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Extended-spectrum β-lactamases in *Shigella flexneri* from Argentina: first report of TOHO-1 outside Japan☆

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This paper is dedicated to the memory of Dr. Alicia Rossi, who directed our Service and guided this work.

Abstract

A 9-year nation wide survey of the presence of extended-spectrum β-lactamases (ESBLs) in *Shigella flexneri* is described. Ten of 9033 (0.1%) isolates produced ESBLs, which were characterized by isoelectric focusing, PCR and DNA sequencing. These were CTX-M-2 (five isolates), TOHO-1 (one isolate), SHV-2 (two isolates) and PER-2 (two isolates, the first report in *S. flexneri* world wide). The emergence of each ESBL type in *S. flexneri* was not restricted to a particular region of Argentina. TOHO-1 showed a more basic isoelectric point (8.4) than that previously found (7.8) and its encoding gene ($bla_{\text{TOHO-1a}}$) harboured a silent change, G825A, relative to the reported $bla_{\text{TOHO-1}}$. All the ESBL-encoding genes were transferred to *Escherichia coli* by conjugation. PFGE analysis indicated that the 10 ESBL-producing *S. flexneri* isolates were subtypes of a unique clone.

Keywords: *Shigella flexneri*; Resistance; β-Lactamases; ESBL; CTX-M-2; TOHO-1

1. Introduction

In Enterobacteriaceae, extended-spectrum β-lactamases (ESBLs) constitute the major mechanism conferring resistance to expanded-spectrum cephalosporins world wide [1]. In Argentina, ESBLs are widely spread among enterobacterial isolates, CTX-M-2 being the most prevalent enzyme by far [2]. CTX-M-enzymes are an expanding family of ESBLs that preferentially hydrolyse cefotaxime over ceftazidime and are inhibited by β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam. Currently, this family comprises 36 enzymes that were grouped in four clusters according to their amino acid identity; namely, the CTX-M-1 cluster; the CTX-M-2 cluster (hereafter referred to as CTXM2c); the CTX-M-8 cluster, and the CTX-M-9 cluster [2]. The first reported enzymes from the CTXM2c were CTX-M-2 and TOHO-1, described in Argentina [3] and Japan [4], re-

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spectively. In spite of the fact that both enzymes differ by only one amino acid change, TOHO-1 was detected only in Japan while CTX-M-2 has been widely spread throughout Argentina, its neighbouring countries, Israel, Japan, France, and also has been recently reported in Italy, Belgium, Turkey and the Republic of South Africa [2]. Moreover, in Argentina, the $bla_{\text{CTX-M-2}}$ gene has been located in transferable plasmids not only in enterobacterial isolates such as *Escherichia coli*, *Salmonella* spp., *Klebsiella* spp., and *Proteus* spp. [2,5], but also in isolates of *Vibrio cholerae* O1 El Tor [6].

Currently, *Shigella* species remain a world wide public health problem. Shigellosis is one of the major causes of bloody diarrhoea associated with high morbidity and mortality, especially in paediatric patients. The adequate antibiotic treatment of *Shigella* infections may limit the clinical course of illness and the duration of faecal excretion of bacteria. Therefore, the emergence of resistance in *Shigella* spp. is a matter of great public health concern, mainly in developing countries [7]. In Argentina, *Shigella flexneri* is the major specie recovered from patients with shigellosis and shows a high incidence of resistance to first line antibiotics, such as ampicillin, trimethoprim-sulphamethoxazole and chloramphenicol [8–10]. Due to this high incidence of resistance, alternative options such as the use of oral expanded-spectrum cephalosporins have been recommended, since the late 1990s, as the empirical antibiotic treatment of shigellosis in paediatric patients [11,12]. However, in spite of the fact that the occurrence of an ESBL enzyme in two *S. flexneri* isolates from Buenos Aires was mentioned in a previous work [2], a nation wide study for addressing resistance to expanded-spectrum cephalosporins in this pathogen has not been reported yet. A national resistance surveillance network (WHONET-Argentina) has been implemented since 1989 and currently includes 55 hospitals throughout the country. From 1995 to date, 9033 clinical isolates of *S. flexneri* have been reported by WHONET-Argentina. Ten (0.1%) of these were suspected of producing ESBLs as they showed synergy in the double-disk diffusion test (cefotaxime/ceftazidime and amoxicillin–clavulanic acid) when performed by routine antibiogram (National Committee for Clinical Laboratory Standards, NCCLS). They were then submitted for further characterization to the Instituto Nacional de Enfermedades Infecciosas—ANLIS 'Dr. Carlos G. Malbrán' (the National Reference Laboratory). In this work, we characterise the ESBLs harboured by those 10 *S. flexneri* isolates describing for the first time a PER-2-producing *S. flexneri* strain and pointing out the emergence of TOHO-1 outside Japan.

