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Original Article

Extended-spectrum β-lactamases, transferable quinolone resistance, and virulotyping in extra-intestinal *E. coli* in Uruguay

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Abstract

Introduction: To characterize extended-spectrum β-lactamases (ESBLs) and plasmid-mediated quinolone resistance (PMQR) genes in *Escherichia coli* isolates obtained from extra-intestinal samples in three Uruguayan hospitals.

Methodology: Fifty-five ESBL-producing *E. coli* isolates were studied. Virulence genes, ESBLs, and PMQR genes were detected by polymerase chain reaction. ESBL-producing isolates were compared by pulsed-field gel electrophoresis. Multi-locus sequence typing was also performed on 13 selected isolates.

Results: Thirty-seven isolates harbored $bla_{\text{CTX-M-15}}$ (67.3%), eight $bla_{\text{CTX-M-2}}$ (14.6%), five $bla_{\text{CTX-M-14}}$ (9.1%), three carried both $bla_{\text{CTX-M-2}}$ and $bla_{\text{CTX-M-14}}$, one $bla_{\text{CTX-M-9}}$, and one $bla_{\text{CTX-M-8}}$. Among the CTX-M-15 producers, 92% belonged to sequence types ST131 and ST405, and carried *aac(6')Ib-cr* as well. Isolates harboring $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-9}}$, or $bla_{\text{CTX-M-8}}$ were found to be genetically unrelated.

Conclusions: The successful dissemination of CTX-M-15-producing *E.coli* isolates seems to be linked to the spreading of high-risk clones and horizontal gene transfer. A trade-off between carrying more antibiotic resistance and less virulence-related genes could partially account for the evolutionary advantages featured by successful clones.

Key words: virulence genes; ESBL; plasmid-mediated quinolone resistance.

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Introduction

Escherichia coli is a versatile pathogen, responsible for intestinal and extra-intestinal infections. Urinary tract infections (UTIs), sepsis, bacteremia, and meningitis are among the latter. The ability of *E. coli* to cause extra intestinal infections relies on a number of virulence factors that include adhesins, tissue-damaging effectors, and factors conferring resistance to the bactericidal activity of serum, among others [1,2].

Identification of extra-intestinal pathogenic *E. coli* strains (ExPEC) can be achieved by detecting at least two of the following genes: *papA*, *papC*, *sfa/foc*, *afa/dra*, *iutA*, or *kpsM II* [3]. The occurrence of these genes is associated with strains belonging to phylogenetic groups B2 and D (associated with ExPEC), and less frequently with groups A and B1, corresponding to commensal *E. coli* strains [4].

The emergence of successful clones such as *E. coli* ST131 or clonal group A (CGA), both distributed worldwide, is a clear example of coexistence between antibiotic multiresistance and virulence factors [5-7].

Co-resistance in *E. coli* to oxyiminocephalosporins (especially mediated by ESBLs) and quinolones is an increasing event worldwide, particularly in Latin America [8]. Yet, the available information concerning

the dissemination of different clones and/or the presence of virulence factors is scarce in South America [9,10].

In Uruguay, the occurrence of *E. coli* strains producing PER-2, CTX-M-15, SHV-5, or CTX-M-2 ESBLs, as well as plasmid-mediated quinolone resistance genes (PMQR) linked to CTX-M-15, has already been reported [11-14]. We recently issued the first report in Latin America of a non-ExPEC strain harboring CTX-M-19 [15]; nevertheless there is no information so far that relates virulence factors in ExPEC, ESBLs, and PMQR among the circulating clones.

In the present work, we describe the ESBLs present in *E. coli* strains obtained from extra-intestinal samples from three hospitals in Uruguay.

Methodology

Bacterial isolates

Fifty-five ESBL-producing extra-intestinal *E. coli* isolates were obtained from three Uruguayan hospitals located in two different cities, between 1 March 2010 and 28 February 2011. Each isolate was designated with letters E, C, or HP according to the hospital of origin. Thirty-six isolates were recovered from urine, seven from blood, five from skin lesions, two from abscesses, three from respiratory samples, and two from peritoneal fluid samples.

Antimicrobial susceptibility and detection of extended-spectrum β-lactamases (ESBLs)

Bacterial identification and antibiotic susceptibility tests were performed using the VITEK2 Compact system (bioMérieux, Marcy l'Étoile, France). Every isolate displaying minimum inhibitory concentration (MIC) values to cefotaxime and/or ceftazidme and/or cepefime > 1 µg/mL underwent ESBL screening and confirmatory tests using ceftazidime and cefotaxime (30 µg) alone and combined with 10 µg of clavulanate, performed by disk diffusion as suggested by Clinical Laboratory Standards Institute (CLSI) guidelines [16]. MIC values to ceftazidime, cefotaxime, and ciprofloxacin were confirmed by Etest (bioMérieux, Marcy l'Étoile, France), following the manufacturer's instructions.

Characterization of ESBL and PMQR genes

Isolates with positive ESBL-screening results were further analyzed by polymerase chain reaction (PCR) for the presence of $bla_{\text{CTX-M}}$, bla_{TEM}, $bla_{\text{PER-2}}$, and bla_{SHV} genes using specific primers [11]; PCR products were sequenced on both strands using the same primers.

The occurrence of genes *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, and *qepA* was also screened by PCR, in isolates with MIC to nalidixic acid > 2 µg/mL [11]. Strains displaying MIC values to amikacin > 2 µg/mL were analyzed for the presence of *aac(6')Ib* and the *cr* variant [11].

