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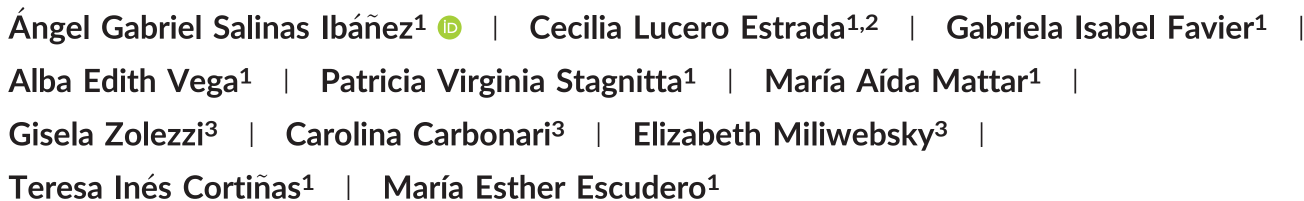
Characterization of Shiga-toxin producing *Escherichia coli* isolated from meat products sold in San Luis, Argentina



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Abstract

Shiga toxin-producing *Escherichia coli* (STEC) in meat products was studied by four procedures which combined enrichment, immunomagnetic separation (IMS), plating, and a *stx* screening polymerase chain reaction. A total of 167 samples (57 ground beef, 58 fresh sausages, and 52 precooked sausages) were collected from retail markets in San Luis, Argentina. The number of *stx*-positive samples represented 11.4% of the total (19/167) distributed as follows: seven for raw ground beef (12.3%), six for precooked sausages (11.5%), and six for fresh sausages (10.3%). The *stx*-positive sample rate increased when IMS was included in detection protocols. The stx_2 gene (16/19) was more frequently detected than stx_1 (4/19). The *eae*, $rfbE_{O157}$, and *fli*Ch7 genes were also investigated in *stx*-positive samples. Three non-O157 STEC strains were isolated from one precooked sausage and one fresh sausage (1.2%, 2/167). Their potential pathogenicity, antimicrobial susceptibility, and genetic diversity were determined. This study highlights meat products as possible vehicles for transmission of STEC in this Argentine region.

Practical applications

Meat products intended for human consumption can be vehicles for the transmission of Shiga toxin-producing *Escherichia coli* (STEC) in this region. STEC strains isolated from sausages in this work represent a hazard for consumers. Further research to continue the STEC surveillance in meat products from this region in Argentina and to improve the STEC detection procedures in these foods and other possible transmission vehicles of this pathogen is required. Additionally, livestock control programs as well as good practices for slaughtering, processing, and manufacturing plants should be implemented to reduce STEC spreading in the food chain. Adequate cooking of ground beef and fresh and precooked sausages is recommended to consumers.

1 | INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC), a known enteric pathogen associated with foodborne illnesses, constitutes an important problem for human health (Tarr, Gordon, & Chandler, 2005). This pathogen can cause nonbloody diarrhea, hemorrhagic colitis, and life-threatening hemolytic uremic syndrome (HUS) (Rivas, Chinen, Miliwebsky, & Masana, 2014). The stx_1 and stx_2 genes encode Shiga toxins which inhibit host protein synthesis, and the *eae* gene encodes the intimin, an outer membrane protein required for the bacterial adhesion to the intestinal epithelium prior to the attaching and effacing (A/E) lesion. Other virulence factors such as an enterohemolysin, adhesins, and proteases contribute to the STEC pathogenicity (Etcheverría & Padola, 2013). Even though antimicrobial therapy is not recommended, the study of antimicrobial susceptibility of STEC strains has epidemiological relevance (Nguyen & Sperandio, 2012).

Ruminants are the main STEC reservoirs, with approximately 52% of outbreaks in humans having been associated with consumption of products of bovine origin (Karmali, Gannon, & Sargeant, 2010), although meat products from other origins such as pork have also been

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confirmed as vehicles for STEC transmission (Ercoli, Farneti, Ranucci, Scuota, & Branciari, 2015). During slaughtering, carcasses might be contaminated by STEC present in the digestive tract and lead to dissemination of this bacterium to the muscles (Petruzzelli et al., 2016). In the United States, undercooked ground beef has been responsible for nearly 75% of human STEC O157 outbreaks (Bonardi et al., 2015). In recent years, non-O157 STEC-related outbreaks and sporadic cases have been reported, some of them in association with consumption of products of bovine origin (Robbins et al., 2014).

Absence of *E. coli* O157:H7 and non-O157 STEC (mainly O26, O103, O111, O145, and O121 serogroups) strains in ground beef sold at retail markets is mandatory in Argentina (Argentinean Food Code, 2017). However, during 2015, 337 HUS cases were reported, and even though *E. coli* O157:H7 was the predominant serotype isolated from patients, non-O157 STEC strains were responsible for 25.1% of STEC infections (Brusa et al., 2017). Consumption of undercooked beef has been identified as a major risk factor, and meat products of different animal origin have been investigated as the most probable STEC transmission vehicles in this country (Brusa et al., 2013, 2017; Chinen et al., 2009; Jure et al., 2015).