2. Materials and methods

2.1. Bacterial strains and patients

The 10 *S. flexneri* isolates analysed in this study were typed as serotype 2 and were recovered from faeces of patients in seven hospitals located in five different geographical areas of the country (Table 1).

S. flexneri M3349 was isolated from a 33-year-old hospitalized woman who was from Mariflores, an aboriginal community in Castelli, Chaco, Argentina. In August 1999, she was admitted at the Hospital Perrando, in Resistencia, Chaco, due to a premature delivery. At that time, the patient presented moderate neutropenia (3400 leukocytes/mm^3), low platelet count (15 000 platelets/mm^3), severe anaemia (7.8 g/dl haemoglobin), chronic diarrhoea and a presumptive bacterial pneumonia. Due to this last presumption, she received a 6-day course of amoxicillin–clavulanate (1500 mg/day). On the sixth day, *S. flexneri* M3349 was recovered from a stool culture and the patient was subsequently treated with ciprofloxacin (750 mg/day) for 3 days; there was a good clinical outcome.

Table 1
Epidemiological data of *S. flexneri* isolates

Isolate	Date of isolation	Htal[a]	City (province)	Region of the country	Patient (age)	Antibiotic treatment (time)[b]
M3331	April-1997	HPP	Posadas (Misiones)	Northeast	Paediatric (20 months)	CTX + AMK (4 days)
M3186	July-1998	GUT	Buenos Aires	BA-Out[c]	Paediatric (1 year)	CXM (11 days)[d]
M3244	January-1999	HMI	Salta (Salta)	Northwest	Paediatric (1 year)	CTX (3 days)
M3349	August-1999	HJP	Resistencia (Chaco)	North	Adult (33 years)	AMC (6 days)
M3354	October-1999	GAR	Buenos Aires	BA-Out	Paediatric (6 years)	Unknown
M3824	September-2001	POS	Haedo (Buenos Aires)	BA-Out	Paediatric (6 months)	AMX (unknown)
M3127	March-1998	GAR	Buenos Aires	BA-Out	Paediatric (2 years)	None
M3260	December-1998	LCA	Córdoba (Córdoba)	Centre	Paediatric (8 months)	None
M3771	March-1995	LCA	Córdoba (Córdoba)	Centre	Paediatric (1 year)	CHL (7 days)
M3748	June-2001	GAR	Buenos Aires	BA-Out	Paediatric (5 years)	None

[a] Hospital abbreviations: GAR, Htal. de Pediatría 'Prof. Dr. Juan P. Garrahan'; GUT, Htal. de Niños 'Dr. Ricardo Gutierrez'; HJP, Htal. General de Agudos 'Dr. Julio C. Perrando'; HMI, Htal. Materno Infantil; HPP, Htal. Público Provincial de Pediatría; LCA, Htal. de Niños de la Santísima Trinidad, and POS, Htal. Nacional 'Prof. Dr. Alejandro Posadas'.

