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Development and validation of two SYBR green PCR assays and a multiplex real-time PCR for the detection of Shiga toxin-producing *Escherichia coli* in meat

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ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) are recognized as food-borne pathogens. We developed and validated two SYBR green PCR (SYBR-PCR) and a real-time multiplex PCR (RT-PCR) to detect stx_1 and stx_2 genes in meat samples, and compared these techniques in ground beef samples from retail stores. One set of primers and one hydrolysis probe were designed for each *stx* gene. For RT-PCR, an internal amplification control (IAC) was used. All PCR intra-laboratory validations were performed using pure strains and artificially contaminated ground beef samples. A total of 50 STEC and 30 non-STEC strains were used. Naturally contaminated ground beef samples (n = 103) were obtained from retail stores and screened with SYBR-PCR and RT-PCR, and *stx*-positive samples were processed for STEC isolation. In the intra-laboratory validation, each PCR obtained a 1×10^2 CFU mL^{-1} limit of detection and 100% inclusivity and exclusivity. The same results were obtained when different laboratory analysts in alternate days performed the assay. The level of agreement obtained with SYBR-PCR and RT-PCR was *kappa* = 0.758 and 0.801 ($P < 0.001$) for stx_1 and stx_2 gene detection, respectively. Two PCR strategies were developed and validated, and excellent performance with artificially contaminated ground beef samples was obtained. However, the efforts made to isolate STEC from retail store samples were not enough. Only 11 STEC strains were isolated from 35 *stx*-positive ground beef samples identically detected by all PCRs. The combination of molecular approaches based on the identification of a virulence genotypic profile of STEC must be considered to improve isolation.

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1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are important foodborne human pathogens that cause mild to bloody diarrhoea, haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS), and can even lead to death (Adamu et al., 2014; Karmali et al., 2010). STEC strains are a heterogeneous group of bacteria carrying stx_1, stx_2, or both genes that codify for cytotoxins, which are considered essential virulence factors for human disease development (Calderwood and Mekalanos, 1987; Krüger and Lucchesi, 2015). *E. coli* O157:H7 is by far the most prevalent serotype associated with large outbreaks and sporadic cases of HC and HUS in many countries (Atkinson et al., 2012; EFSA Panel on Biological Hazards (BIOHAZ), 2013; Leotta et al., 2008). However, there is a subset of STEC serogroups most frequently associated with severe diseases worldwide, such as O26, O45, O103, O111, and O145 (Atkinson et al., 2012; EFSA Panel on Biological Hazards (BIOHAZ), 2013).

Vegetables and sprouts have been frequently associated with outbreaks all over the world (EFSA Panel on Biological Hazards (BIOHAZ), 2013). However, ruminants are the main animal reservoir of STEC currently known, and contaminated foodstuffs derived from cattle are responsible for human illness (EFSA and ECDC (European Food Safety Authority and European Centre for Disease Prevention and Control), 2015; Hussein, 2007; Martin and Beutin, 2011). In Argentina, several studies have been conducted to determine STEC prevalence in cattle and meat samples (Llorente et al., 2014; Masana et al., 2010; Masana et al., 2011; Meichtri et al., 2004; Varela et al., 2008) using end-point PCR for *stx* screening. This technique is a laborious process that requires the separation of amplified DNA by gel electrophoresis. Alternatively, PCR products can be nowadays detected using a DNA binding dye, such as SYBR green PCR (SYBR-PCR), or fluorescent probes (Kagkli et al., 2011; Kagkli et al., 2012). As SYBR-PCR is an automated but not a real-time PCR (RT-PCR), results cannot be analysed until a dissociation curve is constructed at the end of thermal cycling. RT-PCR allows identification of positive samples during cycling, before the run ends. SYBR-PCR is less specific than RT-PCR assays that use hydrolysis probes or

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molecular beacons (Jothikumar and Griffiths, 2002). RT-PCR assays can be automated; they are rapid and most of them include an internal amplification control (IAC) (Anklam et al., 2012; Fratamico et al., 2014). Therefore, RT-PCR is very useful for industrial laboratories because less operator training, infrastructure and facilities are needed, reaction mixtures containing all reagents for PCR are commercially available, and the assay provides fast results.

The demand for standardized rapid analytical screening for the detection of STEC in food, such as PCR-based methods, requires the establishment of an appropriate validation approach (Kagkli et al., 2011). The limit of detection$_{50}$ (LOD_{50}), defined as the analyte concentration at which the probability of detection is equal to 50% (AOAC International, 2012), allows us to predict the capacity of the technique to identify samples with low bacterial initial charges. The dynamic range (DR), i.e., the range of concentrations over which a method performs in a linear manner with acceptable levels of accuracy and precision (Kagkli et al., 2011), is necessary to establish PCR efficiency (Eff), which is considered acceptable between 90 and 110%. The robustness study ensures PCR reliability during normal use, even when applying small but deliberate variations in method parameters (AOAC International, 2012). Inclusivity is calculated with the strains of the target analyte(s) that the method can detect (AOAC International, 2012). For *stx* screening, the greatest amount of Stx subtypes (Stx_{1a}, Stx_{1c}, Stx_{1d} and Stx_{2a} to Stx_{2g}) should be included (Scheutz et al., 2012). The exclusivity value is calculated with the non-target strains, which are potentially cross-reactive and not detected by the method (AOAC International, 2012). All validation protocols should include the analysis of these parameters and a matrix study.