Multi-locus sequence typing (MLST), determination of CGA, and PFGE

MLST was performed on selected isolates, representative of the most frequent pulsetypes, by gene amplification and sequencing of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*), according to the protocol and primers specified at the *E. coli* MLST web site (http://mlst.ucc.ie/mlst/dbs/Ecoli).

*Xba*I PFGE analysis was performed as previously described [17]. Additionally, CGA was determined for isolates characterized by MLST and belonging to phylogenetic group D by single-nucleotide polymorphisms (SNPs) analysis of *fumC*, as described by Johnson *et al*. [18]. PFGE profiles were analyzed with BioNumerics fingerprinting software (Applied Maths, St-Martens-Latem, Belgium). Dendrograms were generated using the unweighted pair-group method (UPGMA) using arithmetic averages, based on the Dice similarity coefficient, with a 2.0% band position tolerance. PFGE profiles sharing > 85% similarity were considered to be genetically related [19].

Transfer of ESBL, conjugation assays, and plasmid characterisation

Twenty-five isolates representative of all pulsetypes were selected for conjugation and plasmid characterization. Conjugation assays were carried out using rifampicin-resistant *E. coli* J53-2 strain as recipient. Transconjugants were selected on MacConkey agar plates (Oxoid, Basingstoke, UK) supplemented with rifampicin (150 mg/L) (Sigma-Aldrich St. Louis, USA) and ceftriaxone (1 mg/L) (Sigma-Aldrich St. Louis, USA) [20].

Conjugative plasmid incompatibility groups (Inc) and addiction systems were determined by PCR-based replicon-typing according to Carattoli *et al*. and Mnif *et al*., respectively, using genomic DNA obtained from transconjugants as a template [21,22].

Phylogenetic group and virulotyping

Classification of isolates into phylogenetic groups was determined by PCR as previously described [4], based on the presence or absence of three DNA fragments: *chuA*–, yjaA, and Tspe4.C2. Additionally, the sub-grouping scheme proposed by Branger *et al*. [23] was used. Briefly, the absence of the three DNA fragments corresponds to subgroup A_0, whereas the occurrence of only *yjaA* corresponds to A_1; on the other hand, $B2_2$ corresponds to the occurrence of *chuA* and *yjaA*, and $B2_3$ is defined by the presence of the three DNA fragments. Finally, subgroup D_1 features the presence of only *chuA*, whereas subgroup D_2 features *chuA* and Tspe4.C2.

Detection of virulence-related genes was performed in two stages. First, all isolates underwent PCR virulence screening (VS) as previously described by Johnson *et al* [3].

Next, isolates carrying two or more of *papA*, *papC*, *sfa/foc*, *afa/dra*, *iutA*, or *kpsM II* genes (*i.e.*, VS+) were further examined by multiplex PCR for another 25 virulence-related genes, as described by Johnson *et al*. [1].

Results

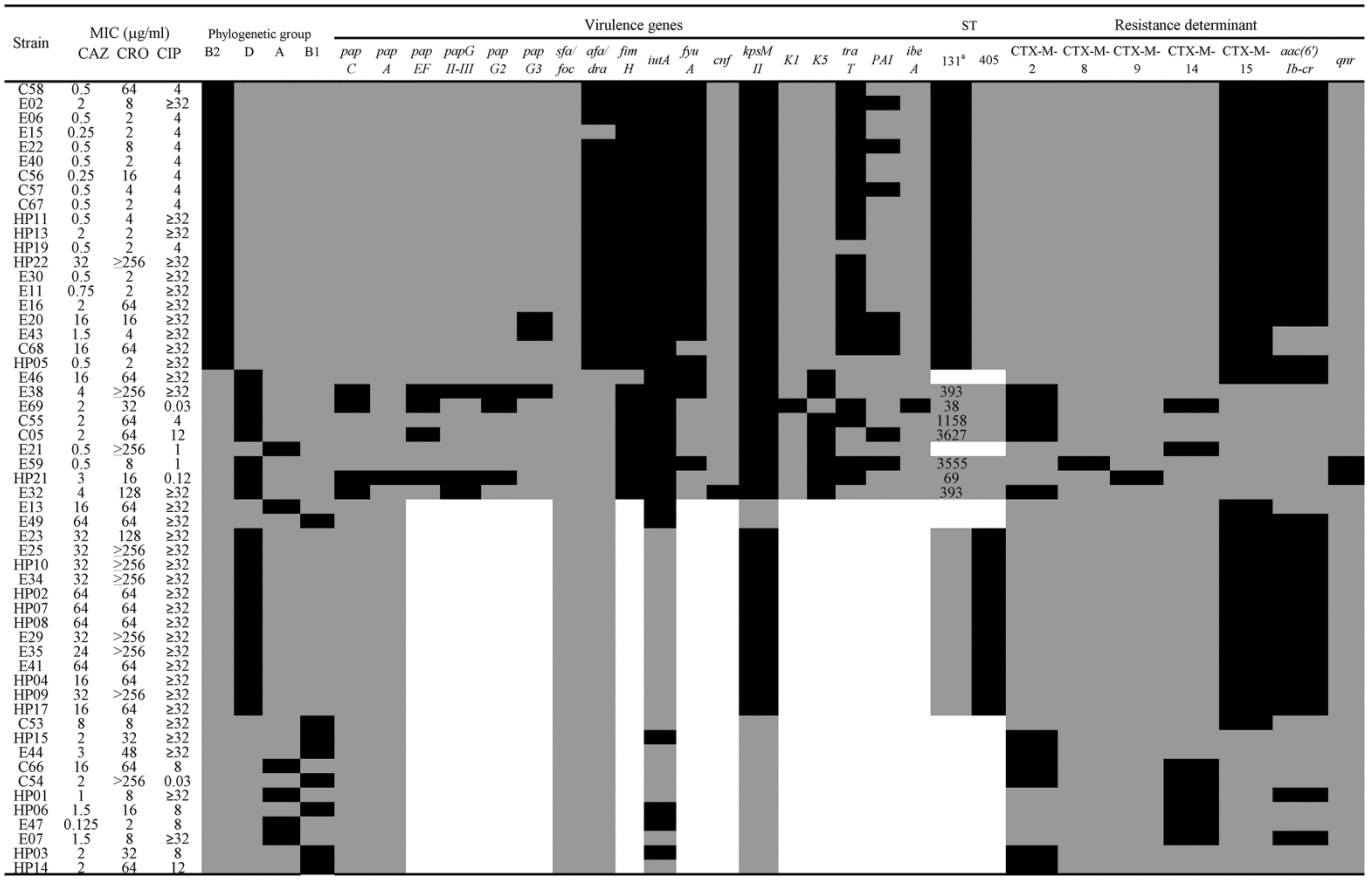
The 55 isolates under study were phenotypically identified as ESBL-producers; however, the proportion of isolates resistant to cefotaxime or to ceftazidime was dissimilar.