[b] Empirical antibiotic treatment received by the patient during the indicated time, just before the stool culture from which the corresponding *S. flexneri* isolate was recovered (AMC, amoxicillin–clavulanic acid; AMK, amikacin; AMX, amoxicillin; CHL, chloramphenicol; CTX, cefotaxime; CXM, cefuroxime).

[c] Buenos Aires City and its outskirts.

[d] An initial *S. flexneri* isolate, susceptible to expanded-spectrum cephalosporins (not available), was recovered from a first stool sample obtained at the hospital admission and just before the administration of CXM.

S. flexneri F-4 (serotype 2), used in the PFGE analysis as a non-related strain, was a Chilean isolate provided by the Laboratory Centre for Disease Control and Pan American Health Organization, as part of a proficiency panel of the Collaborative Surveillance Project.

Nalidixic acid-resistant *E. coli* ER1793 (New England Biolabs, Beverly, MA, USA) was used as the recipient strain for conjugative assays.

2.2. Antimicrobial agents, susceptibility testing and conjugative assays

The antimicrobial agents used in this study were obtained from standard laboratory powders and their sources were as follows: amikacin, aztreonam and cefepime (Bristol-Myers Squibb); amoxicillin (Glaxo SmithKline); ampicillin (Temis Lostaló, Buenos Aires, Argentina); chloramphenicol (Parke Davis, Buenos Aires, Argentina); cefotaxime (Argentia, Buenos Aires, Argentina); cefoxitin and imipenem (Merck Sharp & Dohme); ceftazidime (Glaxo SmithKline); clavulanic acid (Roemmers, Buenos Aires, Argentina); nitrofurantoin, gentamicin, sulfisoxazole and trimethoprim (Schering Plough); cephalothin, piperacillin and tazobactam (Wyeth Pharmaceuticals); tetracycline (Microsules-Bernabó, Buenos Aires, Argentina); meropenem (Astra Zeneca); ciprofloxacin (Bagó, Buenos Aires, Argentina). Nalidixic acid (used in molecular biology applications) was from Sigma (Sigma, St. Louis, MO, USA).

The antimicrobial susceptibility profiles were determined by the agar dilution method according to NCCLS guidelines [13]. When indicated, clavulanic acid (4 mg/l), or tazobactam (4 mg/l), were used in combination with β-lactams.

Biparental conjugations were performed as previously described [14]. Transconjugants were selected on Luria-Bertani agar plates supplemented with nalidixic acid (50 mg/l) plus cefotaxime (20 mg/l), or nalidixic acid plus ceftazidime (1 mg/l), as indicated.

2.3. Isoelectric focusing (IEF)

Preparation of β-lactamases and IEF were performed as described previously [6]. Crude preparations from bacteria producing the following β-lactamases of known isoelectric point (p*I*) were used as standards: TEM-1 (p*I* 5.4), PER-2 (p*I* 5.4), SHV-2 (p*I* 7.6), CTX-M-2 (p*I* 7.9), SHV-5 (p*I* 8.2) and MIR-1 (p*I* 8.9).

2.4. PCR assays and DNA sequencing

Screening for β-lactamase-encoding genes was performed by PCR using the following primers and cycling conditions already described: OT-1/OT-2 (bla_{TEM}) [15], bla_{PER}F/bla_{PER}R (bla_{PER}), and $bla_{CTX\text{-}M}$F/$bla_{CTX\text{-}M}$R (bla_{CTXM2c}) [6]. The primers bla_{SHV}Fb (forward, ATGCGTTATATTCGCCTGTG) and bla_{SHV}Rb (reverse, GTTAGCGTTGCCAGTGCTCG) were used for the screening of bla_{SHV} genes, under described cycling conditions [6], with an annealing temperature of 59 °C. Amplifications from bacterial DNA templates were performed as previously described [6].

PCR amplicons were bidirectionaly sequenced by the method of Sanger et al. [16], with the primers mentioned above and sequence-based primers (when necessary), using the BigDye terminators methodology and an ABI Prism 377 DNA Sequencer (Applied Biosystems/Perkin Elmer, Foster city, CA, USA).