STEC detection is difficult to perform because this pathogen is usually found in very low numbers in the contaminated food. Culture-based methods require at least a 24 hr-enrichment culture of samples, plating on selective media with additional 18 or 24 hr incubation before the suspect colonies can be assayed by biochemical tests and specific methods to identify Shiga-toxin producing strains (Mathusa, Chen, Enache, & Hontz, 2010). In contrast, polymerase chain reaction (PCR)-based molecular methods provide rapid results after plating, and presumptive colonies can be directly confirmed by the detection of virulence genetic markers (Rantsiou, Alessandria, & Cocolin, 2012). Likewise, immunomagnetic separation (IMS) as a concentration step of cells belonging to STEC particular serogroups prior to plating on selective media is a well-established method for STEC detection in food samples (Farrokh et al., 2013).

In the San Luis province, located in the West Central region of Argentina, four to six STEC-HUS cases are annually reported in infants, with 1.86 cases per 100,000 inhabitants detected in 2016 (Ministry of Health of Argentina, 2016). The infection sources, however, have not been clearly identified. Bovine ground meat is a widely used product in the preparation of fast foods and elaborated dishes for sale in retail markets at San Luis city. Fresh sausages ("chorizos") and precooked sausages ("morcillas") are traditional meat products of the Argentine gastronomy. "Chorizos" are prepared with a paste of raw ground beef and pork added with salt and spices, whereas "morcillas" contain a precooked paste of bovine blood, pork fat, ground pigskin, salt, onion, and spices. Both are packed in edible coatings. Since systematic surveillance including STEC monitoring in meat products has not been yet performed in this region, the present study was aimed at (a) knowing the STEC frequency in ground beef and fresh and precooked sausages sold in retail stores in the capital city of the San Luis province, (b) assessing the performance of four procedures to detect *stx*-positive samples and STEC strains that include culturing, O157 IMS and PCR, and (c) determining the serotype, the presence of virulence genes, the antimicrobial susceptibility and the genetic diversity of the isolates.

2 | MATERIALS AND METHODS

2.1 | Sample collection

A total of 167 samples (57 raw ground beef, 58 fresh sausages, and 52 precooked sausages) were collected between April 2012 and August 2013 from 32 commercial establishments (butcher shops and supermarkets) located in San Luis city (33°17′42″S, 66°20′08″W), Argentina. Sample size was calculated for an estimated 10% prevalence of STEC in ground beef in this region (95% confidence level and $<5\%$ precision) (Thrusfield, 1990). Sampling collection was randomly performed and covered all the geographic areas of the city. It was performed during two periods: from April to August 2012 when 78 samples (26 raw ground beef, 27 fresh sausages, and 25 precooked sausages) were collected from 27 retail stores, and from April to August 2013 when 89 samples (31 raw ground beef, 31 fresh sausages, and 27 precooked sausages), were collected from the same 27 establishments and from other five retail stores, making a total of 32 stores for the second year when the IMS was applied. Thus, 27 retail stores and supermarkets were sampled in two opportunities: before and after the IMS technique was included in the STEC detection protocol. After purchase, each sample was transported under refrigeration to our laboratory and immediately processed.

2.2 | Bacterial reference strains

E. coli O157:H7 EDL933 stx_1/stx_2 and *E. coli* ATCC 25922 were used in all assays as positive and negative reference strains, respectively. These organisms were maintained at 4°C on trypticase soy agar slants (TSA, Britania Laboratories, Buenos Aires, Argentina).

2.3 | Isolation and identification of STEC strains

The first step of this study was aimed at detecting stx_1 and/or stx_2 positive samples; the second step was dedicated at isolating and characterizing STEC strains. As described by Rantsiou et al. (2012), 20 g of each sample were mixed with 80 ml of trypticase soy broth (TSB, Britania Laboratories), homogenized in a stomacher (IUL Masticator, Koningswinter, Germany) for 90 s and incubated for 18 hr at 37°C. The following procedures were then applied: (a) 1 ml of TSB enrichment broth was subjected to DNA extraction and duplex PCR targeted to stx_1/stx_2 genes (TSB/PCR) was performed; (b) a loopful of TSB culture was streaked on Sorbitol Mac Conkey agar (SMAC, Britania Laboratories), and incubated 24 hr at 37°C. DNA was extracted from confluent growth zone, from pools of 5 to 10 nonfermenting colonies (probably belonging to O157 STEC) and from pools of five to ten fermenting colonies (probably belonging to non-O157 STEC), and then assayed by duplex PCR; colonies from positive pools were analyzed individually by duplex PCR for the detection of the *stx*-positive colonies (TSB/SMAC/PCR); (c) 1 ml of TSB culture was mixed with 20 μl of immunomagnetic anti-*E. coli* O157 coated beads (1.2×10^7 beads/ml) and IMS was