Although several end-point-PCR-based methods have been used to find STEC in meat products (Etcheverría et al., 2010; Bosilevac and Koohmaraie, 2011; Ju et al., 2012), few RT-PCR protocols were developed and validated for the detection of all *stx* until 2011 (Kagkli et al., 2011). The emergence of atypical *stx*-positive strains associated with severe disease outbreaks (Frank et al., 2011), such as enteroaggregative *E. coli* O104 that acquired the *stx* gene, caused the implementation of *stx* zero-tolerance criteria in the European Union for meat products, and several beef containers have been rejected since 2012 (The Rapid Alert System for Food and Feed. RASFF, 2015). In this context, additional in-house-validated screening alternatives have been needed to detect all *stx* genes and verify the absence of STEC in meat.

The aims of this study were therefore i) to develop and validate one RT-PCR and two SYBR-PCR assays to detect stx_1 and stx_2 genes in meat samples, and ii) to compare these techniques using ground beef samples from retail stores.

2. Materials and methods

2.1. Bacterial strains

The strains used to determine LOD, robustness, inclusivity and exclusivity, and to perform a matrix study are listed in Table 1. They were stored at −70 °C and grown in 4 mL brain heart infusion (BHI) broth (Biokar, Zac de Ther, France) at 37 ± 1 °C for 18–24 h. This culture was streaked onto BHI agar plates and incubated overnight at 37 ± 1 °C. A single colony from each strain was selected and grown overnight in 4 mL BHI broth at 37 ± 1 °C. When necessary, serial strain dilutions were performed in buffered peptone water (BPW) (Biokar) and the inoculum level was confirmed by plating on plate count agar (Britania, Argentina). Plates were incubated at 37 °C for 18–24 h. All strains belong to the strain collection of IGEVET (Instituto de Genética Veterinaria "Ing. Fernando Noel Dulout"; UNLP-CONICET LA PLATA).

2.2. Primer and probe design

One set of primers and one hydrolysis probe were designed for each *stx* gene. The DNA sequences of stx1 (Accession numbers: X07903, M19437, AJ132761, M19473, 16,625, M23980 M21947, L04539, Z36899, Z36900, Z36901, AY170851) and stx2 (Accession numbers: X07865, Z37725, L11078, AF043627, M59432, AB015057, DQ235774, X65949, L11079, AF479828, AF479829, X61283, DQ059012, DQ235775, M36727, M21534, AJ567998, X81417, M29153, AJ010730, AY286000, AB048227, M19437.1, AF329817.1) genes from STEC strains were recovered from GenBank (http://www.ncbi.nlm.nih.gov/genbank) and aligned using PrISM software. For the design, a conserved sequence region was selected, based on multiple alignments of the genes using Primer Express 3.0 software. The primer and hydrolysis probe sequences designed were (5′ to 3′): stx_1 Forward GCAGATAAAT CGCCATTCG, stx_1 Reverse TGTTGTACGAAATCCCCTCTG, stx_2 Forward CATGACAACGGACAGCAGTTA, stx_2 Reverse TCTGGATGCATCTCTGGT CA, stx_1 Probe CY5-AGAGCGATGTTACGGTTTGTTACTG-IABKFQ (stx_1P), and stx_2 Probe FAM-AATGCAAATCAGTCGTCACTCAC-IABKFQ (stx_2P). Both hydrolysis probes were synthesized with a double quencher using ZEN to decrease the background. All primers and probes were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA).

2.3. DNA extraction

To perform DNA extraction from pure culture strains and meat samples, 1 mL BHI and modified *E. coli* broth (mEC) was taken and centrifuged. The pellet was suspended in 150 μL 1% Triton buffer in TE 1× (10 mM Tris: 1 mM Ethylenediaminetetraacetic acid, pH 8) and boiled according to Leotta et al. (2005). For the strain used to determine RT-PCR DR, the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) was used following the manufacturer's instructions.

2.4. SYBR green PCR validation

The validation protocol was carried out according to AOAC International (2012) with minimal modifications. Inclusivity determinations were performed using 19 and 44 STEC strains for SYBR-PCR_1 and SYBR-PCR_2, respectively. The matrix study was performed with three (*stx*$_{1)}$ and seven (*stx*$_{2)}$ STEC strains.