2.5. PFGE

PFGE of *S. flexneri* isolates was performed using either *Xba*I- or *Sfi*I-digested DNA (New England Biolabs) and procedures already described [14]. DNA restriction fragments were separated in a 1% agarose gel (0.5× Tris-borate buffer), using a CHEF-DRIII apparatus (Bio-Rad Laboratories). Electrophoresis was performed for 20 h, at 14 °C, with an electric field of 6 V/cm, and the pulse times were ramped from 2.2 to 54.2 s (*Xba*I), or from 5 to 35 s (*Sfi*I). DNA patterns were compared using Tenover's criteria [17].

2.6. Nucleotide sequence accession number

The nucleotide sequence of $bla_{TOHO\text{-}1a}$ was submitted to the GenBank database under accession no. **AY671905**.

3. Results and discussion

The β-lactam susceptibility profiles of the 10 *S. flexneri* isolates showing synergy in the double-disk diffusion test, are summarized in Table 2. Six isolates showed MICs of cefotaxime that were at least 16 times higher than those of ceftazidime, a phenotype that suggests the presence of a CTX-M-like enzyme [2]. In this group, *S. flexneri* M3349 showed a MIC of cefotaxime at least four times higher than those of the other five putative CTX-M-producers. Conversely, for the remaining four isolates, the MICs of ceftazidime were similar to, or at least 16 times higher than, those of cefotaxime which suggests, from previous studies from our laboratory [6,14,24,25], the presence of SHV-, or PER-related enzymes, respectively. All these β-lactam-resistant phenotypes were transferred to *E. coli* ER1793 by conjugation (Table 2). The presumptive occurrence of these ESBL types was corroborated by IEF and PCR and the identity of each ESBL was established by DNA sequencing of PCR amplicons (Table 2). Remarkably, *S. flexneri* M3349 expressed an enzyme belonging to the CTXM2c with a p*I* of 8.4 (Fig. 1). This enzyme was characterized as TOHO-1 by DNA sequencing of its encoding gene, which was named $bla_{TOHO\text{-}1a}$, since it showed a unique silent change (AG**A** instead of AG**G**) at nucleotide position 825 relative to the reported $bla_{TOHO\text{-}1}$ gene [4]. Interestingly, the purines at position 825 of $bla_{TOHO\text{-}1}$ or $bla_{TOHO\text{-}1a}$ constitute the unique changes (non-synonymous)

Table 2
β-Lactam susceptibility profiles (MIC, mg/l) and ESBL characterization in *S. flexneri* isolates and *E. coli* transconjugants

	S. flexneri clinical isolates				*E. coli* transconjugants[a]			
	CTX-M-like		SHV-like	PER-like	CTX-M-like		SHV-like	PER-like
	M3349	M3331, M3186, M3244, M3354, M3824	M3127, M3260	M3748, M3771	M3349Tc	M3331Tc, M3186Tc, M3244Tc, M3354Tc, M3824Tc	M3127Tc, M3260Tc	M3748Tc, M3771Tc
β-Lactams								
AMP	1024	>1024	512	512	>1024	>1024	512 to >1024	1024
AMC	16	8–16	8	8	8	8–16	8	2–8
PIP	1024	256 to >1024	64–128	32–64	1024	>1024	256–512	64–128
TZP	2	2–8	0.5–2	2	4	2–4	4	2
CEF	>256	>256	64	>256	>256	>256	64–128	128
FOX	4	2–4	2	2–4	4	4–8	4	2–4
CTX	512	64–128	0.5	4–8	1024	128–256	1	8
CTX-CLA	0.06	0.03–0.06	<0.015	<0.015	0.06	0.03–0.06	<0.015	<0.015
CAZ	4	1–4	0.25	128–256	8	4	1	256
CAZ-CLA	0.06	<0.03–0.12	<0.03	<0.03	0.12	0.12	0.06	<0.03–0.06
FEP	8	4–16	0.25	2–8	8	8–16	0.5	2
FEP-CLA	0.015	0.015–0.03	<0.008	0.008	0.03	0.015–0.03	0.015	0.008
ATM	64	8–32	0.25	64	128	32	1–4	128–512
IPM	0.25	0.25	0.25	0.25	0.5	0.25–0.5	0.25–0.5	0.25–0.5
MEM	0.03	0.015–0.03	0.015	0.015–0.03	0.03	0.03	0.015–0.03	0.03
IEF bands	5.4–**8.4**	5.4–**7.9**	(5.4–**7.6**)[b], **7.6**[c]	**5.4**	5.4–**8.4**	5.4–**7.9**	(5.4–**7.6**)[b], **7.6**[c]	**5.4**
PCR[d]	T + CTM2	T + CTM2	(T + S)[b], S[c]	P	T + CTM2	T + CTM2	(T + S)[b], S[c]	P
Sequenced ESBL gene	*toho-1a*	*ctx-M-2*	*shv-2a*[b], *shv-2*[c]	*per-2*	*toho-1a*	*ctx-M-2*	*shv-2a*[b], *shv-2*[c]	*per-2*