According to the CLSI interpretative criteria, 100% of the isolates were resistant to cefotaxime (MIC50 = 64 µg/mL, MIC90 ≥ 256 µg/mL), whereas only 40% (22) were resistant to ceftazidime (MIC50 = 2 µg/mL, MIC90 = 32 µg/mL). On the other hand, 50/55 (90.9%) were resistant to ciprofloxacin (Figure 1, Table 1).

The 55 ESBL-producing isolates were distributed in 12 different resistance patterns; (Table 1) nevertheless, 39/55 (70.9%) were clustered in three major profiles (Table 1).

All of the isolates were susceptible to carbapenems (data not shown).

Figure 1. Main features of 55 *E. coli* strains studied in this work.



Black: positive; **grey**: negative; **white**: not determined. a Sequencetype (ST) other than ST131 and ST405 were depicted in this column.

Detection of ESBLs and transferable quinolone resistance

A total of 58 ESBL genes were detected among the 55 ESBL-producing isolates. Thirty-seven isolates (67.3%) carried $bla_{CTX\text{-}M\text{-}15}$, eight (14.6%) carried $bla_{CTX\text{-}M\text{-}2}$, five (9.1%) carried $bla_{CTX\text{-}M\text{-}14}$, three carried both $bla_{CTX\text{-}M\text{-}2}$ and $bla_{CTX\text{-}M\text{-}14}$ (isolates C54, C66, and E69), one isolate carried $bla_{CTX\text{-}M\text{-}8}$, and another single isolate carried $bla_{CTX\text{-}M\text{-}9}$ (Figure 1). Conversely, no bla_{TEM}, bla_{SHV}, or $bla_{PER\text{-}2}$ ESBL genes were detected.

Fifty of the ESBL-producing isolates were also resistant to ciprofloxacin, 35 of which harbored *aac(6')Ib-cr* (33 carried $bla_{CTX\text{-}M\text{-}15}$ and two carried $bla_{CTX\text{-}M\text{-}14}$). Additionally, *qnrA* was detected in a single isolate, along with $bla_{CTX\text{-}M\text{-}9}$. Furthermore, *qnrB* was detected along with $bla_{CTX\text{-}M\text{-}8}$; however, in both cases, MIC values to ciprofloxacin were within the susceptibility range defined by the CLSI (Figure 1). No *qnrC*, *qnrD*, *qnrS*, or *qepA* genes were detected.

Clonal relationship

PFGE assays were performed on all ESBL-producing isolates; four of them were untypeable (three CTX-M-2 producers and one CTX-M-15 producer).

PFGE analysis of $bla_{CTX\text{-}M\text{-}15}$-bearing isolates indicated that they were clustered in five pulsetypes; two major pulsetypes, named A and B, accounted for 20 and 13 isolates, respectively, whereas pulsetypes C and D comprised two isolates each, and pulsetype E included a single isolate (Figure 2).

On the other hand, isolates carrying $bla_{CTX\text{-}M\text{-}2}$, $bla_{CTX\text{-}M\text{-}14}$, $bla_{CTX\text{-}M\text{-}9}$, and $bla_{CTX\text{-}M\text{-}8}$ belonged to different pulsetypes, named F through O (Figure 2).

MLST analysis showed that all of the isolates in pulsetype A belonged to sequence type ST131, whereas isolates in pulsetype B belonged to ST405.

Isolates belonging to ST131 were detected in the three hospitals included in this study and were recovered from various sources (urine, blood, surgical wound infections, and peritoneal fluid).

Similarly, isolates belonging to ST405 were only recovered in two hospitals (both located in the capital city, Montevideo) from similar sources, including broncheoalveolar lavage, abscesses, and pleural fluid as well.

Table 1. Antibiotics resistance profiles of 55 ESBL producers *E. coli* and distribution of mainly clones.