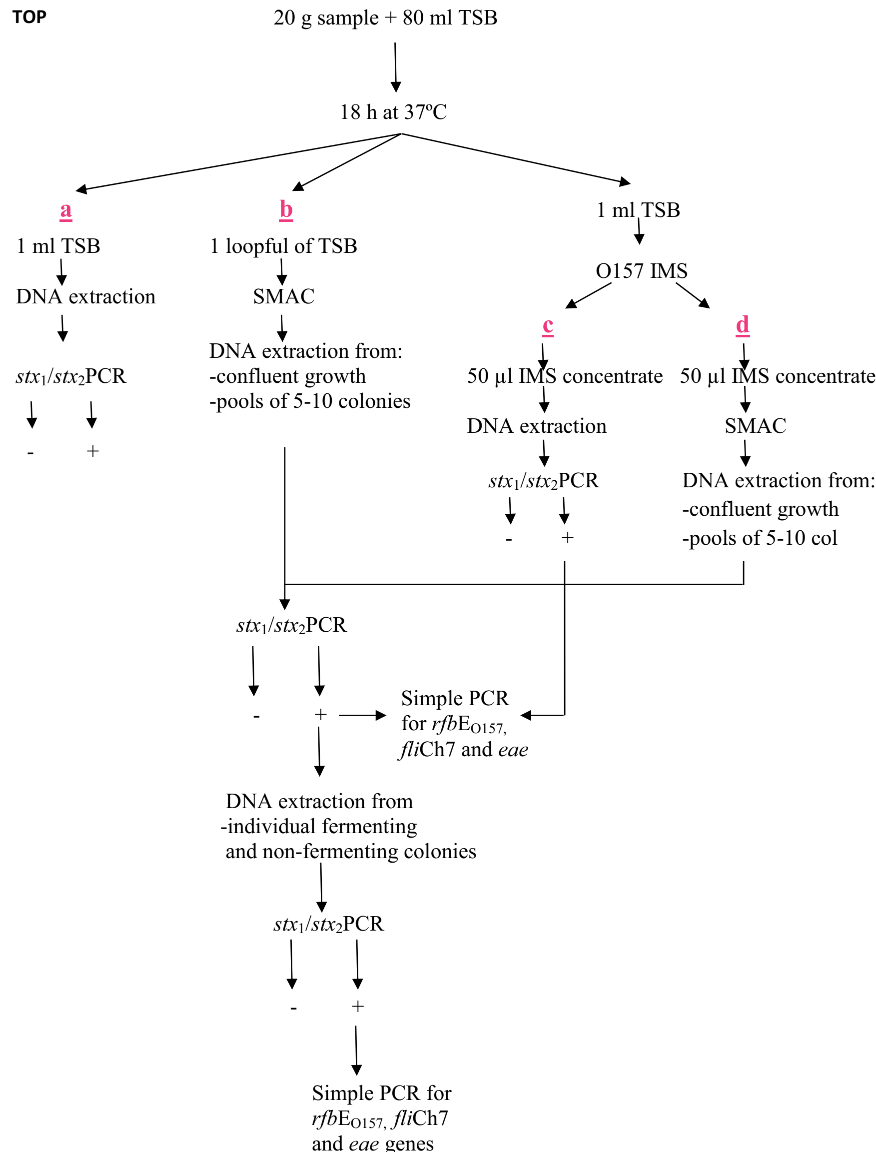

FIGURE 1 Diagram showing four approaches to search STEC in meat samples. a = TSB/PCR; b = TSB/SMAC/PCR; (c) = TSB/IMS/PCR; d = TSB/IMS/SMAC/PCR

performed according to the manufacturer's instructions (Dynabeads M-280; Dynal A/S, Oslo, Norway), 50 µl of the TSB/IMS concentrate were then subjected to DNA extraction for duplex PCR (TSB/O157IMS/PCR); and (d) other 50 µl of the TSB/IMS concentrate were streaked on SMAC and incubated 24 hr at 37°C. Duplex PCR was performed on DNA from the confluent growth zone, from pools of 5 to 10 nonfermenting colonies and from pools of 5 to 10 fermenting colonies (TSB/O157IMS/SMAC/PCR), and if *stx*-amplification was observed, colonies were individually screened by duplex PCR. Screening by single PCRs was also performed for *rfb*E_{O157}, *fli*Ch7, and *eae* genes on DNA from SMAC confluent growth zone, pooled colonies, O157 IMS concentrates, and individual *stx*-positive colonies. Figure 1 shows a diagrammatic representation of the four procedures. As control, a 20-g sample of raw ground beef was artificially contaminated with two to three colonies of the reference STEC strain and subjected to the same procedures as the other samples.

The *stx*-positive isolates obtained on SMAC were submitted to the STEC National Reference Laboratory at the ANLIS-INEI "Dr. Carlos G. Malbran" Institute (Buenos Aires, Argentina) for further determination of Stx production, enterohemolysin, serotype, *eae*, *ehxA*, and *stx*-variant characterization. In addition, a *Xba*I–PFGE-based genetic diversity study was performed.