Strains showing very similar susceptibility profiles were grouped and the corresponding MIC ranges are indicated. Abbreviations: AMP, ampicillin; AMC, amoxicillin–clavulanic acid; CLA, clavulanic acid; ATM, aztreonam; CAZ, ceftazidime; CEF, cephalothin; CTX, cefotaxime; FEP, cefepime; FOX, cefoxitin; IPM, imipenem; MEM, meropenem; PIP, piperacillin; TZP, piperacillin-tazobactam. The p*I* values of bands showing ESBL activity are indicated in bold.

[a] Transconjugant selection was achieved with cefotaxime (CTX-M-like producers), or ceftazidime (SHV- and PER-like producers). Each transconjugant was named with the name of its respective parental *S. flexneri* isolate plus the abbreviation Tc. The susceptibility profile of the recipient strain ER1793 has been already reported [6,14].

[b] Data from *S. flexneri* M3127, or *E. coli* M3127Tc (the GenBank accession no. of reported *shv-2a* gene are X53817, X84314, AF074954 and AF462393).

[c] Data from *S. flexneri* M3260, or *E. coli* M3260Tc.

[d] Positive results in the screening of bla_{CTXM2c} (CTM2); bla_{PER} (P); bla_{SHV} (S), and bla_{TEM} (T), are indicated.

that differentiate these genes from $bla_{\text{CTX-M-2}}$ (AG**C**), determining the occurrence of an Arg at ABL [18] position 274 of TOHO-1, instead of the Ser found in CTX-M-2. Of note, the p*I* of TOHO-1 observed in this work was more basic than that (7.8) previously reported [4], which may be due to differences between the methodologies used for estimating the p*I* value. However, the unique change Arg274Ser (a basic for an uncharged residue) between TOHO-1 and CTX-M-2 would imply a p*I* for the former more basic than that of the latter (7.9), as we found here. Such a difference between the p*I*s of CTX-M-2 and TOHO-1 (0.5 units) may be useful for the proper identification of the later enzyme in a phenotypic characterization of ESBLs. Moreover, comparing the MICs of cefotaxime conferred by TOHO-1 and CTX-M-2, we observed the same increase between either the *S. flexneri* isolates (512 mg/l versus 64–128 mg/l), or their respective *E. coli* transconjugants (1024 mg/l versus 128–256 mg/l). This fact is in agreement with the reported increase in the $k_{\text{cat}}/K_{\text{m}}$ ratio for cefotaxime hydrolysis associated with the presence of the Arg274 in TOHO-1 [19].