Profile	Antibiotype	N	PT/ST/ BLEE (n)
1	OAkGNCS	6	B/405/CTX-M-15$^{(4)}$, A/131/CTX-M-15$^{(1)}$, NT/393/CTX-M-2 $^{(1)}$
2	OAkGNC	1	I/393/CTX-M-2$^{(1)}$
3	OAkNCS	12	A/131/CTX-M-15$^{(11)}$, D/ND/CTX-M-15$^{(1)}$
4	OGNCS	12	B/405/CTX-M-15$^{(6)}$, A/131/CTX-M-15$^{(1)}$, K/ND/CTX-M-2$^{(1)}$, LL/ND/CTX-M-2,CTX-M-14$^{(1)}$, NT/ND/CTX-M-2$^{(1)}$, N/ND/CTX-M-14$^{(1)}$, D/ND/CTX-M-14$^{(1)}$
5	OGNC	2	E/ND/CTX-M-15$^{(1)}$, A/131/CTX-M-15$^{(1)}$
6	OGS	1	O/69/CTX-M-9$^{(1)}$
7	ONCS	15	B/405/CTX-M-15$^{(3)}$, A/131/CTX-M-15$^{(6)}$, NT/ND/CTX-M-2$^{(2)}$, C/ND/CTX-M-15$^{(1)}$, I/ND/CTX-M-2$^{(1)}$, G/ND/CTX-M-14$^{(1)}$, H/ND/CTX-M-14$^{(1)}$
8	ONC	2	F/1158/CTX-M-2$^{(1)}$, F/3627/CTX-M-2$^{(1)}$
9	ONS	1	J/ND/CTX-M-14$^{(1)}$
10	ON	1	M/3555/CTX-M-8$^{(1)}$
11	OG	1	C/38/CTX-M-2,CTX-M-14$^{(1)}$
12	O	1	L/ND/CTX-M-2,CTX-M-14$^{(1)}$

O: oxyiminocephalosporins; Ak: amikacin; G: gentamicin; N: nalidixic acid; C: ciprofloxacin; S: sulfamethoxazole-trimethoprim; PT: pulsetype; ST: sequence type; N: number of isolates displaying each resistance profile, (n) number of isolates belonging to each clone within a particular resistance profile.

Table 2. Correlation between MLST and *fumC* SNPs aimed at the detection of CGA in phylogenetic group D *E. coli* isolates

MLST	No. of isolates	*fumC* SNPs for nucleotidic residues		
		270	271	288
ST 38	1	A	T	C
ST 69	1	**A**	**T**	**T**
ST 393	2	G	C	C
ST 405	13	G	C	C
ST 1158	1	A	T	C
ST3555	1	G	C	C
ST3627	1	A	T	C

Characters in boldface correspond to the only sequence type compatible with CGA, according to the SNP profile.

On the other hand, CTX-M-2-producing isolates were typed as ST393 (n = 2), ST38, ST1158, and ST3627, whereas CTX-M-9 and CTX-M-8-producing isolates were found to belong to ST69 and ST3555, respectively.

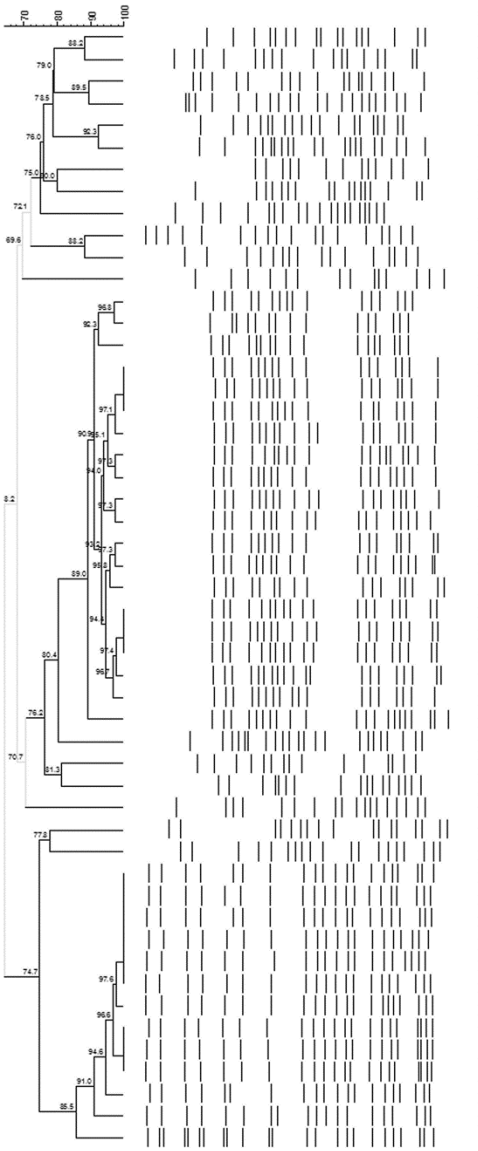
Determination of clonal group A

Phylogenetic group D included 21 isolates, 13 of which belonged to pulsetype B and ST405. Furthermore, another 6 isolates underwent MLST studies. Sequence analyses of *fumC* aimed at the detection of CGA showed that only a single isolate (HP21), belonging to ST69 and carrying $bla_{\text{CTX-M-9}}$/*qnrA*, displayed the SNP C288T (Table 2).

Transfer of ESBL genes and plasmid characterization

Twenty-five isolates representative of each pulsetype, as well as the four untypeable isolates, were selected for conjugation assays: seven isolates harboring $bla_{\text{CTX-M-15}}$, eight $bla_{\text{CTX-M-2}}$, five $bla_{\text{CTX-M-14}}$, three isolates carrying both $bla_{\text{CTX-M-2}}$ and $bla_{\text{CTX-M-14}}$, one carrying $bla_{\text{CTX-M-9}}$, and one with $bla_{\text{CTX-M-8}}$ (Table 3).