2.4 | Duplex PCR targeting STEC stx_1/stx_2 genes

The boiling method for DNA extraction and the PCR protocol proposed by Leotta et al. (2005) with minor modifications were applied. The primer pairs used to amplify particular target regions of stx_1 and stx_2 genes are summarized in Table 1. Duplex PCR was performed in a 25-µl final volume containing 1× PCR buffer, 0.1 mM (each) deoxynucleoside triphosphates, 2 pmol/µl stx_1 primers, 0.4 pmol/µl stx_2 primers (Tecnolab S.A., Buenos Aires, Argentina), 1.5 mM $MgCl_2$, 0.02 U/µl Taq

TABLE 1 Primers used for PCR targeted to STEC genes and amplicon sizes

Gene	Sequence (5′–3′)	Amplicon length (bp)	Reference
stx_1	GAAGAGTCCGTGGGATTACG AGCGATGCAGCTATTAATAA	130	Leotta et al. (2005)
stx_2	TTAACCACACCCCACCGGGCAGT GCTCTGGATGCATCTCTGGT	346	Leotta et al. (2005)
$rfbE_{O157}$	CGGACATCCATGTGATATGG TTGCCTATGTACAGCTAATCC	259	Paton and Paton (1998)
*fli*Ch7	GCGCTGTCGAGTTCTATCGAGC CAACGGTGACTTTATCGCCATTCC	625	Gannon et al. (1997)
eae	CCCGAATTCGGCACAAGCATAAGC CCGGATCCGTCTCGCCAGTATTCG	864	Karch et al. (1993)
ehxA	GGTGCAGCAGAAAAAGTTGTAG TCTCGCCTGATAGTGTTTGGTA	1551	Schmidt et al. (1995)

polymerase (Embiotec SRL, Buenos Aires, Argentina), 2 μl of DNA template, and ultrapure water. An internal amplification control designed in our laboratory (Salinas-Ibáñez et al., 2015) was added to the reaction mix at 0.143 pg/μl per PCR microtube. PCR amplification conditions were 94°C for 5 min, and 30 cycles, each consisting of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 3 min. PCR products were subjected to electrophoresis in a 2% agarose gel stained with Gel Red Acid Gel Stain® (Biotium, Hayward, CA) at 80 V for 40 min, and visualized in an UV transilluminator (UVP, Upland, CA). A 100 bp DNA ladder (Embiotec SRL) was included as a molecular mass reference.

2.5 | Phenotypic characterization

The stx_1 or/and stx_2-harboring isolates were confirmed as *E. coli* by Gram staining and classical biochemical tests, including sorbitol fermentation and the β-glucuronidase test. Serotyping was performed with Staten Serum Institute anti-"O" sera set (O1-O181) (Statens Serum Institut, Copenhagen, Denmark) for the somatic antigen and Denka Seiken anti-"H" sera set (Denka Seiken Co., Ltd., Tokyo, Japan). To determine Stx production, bacterial supernatant fluids, and periplasmic cell extracts were used in cytotoxicity assays on Vero cells (Karmali, Petric, Lim, Cheung, & Arbus, 1985). Enterohemolysin production was determined on sheep blood agar plates (Beutin et al., 1989). The antimicrobial susceptibility of the STEC isolates was assessed by the disk diffusion method (Britania Laboratories). The following antibiotic disks (Britania Laboratories) were assayed: aztreonam, 30 μg; erythromycin, 15 μg; cefotaxime, 30 μg; chloramphenicol, 30 μg; gentamicin, 10 μg; nitrofurantoin, 300 μg; ofloxacin, 5 μg; piperacillin 100 μg; and trimethoprim-sulfamethoxazole, 25 μg. Zones of growth inhibition were evaluated according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2014).

2.6 | Genotypic characterization

The *fli*Ch7, *eae*, $rfbE_{O157}$, and *ehxA* genes were studied in the DNA of all *stx*-positive samples and all STEC strains by single PCR using the Gannon et al. (1997), Karch et al. (1993), Paton and Paton (1998), and Schmidt, Beutin, and Karch (1995) primers, according to previously standardized protocols. The primer sequences and the fragment sizes amplified by PCR are summarized in Table 1. Gel electrophoresis and visualization of products were performed as described.

2.7 | Subtyping of STEC strains

Genotyping of stx_2 variants was done by a restriction fragment length polymorphism analysis of the B-subunit-encoding DNA fragments obtained by PCR (Tyler, Johnson, Lior, Wang, & Rozee, 1991). The genetic diversity study was performed by using *Xba*I-PFGE according to Pulse-Net 24 hr-standardized protocol for *E. coli* O157 (Centers for Disease Control & Prevention, 1998). The analysis of DNA electrophoretic patterns was done with the program Bionumerics Software Package 5.1 (Applied Maths, Belgium) using Dice index and UPGMA to generate dendrograms.