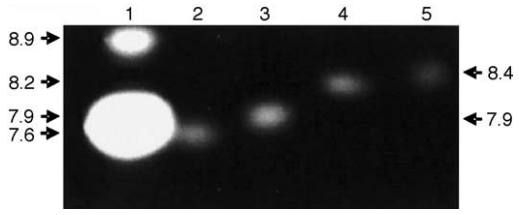

Fig. 1. IEF of CTX-M-like enzymes. Lane 1, MIR-1 (p*I* 8.9) plus CTX-M-2 (p*I* 7.9); lane 2, SHV-2 (p*I* 7.6); lane 3, *S. flexneri* M3331 (the analysis of isolates M3186, M3244, M3354 and M3824 showed the same active band as M3331); lane 4, SHV-5 (p*I* 8.2), and lane 5, *S. flexneri* M3349. After isoelectric focusing, the gel was subjected to the iodometric method, using cefotaxime plus ceftazidime as developing substrates (only the relevant part of the gel is shown). The p*I*s of known β-lactamases are indicated by numbers on the left while the p*I*s of the β-lactamases produced by the *S. flexneri* isolates are shown on the right.

Regarding the other two ESBLs characterized herein, this is the first report of PER-2 in *S. flexneri* worldwide, while SHV-2 was previously described in a clinical isolate of this pathogen in France [20]. Of note, the MICs of cefotaxime and ceftazidime of the SHV-2-producing isolates M3127 and M3260 were in the range of susceptibility described by the NCCLS guidelines (see Table 2). However, both isolates

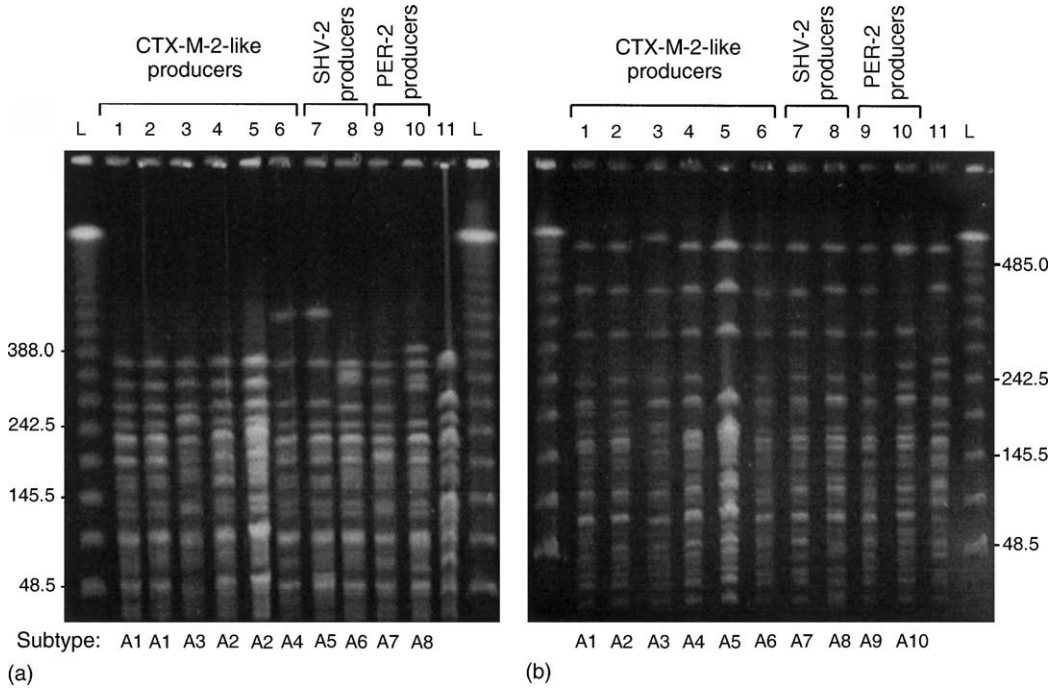

Fig. 2. PFGE patterns of *S. flexneri* isolates. Chromosomal DNA was digested with *Xba*I (a) or *Sfi*I (b). Lanes L, λ ladder (numbers indicate sizes in kb); lanes 1–10, *S. flexneri* isolates M3349, M3331, M3354, M3824, M3244, M3186, M3127, M3260, M3748, and M3771, respectively; lane 11, *S. flexneri* F-4. Subtypes were separately defined for each PFGE assay, according to the number of differences observed among the patterns.