Figure 2. PFGE patterns and main features of ESBL-producing ExPEC strains.



Strain	Sample	Pulsotype	ST	ESBL	*PMQR*	FG	Virulence profile
E13	urine	C		CTX-M-15	-	A_1	nd
E69	blood	C	ST38	CTX-M-2/14	-	D_1	*papC,papEF,papGII,fimH,iutA,kpsMII,K1,traT,ibeA*
C05	urine	F	ST3627	CTX-M-2	-	D_1	*papEF,fimH,iutA,kpsMII,K5,* PAI
C55	urine	F	ST1158	CTX-M-2	-	D_1	*fimH,iutA,kpsMII,k5,traT*
E49	urine	D		CTX-M-15	*aac(6')Ib-cr*	A_1	nd
HP06	urine	D		CTX-M-14	-	A_1	nd
E47	urine	G		CTX-M-14	-	$B1_1$	nd
HP01	urine	H		CTX-M-14	*aac(6')Ib-cr*	A_1	nd
E46	blood	E		CTX-M-15	*aac(6')Ib-cr*	D2	*iutA, fyuA, kpsMII,K5,*
E38	urine	I	ST393	CTX-M-2	-	D_1	*papC,papEF, papGII-III,papGII,papGIII, fimH,iutA,fyuA,kpsMII,K5*
HP15	urine	I		CTX-M-2	-	$B1_1$	nd
E21	WE	J		CTX-M-14	-	A_1	*fimH,iutA,kpsMII,k5*
C68	urine	A		CTX-M-15	-	$B2_3$	*afa/dra, fimH, iutA, kpsMII, traT,* PAI
HP13	urine	A		CTX-M-15	*aac(6')Ib-cr*	$B2_3$	*afa/dra, fimH, iutA,fyuA, kpsMII, traT*
C57	urine	A		CTX-M-15	*aac(6')Ib-cr*	$B2_3$	*afa/dra, fimH, iutA,fyuA, kpsMII, traT,* PAI
E11	urine	A	ST131	CTX-M-15	*aac(6')Ib-cr*	$B2_3$	*afa/dra, fimH, iutA,fyuA, kpsMII, traT*
E20	urine	A	ST131	CTX-M-15	*aac(6')Ib-cr*	$B2_3$	*papGIII, afa/dra, fimH, iutA,fyuA, kpsMII, traT,* PAI
E22	urine	A		CTX-M-15	*aac(6')Ib-cr*	$B2_3$	afa/dra, fimH, iutA,fyuA, kpsMII, traT,*PAI*
E43	urine	A	ST131	CTX-M-15	*aac(6')Ib-cr*	$B2_3$	papGIII, afa/dra, fimH, iutA,fyuA, kpsMII, traT,*PAI*
C58	urine	A		CTX-M-15	*aac(6')Ib-cr*	$B2_3$	*afa/dra, fimH, iutA,fyuA, kpsMII, traT*
C67	urine	A		CTX-M-15	*aac(6')Ib-cr*	$B2_3$	*afa/dra, fimH, iutA,fyuA, kpsMII, traT*
E02	blood	A	ST131	CTX-M-15	*aac(6')Ib-cr*	$B2_3$	*afa/dra, fimH, iutA,fyuA, kpsMII, traT,* PAI
E06	urine	A		CTX-M-15	*aac(6')Ib-cr*	$B2_3$	*afa/dra, fimH, iutA,fyuA, kpsMII, traT*
C56	urine	A		CTX-M-15	*aac(6')Ib-cr*	$B2_3$	*afa/dra, fimH, iutA,fyuA, kpsMII, traT,* PAI
HP05	urine	A		CTX-M-15	*aac(6')Ib-cr*	$B2_3$	*afa/dra, fimH, iutA,fyuA, kpsMII*
HP19	urine	A		CTX-M-15	*aac(6')Ib-cr*	$B2_3$	*afa/dra, fimH, iutA,fyuA, kpsMII*
E15	WE	A		CTX-M-15	*aac(6')Ib-cr*	$B2_3$	*afa/dra, fimH, iutA,fyuA, kpsMII, traT*
E30	urine	A		CTX-M-15	*aac(6')Ib-cr*	$B2_3$	*afa/dra, fimH, iutA,fyuA, kpsMII, traT*
E40	SWI	A		CTX-M-15	*aac(6')Ib-cr*	$B2_3$	*afa/dra, fimH, iutA,fyuA, kpsMII,*
E16	urine	A		CTX-M-15	*aac(6')Ib-cr*	$B2_3$	*afa/dra, fimH, iutA,fyuA, kpsMII,*
HP11	urine	A		CTX-M-15	*aac(6')Ib-cr*	$B2_3$	*afa/dra, fimH, iutA,fyuA, kpsMII,*
PA02	peritoneal	A		CTX-M-15	*aac(6')Ib-cr*	$B2_3$	*afa/dra, fimH, iutA,fyuA, kpsMII,*
E44	peritoneal	K		CTX-M-2	-	$B1_1$	nd
C54	urine	L		CTX-M-2/14	-	$B1_1$	nd
C66	blood	LL		CTX-M-2/14	-	A_1	nd
E59	urine	M	ST3555	CTX-M-8	*qnrB*	D_1	*fimH, iutA,fyuA, kpsMII,K5,traT,* PAI,
E07	urine	N		CTX-M-14	-	A_1	nd
HP21	blood	O	ST69	CTX-M-9	*qnrA*	D_1	*papC,papA,papEF, papGII-III,papGII,fimH,iutA,kpsMII,k5,traT*
E34	urine	B		CTX-M-15	*aac(6')Ib-cr*	D_2	nd
E35	BAL	B	ST405	CTX-M-15	*aac(6')Ib-cr*	D_2	nd
HP17	urine	B		CTX-M-15	*aac(6')Ib-cr*	D_2	nd
HP02	pleural fluid	B		CTX-M-15	*aac(6')Ib-cr*	D_2	nd
HP07	blood	B		CTX-M-15	*aac(6')Ib-cr*	D_2	nd
HP08	TA	B		CTX-M-15	*aac(6')Ib-cr*	D_2	nd
HP10	Abd. Absc	B	ST405	CTX-M-15	*aac(6')Ib-cr*	D_2	nd
E23	urine	B		CTX-M-15	*aac(6')Ib-cr*	D_2	nd
E25	blood	B		CTX-M-15	*aac(6')Ib-cr*	D_2	nd
E29	urine	B		CTX-M-15	*aac(6')Ib-cr*	D_2	nd
E41	urine	B		CTX-M-15	*aac(6')Ib-cr*	D_2	nd
HP04	SWI	B		CTX-M-15	*aac(6')Ib-cr*	D_2	nd
HP09	skin absc	B		CTX-M-15	*aac(6')Ib-cr*	D_2	nd