2.8 | Sequencing of PCR products

To confirm the specificity of the stx_2 amplification product from the positive sample detected in 2012, the DNA band was sequenced by Dr. A. Puebla at the Genomic Unit, Biotechnology Institute, National Institute of Agronomic Technology, Hurlingham, Buenos Aires, Argentina.

2.9 | Statistical analysis

Statistical analysis on STEC frequency by sampling year, type of meat product, the detection procedure and virulence gene profiles was performed by using Chi square test (Analytical Software, Tallahassee FL). Calculations were based on confidence level equal to or higher than 95% ($p \leq .05$ was considered statistically significant).

3 | RESULTS

The frequency of *stx*-detection related to the sampling period and type of samples can be seen in Table 2. Samples were considered *stx*-positive when a signal for one or both *stx* genes was obtained by PCR after

TABLE 2 Detection of *stx*-positive samples related to sampling year and type of meat products in San Luis city, Argentina (2012–2013)

Type of meat product	Sampling year 2012 Total samples	*stx*-positive samples (n)	*stx*-detection (%)	95% CI[a]	2013 Total samples	*stx*-positive samples (n)	*stx*-detection (%)	95% CI	2012–2013 Total samples	*stx*-positive samples (n)	*stx*-detection (%)	95% CI
Raw ground beef	26	1	3.8	11.4–0	31	6	19.3	34.8–3.9	57	7	12.3	21.2–3.3
Fresh sausages	27	0	0	-	31	6	19.3	34.8–3.9	58	6	10.3	18.6–2.1
Precooked sausages	25	0	0	-	27	6	22.2	40–4.4	52	6	11.5	20.8–2.3
Total	78	1	1.3	3.8–0	89	18	20.2	29.6–10.9	167	19	11.4	16.5–6.2

[a]95% CI = 95% confidence interval.

applying the TSB/PCR, TSB/SMAC/PCR, TSB/O157 IMS/PCR, or TSB/O157 IMS/SMAC/PCR procedures. Overall, a *stx*-detection frequency of 11.4% (19 *stx*-positive samples/167 total samples) was observed, with 12.3% (7/57) for raw ground beef, 11.5% (6/52) for precooked sausages, and 10.3% (6/58) for fresh sausages. The detection rate increased in 2013 (20.2%, 18/89) as compared to 2012 (1.3%, 1/78) coinciding with the O157 IMS introduction in the detection protocols. During 2012, only one stx_2 positive sample from raw ground beef was detected by TSB/SMAC/PCR, but the STEC strain could not be recovered. The identity of this stx_2 amplification fragment was confirmed by DNA sequencing. Due to negative results obtained by TSB/PCR, this procedure was not applied in 2013. During 2013, *stx* genes were detected in 18 samples, with a distribution of 22.2% (6/27) for precooked sausages, 19.3% (6/31) for raw ground beef and 19.3% (6/31) for fresh sausages (Table 2).

Each *stx*-positive sample could be detected after applying one or more procedures. Eleven samples were detected by one procedure, five samples were detected by two procedures, and three samples were detected by three procedures. Results related to the type of samples, STEC detection procedures, genetic markers, and number of isolates for 19 *stx*-positive samples are summarized in Table 3. The TSB/O157IMS/PCR procedure yielded the highest *stx* detection frequency (79%, 15/19 samples) followed by TSB/SMAC/PCR (42.1%, 8/19) and TSB/O157IMS/SMAC/PCR (36.8%, 7/19) ($p \leq .05$). One precooked sausage (1.9%, 1/52) was positive by both TSB/O157 IMS/PCR and TSB/O157 IMS/SMAC/PCR procedures, and one strain (STEC 1) was recovered from this sample. One fresh sausage sample (1.7%, 1/58) was *stx*-positive by the three procedures, and two strains (STEC 2 and STEC 3) were isolated from this sample by using TSB/O157IMS/SMAC/PCR.

The stx_2 gene (84.2%, 16/19) was more frequently detected than stx_1 (21.1%, 4/19) ($p \leq .05$) when duplex PCR targeting stx_1/stx_2 genes was used as a screening technique. Both genes were simultaneously detected only in one ground beef sample. Based on the *stx*-positive samples, other genes (*eae*, $rfbE_{O157}$, and *fli*Ch7) were also screened in the DNA extracted from SMAC confluent growth zone, pooled colonies or O157 IMS concentrates. By different single PCRs, the *eae* virulence gene was detected in two stx_2-positive samples (10.5%, 2/19) including one fresh sausage and one precooked sausage; the $rfbE_{O157}$ gene was detected in two stx_2-positive samples (10.5%, 2/19) including one ground beef sample and the *eae*-positive precooked sausage; and the *fli*Ch7 gene was detected in eleven samples (58%, 11/19) including all the types of samples (Table 3).

Three STEC strains were isolated, STEC 1 from precooked sausage, and STEC 2 and STEC 3 from fresh sausage (Table 4). These two samples represented 1.2% of the total samples. STEC 1 was characterized as ONT:H7 (NT: not typeable)/stx_{2c}/*fli*Ch7, and STEC 2 and STEC 3 were identified as ONT:HNT/stx_{2c} and O112:H19/stx_{2c}, respectively. The three isolates were Stx2 producers when cytotoxicity was assayed by the Vero cell test and they did not show enterohemolytic activity.