were suspected of producing ESBLs when a cefotaxime-containing disc was used in the routine antibiogram (data not shown), when the NCCLS breakpoint for *Klebsiella pneumoniae*, *K. oxytoca* and *E. coli* was considered. These findings are additional evidence points to the need for a revision in the NCCLS recommendations regarding both phenotypic screening and confirmatory test for ESBL-producing enterobacterial isolates. Therefore, based on the ESBL epidemiology in our country and the recent emergence and spread of non-TEM-derived ESBLs in other regions of the world, clinical laboratories should evaluate more than one expanded-spectrum cephalosporin (i.e. cefotaxime/ceftriaxone and ceftazidime), in order to achieve the full detection of ESBL-producing enterobacterial isolates.

The genetic relatedness of the 10 ESBL-producing *S. flexneri* isolates was firstly examined by PFGE of *Xba*I-digested DNA (Fig. 2(a)). Since comparison between all the PFGE profiles resulted in six, or fewer, differences (i.e. variants of a unique clone in an extended period of time, with Tenover's criteria [17]), a subsequent analysis of *Sfi*I-digested DNA was carried out (Fig. 2(b)). The combined results of both PFGE assays showed that the 10 ESBL-producing isolates from Argentina were subtypes of a unique clone, while the Chilean F-4 isolate was genetically unrelated. This finding, in addition to the epidemiological data of *S. flexneri* isolates (see Table 1), suggests the presence of an endemic clone of this pathogen in our country. However, due to the low number of isolates analysed here and the lack of another PFGE study from Argentina, we cannot discard the possibility that there are coexistent circulating clones.

Even though selection of transconjugants was only achieved with cefotaxime or ceftazidime (plus nalidixic acid), several determinants of resistance to non-β-lactam antibiotics were cotransferred to *E. coli* with the detected ESBLs (Table 3). This fact appears to be associated with the occurrence of the ESBL-encoding gene. For example, in all six isolates producing a CTX-M-2-like enzyme (CTX-M-2 or TOHO-1), resistance to gentamicin and sulfisoxazole, and the resistance/decreased susceptibility to amikacin were cotransferred with the cefotaxime-resistant phenotype. Conversely, both in the SHV-2- and PER-2-producing isolates, different non-β-lactam resistance determinants were cotransferred with the ceftazidime-resistant phenotype (Table 3). This finding suggests that $bla_{\text{CTX-M-2}}$ and $bla_{\text{TOHO-1a}}$ are located in mobile genetic elements, which share the same resistance determinants. Of note, $bla_{\text{CTX-M-2}}$ has been found in conjugative megaplasmids of enterobacterial isolates from Argentina, as a cassette of an unusual class 1 integron which also contains amikacin or gentamicin resistance genes (*aac*(6′)-*Ib* or *ant*(2″)-*Ia*, respectively), in addition to the typical *sulI* gene located in the 3′-conserved segment [14,21–23]. Conversely, so far as we know, the Japanese $bla_{\text{TOHO-1}}$ allele has not been associated with integrons yet. Moreover, in the characterization of the TOHO-1 enzyme from Japan, Ishii et al. reported the horizontal transference of $bla_{\text{TOHO-1}}$ separately from a gentamicin resistance determinant [4]. These facts, in addition to the epidemiological data from *S. flexneri* M3349 (recovered from a patient who lives in a close aboriginal community of our country) and the clonal relatedness between the isolate M3349 and the CTX-M-2 producers, support the

Table 3
Non-β-lactam susceptibility (MIC, mg/l) of *S. flexneri* isolates and *E. coli* transconjugants