PMQR: plasmid mediated quinolone resistance; PG: phylogenetic group; VS: virulence screening; WE: wound exudate; SWI: surgical wound infection; TA: tracheal aspirate; BAL: bronchoalveolar lavage; abd. absc: abdominal abscess.

Table 3. Main features of the 25 selected extra-intestinal pathogenic *E. coli* and transconjugants strains under study

Strain	Resistance genes	Pulsetype	ST	CTX	CAZ	CIP	AK	Inc	Addiction system
C05	$bla_{CTX-M-2}$	F	3627	64	2	12	4		
E7	$bla_{CTX-M-14}$ /*aac(6')Ib-cr*	N		8	1.5	≥ 32	16		
TcE7	$bla_{CTX-M-14}$	-	-	8	1.5	0.03	0.38	I1	*pndAC*
E13	$bla_{CTX-M-15}$	C		64	16	≥ 32	4		
TcE13	$bla_{CTX-M-15}$	-	-	24	8	.06	1	F -FIA-FIB	*vagCD*/*pemKI*/*ccdAB*/*srnBC*
E20	$bla_{CTX-M-15}$/ *aac(6')Ib-cr*	A	131	16	16	≥ 32	8		
TcE20	$bla_{CTX-M-15}$/ *aac(6')Ib-cr*	-	-	16	8	0.12	2	F-FIA-FIB	*vagCD*/*pemKI*/*ccdAB*/*srnBC*
E21	$bla_{CTX-M-14}$	J		> 256	0.5	1	3		
TcE21	$bla_{CTX-M-14}$	-	-	16	2	0.015	0.5	I1	*pndAC*
E32	$bla_{CTX-M-2}$	NT	ST393	128	4	≥ 32	≥ 32		
E35	$bla_{CTX-M-15}$/ *aac(6')Ib-cr*	B	ST405	> 256	24	≥ 32	6		
E38	$bla_{CTX-M-2}$	I	ST393	> 256	4	≥ 32	24		
E44	$bla_{CTX-M-2}$	K		48	3	≥ 32	1,5		
TcE44	$bla_{CTX-M-2}$	-	-	32	3	0.015	0.125	F-FIB, A/C	*hok-sok*
E46	$bla_{CTX-M-15}$/ *aac(6')Ib-cr*	E		64	16	≥ 32	8		
E47	$bla_{CTX-M-14}$	G		2	0.125	8	3		
TcE47	$bla_{CTX-M-14}$	-	-	6	1	0.015	0.25	K, B/O	
E49	$bla_{CTX-M-15}$/ *aac(6')Ib-cr*	D		64	64	≥ 32	16		
C53	$bla_{CTX-M-15}$	NT		8	8	≥ 32	32		
C54	$bla_{CTX-M-2/14}$	L		> 256	2	0.03	3		
TcC54	$bla_{CTX-M-14}$	-	-	32	6	0.015	0.35	I1	*pndAC*
C55	$bla_{CTX-M-2}$	F	ST1158	64	2	4	3		
E59	$bla_{CTX-M-8}$/*qnrB*	M	ST3555	8	0.5	1	6		
TcE59	$bla_{CTX-M-8}$	-	-	2	0.75	0.015	0.125	I1	*pndAC*
C66	$bla_{CTX-M-2/14}$	LL		64	16	8	2		
TcE66	$bla_{CTX-M-14}$	-	-	12	1.5	0.015	0.25	F I1	*pndAC*/ *hok-sok*
E69	$bla_{CTX-M-2/14}$	C	ST38	32	2	0.03	4		
TcE69	$bla_{CTX-M-14}$	-	-	16	1.5	0.015	0.38	F I1	*pndAC*/ *hok-sok*
HP01	$bla_{CTX-M-14}$/ *aac(6')Ib-cr*	H	Nd	8	1	≥ 32	1		
TcHP01	$bla_{CTX-M-14}$			8	2	0.015	0.25	I1	*pndAC*
HP03	$bla_{CTX-M-2}$	NT	Nd	32	2	8	2		
HP06	$bla_{CTX-M-14}$	D	Nd	16	1.5	8	2	I1	
TcHP06	$bla_{CTX-M-14}$	-	-	8	1.5	0.008	0.125		*pndAC*
HP13	$bla_{CTX-M-15}$	A	ST131	2	2	≥ 32	16		
HP14	$bla_{CTX-M-2}$	NT	Nd	64	2	12	4		
HP15	$bla_{CTX-M-2}$	I	Nd	32	2	≥ 32	4		
HP21	$bla_{CTX-M-9}$/*qnrA*	O	ST69	16	3	0.12	8		
TcHP21	$bla_{CTX-M-9}$	-	-	4	1	0.03	0.25	HI1-HI2	

