceptibility to AMC (MIC=16/8 μg/ml) and susceptible to FOX (Table 2). The $bla_{TEM-163}$ gene presents more than 99% identity

Table 3 Characterization of β-lactamases produced by studied isolates

Strains	β-lactamase production[a]	pI	PCR results			Sequence of bla_{TEM} gene	Phenotype
	X ± SD		bla_{TEM}	bla_{SHV}	bla_{OXA-2}		
2772	2.10 ± 0.06	5.4	+	-	-	bla_{TEM-1b}	TEM-1 overproducer
3184	3.16 ± 0.55	5.4	+	-	-	bla_{TEM-1b}	TEM-1 overproducer
3637	1.81 ± 0.08	5.4	+	-	-	bla_{TEM-1b}	TEM-1 producer
3313	2.50 ± 0.44	5.4	+	-	-	bla_{TEM-1b}	TEM-1 overproducer
41002	4.44 ± 0.53	5.4	+	-	-	bla_{TEM-1b}	TEM-1 overproducer
2754	3.15 ± 0.52	5.4	+	-	-	bla_{TEM-1b}	TEM-1 overproducer
3317	1.27 ± 0.17	5.4	+	-	-	bla_{TEM-1b}	TEM-1 producer
40952	2.88 ± 0.42	5.4	+	-	-	bla_{TEM-1b}	TEM-1 overproducer
3343	6.14 ± 1.42	5.4	+	-	-	$bla_{TEM-163}$	TEM-163 overproducer
40479	3.16 ± 1.61	5.4	+	-	-	bla_{TEM-1b}	TEM-1 overproducer
2617	ND	5.4 - ≈ 7.2	+	-	+	bla_{TEM-1b}	TEM-1 + OXA-2-like
2574	12.02 ± 4.02	5.4	+	-	-	bla_{TEM-1b}	TEM-1 overproducer
2875	ND	5.4 - ≈ 7.2 - >8	+	+	+	bla_{TEM-1b}	TEM-1 + SHV-like + OXA-2-like
3400	ND	5.4 - ≈ 7.2	+	+	-	bla_{TEM-1a}	TEM-1 + SHV-like
41362	ND	5,4 - ≈ 7.6	+	+	+	bla_{TEM-1b}	TEM-1 + SHV-like + OXA-2-like
41318	ND	5.4 - ≈ 7.6	+	+	+	bla_{TEM-1b}	TEM-1 + SHV-like + OXA-2-like
1130	ND	5.4 - ≈ 7.2	+	-	+	bla_{TEM-1b}	TEM-1 + OXA-2-like
1132	ND	5.4 - ≈ 7.2	+	-	+	bla_{TEM-1b}	TEM-1 + OXA-2-like

[a] Expressed as the ratio of specific activity between each strain vs *E. coli* ATCC 35218 (β-lactamase activity average = 121.1 nmol/min.mg). ND: not determined.

with $bla_{TEM-122}$ (previously considered IRT)[10]. $bla_{TEM-122}$ variant (accession number AY307100) could be considered a bla_{TEM-1b}-derived gene that has a G→A transition at base 812, leading to Arg→Gln amino acid change at position 275 (numbering according to Ambler *et al.*[1]). A new mutation (A→T transversion) at base 854 was detected in $bla_{TEM-163}$ producing a His→Leu amino acid substitution at position 289 in the mature protein of TEM-163, which has not been previously reported (Table 4). Distinctiveness of these mutations was verified by sequencing both the coding and non-coding strands from two independent PCR reactions.

Table 4 Nucleotide mutations and amino acid substitutions in *E. coli* isolate encoding new β-lactamase

	Position and character of altered base[a] (amino acid)[b] in the coding region of the bla_{TEM} gene				
bla_{TEM} *gene*	18	228	396	812 (275)	854 (289)
bla_{TEM-1a}	C	C	G	G(Arg)	A (His)
bla_{TEM-1b}	T	T	T	G(Arg)	A(His)
$bla_{TEM-122}$	C	T	T	A(Gln)	A (His)
$bla_{TEM-163}$	C	T	T	A(Gln)	T (Leu)

The sequences are compared to those of bla_{TEM-1a} and bla_{TEM-1b}.
[a] Numbering according to start codon.
[b] Numbering according to Ambler *et al.*[1].

Discussion

We have focused the study on β-lactamases present in different *Enterobacteriaceae* with phenotypic resistance to β-lactam/β-lactamase inhibitors but remaining susceptible to cefoxitin and extended-spectrum cephalosporins. During the 5-month period of our study the overall frequency of ampicillin-sulbactam-resistant isolates (all non-inducible AmpC producing enterobacteria) remained at about 6.4%. Among them, we selected those that also showed a reduced inhibition zone to AMC by the disk diffusion test to describe the enzymatic mechanisms that are poorly known in our country. AMC resistance in *Enterobacteriaceae* is a complex phenomenon with heterogeneous clinical implications.