Strains	Non-β-lactam antibiotics							
	AMK	GEN	CHL	TMP	SFI	TET	NIT	CIP
CTX-M-2-like producers								
M3349	**32**	**512**	>**128**	>512	>**1024**	128	8	0.008
M3349Tc	**8**	**64**	>**128**	<0.25	>**1024**	0.5	4	0.25
M3331	**32**	>**512**	**128**	>512	>**1024**	128	8	0.008
M3331Tc	**8**	**64**	>**128**	<0.25	>**1024**	0.5	4	0.25
M3186	**32**	>**512**	1	>512	>**1024**	128	4	0.008
M3186Tc	**4**	**64**	4	<0.25	>**1024**	0.5	8	0.25
M3244	**64**	>**512**	64	>512	>**1024**	128	8	0.008
M3244Tc	**4**	**128**	4	<0.25	>**1024**	0.5	8	0.25
M3354	**64**	>**512**	>**128**	>512	>**1024**	128	16	0.008
M3354Tc	**8**	**64**	>**128**	<0.25	>**1024**	0.5	4	0.25
M3824	**32**	**512**	**128**	>512	>**1024**	64	16	0.008
M3824Tc	**4**	**64**	>**128**	<0.25	>**1024**	0.5	8	0.12
SHV-2 producers								
M3127	**64**	4	1	<0.25	>1024	0.5	4	0.008
M3127Tc	**8**	0.25	4	<0.25	8	0.5	8	0.25
M3260	8	1	128	>**512**	>**1024**	128	8	0.008
M3260Tc	0.25	<0.125	8	**1024**	>**1024**	0.5	8	0.25
PER-2 producers								
M3748	>**128**	**32**	**128**	>**512**	>**1024**	128	16	0.008
M3748Tc	**4**	**2**	>**128**	**64**	>**1024**	0.5	4	0.06
M3771	8	2	32	>512	>**1024**	32	8	0.008
M3771Tc	0.25	<0.125	4	<0.25	>**1024**	0.5	8	0.06
E. coli ER1793	0.25	<0.125	4	<0.25	4	1	8	0.25

Abbreviations: AMK, amikacin; CHL, chloramphenicol; CIP, ciprofloxacine; GEN, gentamicin; NIT, nitrofurantoin; SFI, sulfisoxazole; TET, tetracycline; TMP, trimethoprim. Transconjugants were named as in Table 2. MIC values of resistance determinants cotransferred with the bla_{ESBL} genes are indicated in bold.

conclusion that $bla_{TOHO-1a}$ has emerged in Argentina as a result of a single point mutation (C825A) in a pre-existing $bla_{CTX-M-2}$ gene.

In conclusion, this is the first report of a 9-year national multicentre survey of ESBL-producing *S. flexneri* in Argentina. The survey found 6 of 10 isolates were producing CTX-M-2 or TOHO-1, two others SHV-2 and two isolates produced PER-2. In addition, the available epidemiological data indicated that the emergence of each ESBL type in *S. flexneri* was not restricted to a particular region of the country (see Table 1). These results agree with previous studies carried out with other species of the Enterobacteriaceae [24–27], which may reflect the broad horizontal dissemination of such ESBL genes among clinical enterobacterial isolates in Argentina. Furthermore, a *S. flexneri* isolate susceptible to expanded-spectrum cephalosporins was recovered previously to M3186, from a stool culture taken before the administration of cefuroxime (see Table 1). This evidence, in addition to the low incidence of resistance to expanded-spectrum cephalosporins in this pathogen suggests an intra-treatment acquisition of the $bla_{CTX-M-2}$ gene by *S. flexneri* M3186, perhaps from a nosocomial enterobacterial source.

Although the prevalence of resistance to expanded-spectrum cephalosporins in *S. flexneri* is still low, our findings on its emergence and dissemination throughout several provinces of Argentina emphasise the need for continuous resistance surveillance, especially among paediatric and maternity patients with shigellosis, for whom antibiotic the therapeutic options are scarce.

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