STEC strains were sensitive to all antimicrobials assayed, except for erythromycin. By *Xba*I-PFGE, STEC 1 and STEC 3 exhibited 72.1% similarity (Figure 2). STEC 2 was untypeable.

TABLE 3 Positive *stx*-samples, STEC genetic markers detected by PCR in SMAC confluent growth zone, pooled colonies or O157-IMS concentrates, and number of STEC isolates according to STEC detection procedures applied during 2012–2013

			Positive samples according to detection procedure				STEC genetic markers detected in SMAC confluent growth zone, pooled colonies or O157-IMS concentrates					
Year	Month	Positive samples	a	b	c	d	stx_1	stx_2	*eae*	$rfbE_{O157}$	*fliCh7*	Number of STEC isolates
2012	August	Ground beef	–	+	NP	NP	–	+	–	–	+	–
2013	April	Fresh sausage	NP	–	+	–	–	+	–	–	–	–
	April	Precooked sausage	NP	–	+	–	–	+	–	–	+	–
	April	Ground beef	NP	–	+	–	–	+	–	–	–	–
	April	Fresh sausage	NP	+	+	+	–	+	–	–	–	2
	April	Precooked sausage	NP	–	+	+	–	+	–	–	+	1
	April	Ground beef	NP	–	+	–	–	+	–	+	+	–
	May	Ground beef	NP	+	+	+	+	+	–	–	+	–
	May	Fresh sausage	NP	–	+	–	–	+	+	–	–	–
	May	Precooked sausage	NP	–	+	–	–	+	–	–	–	–
	May	Ground beef	NP	–	+	–	–	+	–	–	+	–
	May	Fresh sausage	NP	–	+	–	–	+	–	–	–	–
	May	Fresh sausage	NP	+	–	–	–	+	–	–	+	–
	May	Precooked sausage	NP	+	+	+	–	+	–	–	+	–
	May	Fresh sausage	NP	–	+	+	+	–	–	–	+	–
	May	Precooked sausage	NP	–	+	+	+	–	–	–	+	–
	July	Precooked sausage	NP	+	–	–	–	+	+	+	–	–
	August	Ground beef	NP	+	+	–	+	–	–	–	+	–
	August	Ground beef	NP	+	–	+	–	+	–	–	–	–
	Total	19	0	8	15	7	4	16	2	2	11	3

a = Trypticase soy broth/polymerase reaction chain (TSB/PCR); b = Trypticase soy broth/Sorbitol Mac Conkey agar/polymerase reaction chain (TSB/SMAC/PCR); c = Trypticase soy broth/IMS/polymerase reaction chain (TSB/IMS/PCR); d = Trypticase soy broth/IMS/Sorbitol Mac Conkey agar/polymerase reaction chain TSB/IMS/SMAC/PCR; NP = nonperformed.

4 | DISCUSSION

In this study, STEC presence in meat products intended for human consumption in San Luis city was evaluated by four detection procedures. During a 2-year period, 11.4% of meat product samples were *stx*-positive. The low frequency of *stx*-positive results observed in 2012 might be attributed to the poor performance of TSB/PCR and TSB/SMAC/PCR procedures. Since organic compounds of the food matrix could have interfered in the PCR performance, the TSB/PCR procedure was not applied in 2013. A slight improvement in *stx* gene detection was observed when SMAC plating was introduced after TSB enrichment and prior to PCR. Significant increases of *stx*-detection in ground beef (19.3%), fresh sausages (19.3%), and precooked sausages (22.2%) were observed when O157 IMS was introduced in detection protocols. Interestingly, the use of O157 IMS led to increased *stx* gene detection in samples as seen by using TSB/O157IMS/PCR (15/19) procedure. However, no STEC O157 strains were recovered. This could be explained by the cross-reactivity between the O157 immunomagnetic beads and antigens of *E. coli* non-O157 strains present in samples. Although anti-*E. coli* O157 antibodies are attached to immunomagnetic beads, bindings with strains corresponding to other STEC serogroups might occur. The degree of nonspecificity might depend on the source of IMS beads. Cernicchiaro et al. (2013) reported a degree of cross-reactivity with Dynabeads (Invitrogen, Carlsbad, CA), when different STEC serogroups isolated from bovine feces were determined. As reported by Wang, Chen, and Jiang (2016), the IMS capture efficiency can vary among different non-O157 STEC serotypes in dairy compost.