Tc: transconjugant; NT: untypeable; Nd: not determined; CTX: cefotaxime; CAZ: ceftazidime; CIP: ciprofloxacin; AK: amikacin; Inc: incompatibility group; Incompatibility group and addiction systems only were detected in transconjugants.

Thirteen transconjugants were obtained. All of the $bla_{CTX\text{-}M\text{-}14}$, $bla_{CTX\text{-}M\text{-}9}$, and $bla_{CTX\text{-}M\text{-}8}$ genes were encoded in conjugative plasmids; however, only two $bla_{CTX\text{-}M\text{-}15}$ alleles (carried by isolates E13 and E20, pulsetypes A and C, respectively) as well as a single $bla_{CTX\text{-}M\text{-}2}$ allele (isolate E44, pulsetype K) were encoded in conjugative plasmids.

Seven of the $bla_{CTX\text{-}M\text{-}14}$ alleles were encoded in IncI1 plasmids featuring a *pndAC* addiction system. In contrast, plasmids carrying $bla_{CTX\text{-}M\text{-}2}$ (IncF-FIB, IncA/C) and $bla_{CTX\text{-}M\text{-}9}$ (IncHI1-HI2) featured no addiction systems. Nevertheless, both of the $bla_{CTX\text{-}M\text{-}15}$-bearing plasmids featured several addiction systems, specifically *vagCD*, *pemKI*, *ccdAB*, and *srnBC* (Table 3).

Phylogenetic group and virulotyping of ESBL-producing strains

Forty-one of the 55 ESBL-producing isolates belonged to phylogenetic groups B2 and D ($B2_3$ = 20; D_2 = 13; D_1 = 8). However, only 29 of such isolates were VS+, namely the twenty strains belonging to phylogenetic group $B2_3$ (pulsetype A, ST131, CTX-M-15 producers), eight isolates belonging to phylogenetic group D_1, and one isolate belonging to phylogenetic group A_1 (Figure 1).

Isolates belonging to phylogenetic group D_1 were found to be genetically unrelated; five such isolates were CTX-M-2 producers and were typed as ST393 (n = 2), ST38, ST1158, and ST3627. Other D_1 isolates included a CTX-M-9 and QnrA-producer (ST69), as well as another isolate belonging to the new sequence type ST3555, which harbored both *qnrB* and $bla_{CTX\text{-}M\text{-}8}$.

Isolates carrying large numbers of virulence genes (between 9 and 10), namely isolates E38, E69, and HP21, belonged to phylogenetic group D_1; nevertheless, they were not among the most disseminated strains.

On the other hand, the 14 isolates belonging to phylogenetic group D_2 and ST405 were VS-.

Discussion

In the present study, the 55 ESBL-producing isolates were distributed in 12 different resistance patterns, the most frequent being oxyiminocephalosporins / nalidixic acid / ciprofloxacin / sulfamethoxazole-trimethoprim (n = 15), oxyiminocephalosporins / gentamicin / nalidixic acid / ciprofloxacin / sulfamethoxazole-trimethoprim (n = 12), and oxyiminocephalosporins / amikacin / nalidixic acid / ciprofloxacin / sulfamethoxazole-trimethoprim (n = 12).

Co-resistance to fluoroquinolones was observed in 90.9% (50/55) of the studied isolates; co-resistance to sulfamethoxazole trimethoprim was detected in 85.5% (47/55), co-resistance to gentamicin in 41.8% (23/55), and co-resistance to amikacin in 34.5% (19/55).

Recent studies have reported susceptibility results and molecular characterization of *E. coli* isolates in India, albeit with some differences; while co-resistance levels to sulfamethoxazole-trimethoprim and to ciprofloxacin were similar (81.6% and 93.9%, respectively), co-resistance to amikacin was higher (57.1%), and resistance to carbapenems was even detected (20.4%) [24].

Sixty percent of the isolates (33/55) were clustered in two clones, namely $B2_3$-ST131 and D_2-ST405. Accordingly, these clones accounted for 56.5% of co-resistance to gentamicin (13/23 isolates) and 66% of co-resistance to ciprofloxacin (33/50); however, they were responsible for 84.2% of co-resistance to amikacin (16/19 isolates).

Although both clones have been widely reported worldwide [25-28], their presence in South America is still scarcely reported [9,10,29].

In the present study, 41/55 isolates (74.5%) corresponded to phylogenetic groups B2 and D; nevertheless, only 28 (50.9%) could be classified as ExPEC based on VS. Additionally, all of the isolates corresponding to phylogenetic group B2 belonged to ST131. Our results are in accordance with those of Brisse *et al*., who found that CTX-M-production in ExPEC was exceptional in non-ST131 B2 strains [30]. Conversely, Roy *et al*. recently reported that over a third of CTX-M-15-producing *E. coli* B2 strains did not belong to ST131 [24]. These discrepancies highlight the importance of knowing the local epidemiology, in order to determine the circulating clones in each region.