The overproduction of TEM-like enzymes followed by TEM-1 production in combination with OXA-2-type and/or SHV-type β-lactamases were the main mechanisms identified.

In agreement with literature data, genes encoding TEM-1 were detected in the majority of the isolates examined, being bla_{TEM-1b} the predominant allelic variant (16/18) identified so far, and only one bla_{TEM-1a} harboring strain (*K. pneumonia* 3400) was described. Consistently, other bla_{TEM-1} variants (i.e. bla_{TEM-1c}, bla_{TEM-1d}, bla_{TEM-1e}), rarely detected in enterobacteria[13,17], were not found.

The presumed overproduction of TEM-1 β-lactamase in *E. coli*, which was present in 7/11 isolates with reduced susceptibility to AMC, was the main mechanism responsible for that resistance profile. These data are similar to those detected in *E. coli* by Pérez-Moreno *et al.* in Spain[20], although other researchers have observed a lower proportion[19,24].

At least two mechanisms may lead to TEM overproduction: 1- multiple copies of the bla_{TEM} gene due to the presence of multiple copies of a plasmid per cell have been reported to cause overproduction of β-lactamase or, 2- different promoter regions have been widely reported in various isolates. Even though bla_{TEM-1a} and bla_{TEM-1b} alleles were initially associated with a weak promoter[7] (called *P3*), it is known that stronger promoters could be present (i.e., *Pa*/*Pb*, *P4*, *P5*) upstream of bla_{TEM}-like genes, which would explain the higher levels of production of TEM enzymes[11-13].

Two *E. coli* isolates producing a basal or slightly increased level of TEM-1 as a single resistance mechanism showed reduced susceptibility to AMC and CTN, being the reduced permeability or increased efflux to β-lactam antibiotics the possible resistance mechanisms involved[4,20].

In the present study, OXA-2-type enzymes were mainly associated with *K. pneumoniae* and *P. mirabilis*, while the OXA-1 and OXA-10 groups were not detected. OXA-2-like enzymes, known to be more poorly inhibited by clavulanic acid than class A β-lactamases, accounted for 33% (6/18) of the isolates analyzed, but when *E. coli* determinants were compared with other studies[13,19,20,24], the frequency of OXA-producing *E. coli* remained low (1/11).

Genes encoding SHV-1 and other close related enzymes have been shown to be natural in *K. pneumoniae* chromosome[9]; therefore bla_{SHV} detection in this species is always expectable.

Contribution of IRT β-lactamases to AMC resistance in *Enterobacteriaceae* appear to be different in isolates from diverse countries. In this study, IRT-like enzymes were not detected among isolates having intermediate susceptibility and also among those that were clearly resistant. To the best of our knowledge, the presence of IRT-producing isolates appears to be much less common in the American continent than in Europe[14]. Nevertheless, amplification and direct sequencing of bla_{TEM} has contributed to discover a new enzymatic variant called TEM-163 present in an *E. coli* strain having intermediate susceptibility to AMC (MIC= 16/8 mg/l). The predicted amino acid sequence of the latter contained the same substitution present in TEM-122[10] ($Arg_{275}Gln$), and also provided an extra mutation ($His_{289}Leu$), which has not been previously described.

An *in silico* model of TEM coevolution network and its communities was constructed by Guthrie *et al.*, and the two mutant positions described in this work (275, 289) are included within the inhibitor-resistant β-lactamase community. Interestingly, position 289 is located at the interface between the two communities[8] (extended-spectrum and inhibitor resistance).

The amino acid substitution at position 275 (located at the C-terminal of the α-11 helix) was previously considered important to confer resistance to β-lactamase inhibitors. However, the same substitution ($Arg_{275}Gln$) was involved in TEM-45 (IRT-14), TEM-82 and TEM-83 but these enzymes contained at least a second mutation in residue 69[10,13], which was highly associated with IRT enzymes[3,9].

The absence of a mutation in residue 69 on this new β-lactamase in agreement with its low IC_{50} to clavulanic

acid allow us to speculate that TEM-163 is more clearly a new variant of broad spectrum β-lactamase than an IRT enzyme (IC_{50}>10 μM is considered a probable IRT enzyme[20]). In conclusion, amoxicillin-clavulanate resistance in different species of non-inducible AmpC *Enterobacteriaceae* isolated in a teaching hospital from Buenos Aires seems to be mainly associated with TEM-1 overproduction or TEM-1, OXA-2-like and/or SHV-like β-lactamase co-production.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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