Screening of *stx* by PCR is useful for the presumptive diagnosis of clinical infections triggered by STEC (Parsons, Zelyas, Berenger, & Chui, 2016), identification of reservoirs (Brusa et al., 2013) and monitoring of STEC contamination in foods (Etcheverría et al., 2010). Likewise, considering the importance of STEC illnesses in public health, the isolation, characterization and typing of strains is highly recommended for epidemiological purposes (Jure et al., 2015); therefore, effective protocols

TABLE 4 Phenotypic and genotypic characteristics of STEC strains isolated from precooked and fresh sausages

		Serotype		Phenotype				Genotype						
Type of meat product	Strain	O	H	*β-glu*	Sor	E-Hly	Stx	stx_1	stx_2	*eae*	*ehxA*	$rfbE_{O157}$	*fliCh7*	stx_2 variant
Precooked sausage	STEC 1	NT	7	+	+	–	+	–	+	–	–	–	+	Stx2c
Fresh sausage	STEC 2	NT	NT	+	+	–	+	–	+	–	–	–	–	Stx2c
	STEC 3	112	19	+	+	–	+	–	+	–	–	–	–	Stx2c

β-glu = beta-glucuronidase; E-Hly = enterohemolysin; H = flagellar antigen; O = somatic antigen; Sor = sorbitol fermentation; Stx = Shiga toxin.

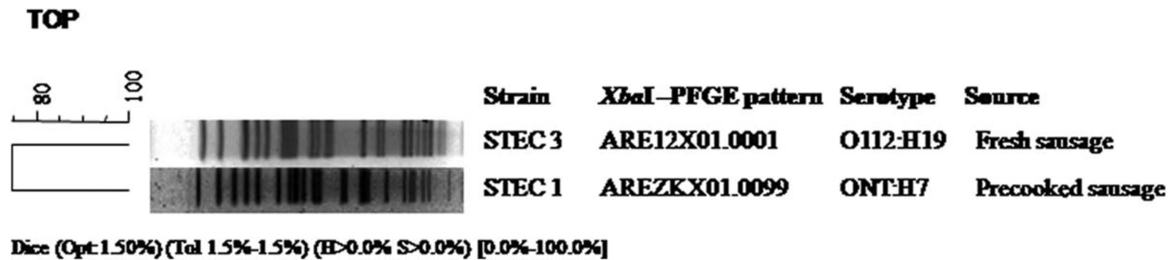

FIGURE 2 Clonal relatedness of STEC strains isolated from meat products in San Luis, Argentina

for STEC isolation and identification should be applied to food samples with *stx*-positive PCR signal (U.S. Food and Drug Administration, 2016). The duplex PCR protocol applied in the present study yielded positive results in the search of *stx*-positive pediatric feces until a detection limit of 10^4 STEC CFU g^{-1} of stools (Salinas-Ibáñez et al., 2015). The same PCR protocol including primers targeted to the *rfb*E_{O157} gene was used by Brusa et al. (2017) to characterize non-O157 STEC strains in beef carcasses, cuts and trimmings of abattoirs in Argentina. These authors used Mac Conkey agar and Levine-eosin-methylene blue agar instead of SMAC to isolate STEC colonies. PCR screening for *stx* genes was very helpful in our research, but even though 19 of 167 samples were *stx*-positive, the isolating steps require adjustments to increase the STEC recovery. Besides, the results of PCR screening for genes such as *rfb*E_{O157} (2/19 samples), *fli*Ch7 (11/19), and *eae* (2/19) in *stx*-positive samples may indicate that STEC strains carrying these genes were present but they could not be recovered.

As regards the ground meat, in this study 7 of 57 (12.3%) samples were *stx*-positive by PCR, but STEC strains could not be recovered. This result was significantly different to the percentage of *stx*-positive samples (36.1%) and the number of STEC strains (57) obtained by Llorente, Barnech, Irino, Rumi, and Bentancor (2014) after the analysis of 252 ground beef samples collected in markets from an urban area of Buenos Aires Province (Eastern Argentina) by using broth enrichment, IMS, and plating on selective media. In another study carried out by Etcheverría et al. (2010) in a farming area located in Buenos Aires, the detection of the *stx* gene was also used as an indicator of STEC carriage by meat. These authors observed that 25% of retail beef cuts were STEC-positive with significant differences among the different cuts (12.12% in chuck and rump roast and 40.74% in ground beef). STEC prevalence in bovine ground meat may be related to the carriage of STEC in cattle. As known, the Buenos Aires Province belongs to the main beef-producing area in Argentina where 39–44% STEC carriage has been reported from stools of healthy young steers and adult bovines (Meichtri et al., 2004). In contrast, a low level of STEC carriage has been observed in San Luis cattle where stx_1/stx_2 genes have been detected by PCR in 2.3% bovine stools (Favier, Lucero Estrada, Cortiñas, & Escudero, 2014).

In this work, two STEC strains were isolated from fresh sausage samples, in agreement with results reported for other regions of the country (Jure et al., 2015). Therefore, this food could represent a human health risk when appropriate cooking temperatures are not applied. Although bovine ground beef is the major ingredient of fresh sausages, ground pork is also included in the preparation. Considering that 4.6% of pork meat in Argentina has been demonstrated to be STEC-positive (Colello et al., 2016), it can be speculated that pork may be a vehicle for STEC transmission in fresh sausages.