Multiresistant clone $B2_3$-ST131 has been previously associated with a high number of virulence genes [27,31]; in this work, the number of virulence genes associated with this clone was between five and eight. Regarding the clone D_2-ST405, none of the antibiotic-resistant isolates could be classified as ExPEC (based on VS). Conversely, only a single isolate belonging to CGA was detected, which suggests that this group does not constitute a problem, at least within the studied hospitals. This isolate, however, harbored a large number (10) of virulence-related genes. Accordingly, this ST69 isolate (HP21) along with isolates E38 and E69 (ST393 and ST38,

respectively) carried large numbers of virulence genes and were not disseminated.

In this sense, the multiresistant clone $B2_3$-ST131 could have reached an optimal balance between virulence and antibiotic resistance, allowing it to competitively colonize, to cause infection, and to disseminate among different hosts.

On the other hand, we recently reported that a group of non-ExPEC *E. coli* isolates (based on VS) formed intracellular niches in urothelial cells; this finding was linked to the occurrence or recurrent UTIs [32]. Taking those findings into consideration, it is possible that the diversity of virulence-related genes in ExPEC is greater than the VS employed during the present study.

With respect to ESBL production, this is the first study to show a clear predominance in our region of CTX-M-15 in *E. coli* strains (60%), followed by CTX-M-2 (20%). Until recently, and particularly in Uruguay, the most frequently detected ESBL (in varying proportions) was CTX-M-2 [12,33] CTX-M-15 was first detected in 2006 in a general hospital [13], and in 2009, this ESBL was detected in the pediatric hospital [11]. However, until now, there was no information on the sequence types of ESBL-producing *E. coli* strains. The presence of CTX-M-15-producing pandemic clones such as ST131 and ST405 has already been reported in Colombia [10] and Argentina (ST131) [9]; nevertheless, there is no information about the sequence types associated with other CTX-M enzymes in our continent.

The CTX-M-9 and QnrA1-producing strain was typed as ST69, which has already been associated with ESBL and non-ESBL-producing strains [34,35]. In our study, this was the only isolate that belonged to CGA. Strains belonging to CGA have been widely reported around the world, even in South America [7,18]; nevertheless, in general, there is no information regarding sequence types of CGA strains.

On the other hand, CTX-M-2-producing strains were associated with various sequence types, which included clones already linked to CTX-M enzymes such as ST38 and ST393 [25,34-36]. Furthermore, CTX-M-2 was detected in clones so far unrelated with antibiotic resistance, *i.e.*, ST1158 (allelic profile 18, 3, 17, 6, 5, 5, 4) and in the novel sequence type ST3627 (allelic profile 13, 26, 39, 25, 5, 31, 19). Additionally, the isolate carrying *qnrB*/$bla_{\text{CTX-M-8}}$ also belonged to the novel sequence type ST3555 (allelic profile 101, 19, 97, 108, 26, 79, 19).

CTX-M-8 was first described in Brazil in 2000, but was only recently detected in other South American countries including Uruguay and Argentina [9,11]. Additionally, this ESBL seems to be slowly disseminating in Europe, Africa, and Asia as well [37-39].

In our country, the dissemination of CTX-M-15 may be the result of two mechanisms: either the dispersion of successful clones such as ST131 and ST405, or the acquisition of conjugative plasmids featuring multiple addiction systems.

On the other hand, the expansion of $bla_{\text{CTX-M-14}}$ could be mainly due to cross-species dissemination of IncI1 plasmids featuring the *pndAC* addiction system. In this regard, we recently reported the occurrence of $bla_{\text{CTX-M-14}}$ in an isolate of *Salmonella enterica* serovar Enteritidis, encoded in a similar plasmid [40]. Additionally, in our continent, IncI1 plasmids harboring $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-19}}$ (both belonging to group 4 in the CTX-M family) have already been described in *E. coli* strains in our country and in Bolivia [14,36].

Furthermore, only one of the 11 isolates encoding $bla_{\text{CTX-M-2}}$ carried such gene in a conjugative plasmid. Incidentally, such plasmid had no addiction systems.

Recently, Bartoloni *et al*., in a 20-year study, reported a shift in the epidemiology of ESBLs in Bolivia and Perú, describing an increase in the prevalence of CTX-M-15 and CTX-M-14, and a decrease of CTX-M-2 [41]. A similar scenario was also described in Argentina [9].

Conclusions

In this study, we propose some elements that could account for this epidemiologic change. For instance, the association of CTX-M-15 with successful clones or with plasmids carrying addiction systems, and/or the dissemination $bla_{\text{CTX-M-14}}$ in conjugative IncI1 plasmids with *pndAC*, contrasts with the small proportion of $bla_{\text{CTX-M-2}}$-encoding conjugative plasmids lacking detectable addiction systems.

Additionally, the link between CTX-M-15 (which confers higher resistance levels to ceftazidime) and PMQR such as *aac(6')Ib-cr*, creates better chances for co-selection of those isolates carrying both resistance mechanisms, in relation to those carrying only CTX-M-2.

The fact that these changes in the epidemiology of ESBLs go hand in hand with changes in genes conferring resistance to other antimicrobials (such as PMQR) highlights the importance of molecular and epidemiological studies.

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