The heat treatment applied during the elaboration of precooked sausages destroys viable bacteria, and the survival of STEC in the manufactured product should not be possible. In the present study, however, one STEC strain (1.9%) was recovered from precooked sausages. In an investigation performed in Argentina, Oteiza, Chinen, Miliwebsky, and Rivas (2006) also detected STEC in precooked sausages and reported that the samples studied contained *Enterobacteriaceae* (100%) and fecal coliforms (81%), which indicate inadequate application of the thermal treatment, deficient hygiene conditions during the elaboration, and transportation of the product or low microbiological quality of raw materials. It is important to emphasize that this type of food can be consumed without additional cooking, so good manufacturing practices (GMP) and the determination of critical control points along the production and distribution chain are required to avoid possible infections in humans.

Etcheverría et al. (2010) detected the stx_2 gene in 70% of the retail beef cuts studied in their work. Similarly, the present study showed that this gene was the most frequent virulence marker (84.2%) in 19 *stx*-positive samples of meat products in San Luis, followed by the stx_1 gene (21.0%). It is well known that STEC strains carrying stx_2 gene constitute a great risk to public health because they frequently lead to extraintestinal complication like HUS (Etcheverría & Padola, 2013). Coincidently, the stx_2 genotype has been the most frequently identified one (90.3%) among STEC strains isolated from symptomatic patients in Argentina (Rivas et al., 2006).

Regarding the serotypes and genotypes of the STEC strains isolated in San Luis, one strain recovered from precooked sausage was ONT:H7 stx_{2c}/*fli*Ch7, while two isolates from fresh sausage were ONT:HNT and O112:H19 and both were stx_{2c} positive. No STEC strain was *eae* positive. Subtypes such as stx_{2c} and stx_{2a} have been more frequently associated to HUS than others (Iyoda et al., 2014).

To date, the O112 serotype has not been involved in STEC outbreaks or sporadic cases in humans in our region. This serotype has been previously identified in cattle from Argentina (Meichtri et al., 2004); thus, its presence in fresh sausages could imply a risk for the public health. The remaining STEC strains recovered in this study might also be considered a hazard for consumers since they are Shiga toxin producers. In a study performed in 2014, Brusa et al. (2017) obtained 6% prevalence of non-O157 STEC in beef carcasses, cuts, and trimmings from eight Argentinean abattoirs. Previously, Masana et al.

(2011) observed 9% non-O157 STEC contamination in beef carcasses from Argentina. The main serotypes identified by these authors were O178:H19, O8:H19, O130:H11, and O113:H21, all of which have produced sporadic cases of HUS in Argentina and worldwide.

Intervention strategies to avoid the contamination of meat products and consequently, to reduce the STEC incidence in humans, have been focused on live cattle on the farm, on slaughter plants, and on the final product at retail (Brashears & Chaves, 2017; Leotta et al., 2016). The National Service of Agrifood Health and Quality of Argentina (SENASA, for its Spanish acronym) applies intervention measures in order to reduce these bacteria on carcasses (SENASA, 2014). Thus, the use of physical (water vapor and vacuum) or chemicals (diluted organic acids) decontamination agents is recommended to reduce the bacterial load on carcasses. Other strategies proposed for STEC control along the food chain are new vaccine formulations (McNeilly et al., 2015), probiotics, and modifications in the production practices and process control based on GMP and Hazard Analysis and Critical Control Point (Brashears & Chaves, 2017).

Antimicrobials intended for veterinary use could be a risk factor for the emergence of resistance in STEC isolates of animal origin. Ramoneda et al. (2013) studied antimicrobial susceptibility in *E. coli* O157 isolated from bovine carcasses in Spain and observed that 32% of strains were susceptible to all antimicrobials tested, but the remaining isolates showed high degree of resistance, particularly to sulfonamide, tetracycline, streptomycin, and trimethoprim/sulfamethoxazole. In contrast, the STEC strains analyzed in the present study were susceptible to all the antimicrobials assayed except for erythromycin.

Subtyping techniques such as *Xba*I-PFGE allow establishing epidemiological relationships between STEC strains isolated from different sources and controlling the spread of circulating clones in a geographical area (Masana et al., 2010). In the present study, *Xba*I-PFGE analysis revealed different genetic profiles of STEC strains according to the isolation source and the strain serotype.

This study demonstrated that meat products intended for human consumption in this region can be vehicles for the transmission of STEC to humans. STEC strains isolated from sausages in this work represent a hazard for consumers. Survival of STEC at cooking temperatures below 160 °F/71.1°C, its acid resistance and its low infective dose (<100 cells) contribute to the pathogenic potential of this bacterium. The results of this research encourage further studies to continue STEC surveillance in meat products from this Argentine region and to improve the detection procedures in these foods and other possible transmission vehicles of this pathogen. Additionally, livestock control programs as well as good practices for slaughtering, processing, and manufacturing plants should be implemented to reduce STEC spreading in the food chain. Adequate cooking of ground beef and fresh and precooked sausages is recommended to consumers.

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