2.4.1. SYBR green PCR conditions

Two SYBR-PCRs were developed. Each set of primers was used to validate stx_1 SYBR green PCR (SYBR-PCR_1) and stx_2 SYBR green PCR (SYBR-PCR_2). The Agilent Mx 3005P qPCR system (Agilent Technologies, Waldbronn, Germany) was used to perform both SYBR-PCRs in a total volume of 25 μL. The optimal primer concentration was defined using a set of primer concentrations ranging from 0.1 to 1 μM for each PCR, selecting those concentrations with amplifications and lower primer dimers. The melting temperature (Tm) of each SYBR-PCR was determined experimentally using IGEVET strain 40 (Table **1**). The tested samples were considered positive when they generated the same melting curve as the positive control. The reaction mixture contained 10 μL PerfeCTa® SYBR® Green SuperMix (Biosciences, Gaithersburg, Maryland, USA), 0.8 μM of each set of stx_1 or stx_2 primers according to the SYBR-PCR performed, 5.6 μL molecular biology-grade water, and 4 μL DNA extraction. IGEVET strain 40 (Table **1**) and molecular biology-grade water were included as external positive and no-template controls in each run. Thermal cycling was as follows: 95 **°C** for 10 min and 40 cycles at 95 **°C** for 10 s and at 56 **°C** for 30 s. The product melt-curve analysis protocol was 95 **°C** for 1 min, 55 **°C** for 30 s, and then an increase of 0.5 **°C**/s to 95 **°C** for 30 s. Fluorescence data were recorded after each 0.5 **°C** increase.

2.4.2. SYBR-PCR limit of detection and robustness

Six stx_1 and six stx_2 STEC strains (Table 1) were used to determine the LOD and robustness of each SYBR-PCR. All stx_1 (stx_{1a}, stx_{1c}, stx_{1d}) and six stx_2 subtypes (stx_{2a}, stx_{2b}, stx_{2c}, stx_{2d}, stx_{2e}, stx_{2g}) were amplified. Our bioinformatic analysis showed that stx_{2f} subtype was not amplified

Table 1
Strains used for $SYBR\text{-}PCR_1$, $SYBR\text{-}PCR_2$ and RT-PCR intra-laboratory validations.

Bacteria	Strain	Source	Serotype	*stx* variant	LOD and R			I	E	MS
					SYBR PCR_1	SYBR PCR_2	RT-PCR			
E. coli	IGEVET 31	U	O11:HNM	stx_{1b}/$stx_{2c(vh-a)}$				*		
E. coli	IGEVET 33	H	O26:H11	stx_{1b}	*		*	*		*
E. coli	IGEVET 36	H	O111:HNM	stx_{1b}	*		*	*		*
E. coli	IGEVET 40	U	O157:H7	stx_{1b}/$stx_{2c(vh-a)}$	*	*	*	*		*
E. coli	IGEVET 47	C	O13:H16	stx_{1b}/$stx_{2c(vh-a)}$				*		
E. coli	IGEVET 59	H	O91:H21	stx_{2a}/stx_{2d}			*	*		*
E. coli	IGEVET 63	H	O145:HNM	$stx_{2c(vh-a)}$		*	*	*		*
E. coli	IGEVET 67	U	O103:H25	$stx_{2c(vh-a)}$		*	*	*		*
E. coli	IGEVET 85	C	O8:H2	$stx_{2d-OUNT}$			*	*		
E. coli	IGEVET 86	C	O112:H2	$stx_{2d-OUNT}$			*	*		
E. coli	IGEVET 103	U	O121:H19	$stx_{2c(vh-a)}$		*	*	*		*
E. coli	IGEVET 34	C	O26:H11	stx_{1b}	*			*		
E. coli	IGEVET 536	E	O130:H11	stx_{1a}/stx_{2only}				*		
E. coli	IGEVET 589	E	O130:H21	stx_{1a}/stx_{2only}	*		*	*		
E. coli	IGEVET 627	H	O157:H7	$stx_{2+2c(vh-a)}$				*		
E. coli	IGEVET 636	E	O79:H19	$stx_{2+2c(vh-b)}$				*		
E. coli	IGEVET 641	F	O174:H21	stx_{1c}/$stx_{2c(vh-b)}$	*			*		
E. coli	IGEVET 642	F	O157:H7	$stx_{2+2c(vh-a)}$				*		
E. coli	IGEVET 650	F	O8:H19	stx_{2only}				*		
E. coli	IGEVET 651	F	O8/O60:H19	stx_{1a}/stx_{2only}				*		
E. coli	IGEVET 702	E	O116:H21	stx_{2only}				*		
E. coli	IGEVET 727	E	O178:H19	$stx_{2c(vh-a)}$		*	*	*		
E. coli	IGEVET 731	F	O181:H49	stx_{1a}/$stx_{2+2c(vh-b)}$				*		
E. coli	IGEVET 763	F	O174:H28	$stx_{2+2c(vh-b)}$				*		
E. coli	IGEVET 771	F	O41:H14	$stx_{2c(vh-b)}$				*		
E. coli	IGEVET 817	E	O109:H25	stx_{2only}				*		
E. coli	IGEVET 844	E	O174:HNM	$stx_{2c(vh-b)}$				*		*
E. coli	IGEVET 848	F	O64:H20	$stx_{2c(vh-b)}$				*		
E. coli	IGEVET 883	F	O141:H49	$stx_{2c(vh-b)}$				*		
E. coli	IGEVET 932	E	ONT:H21	$stx_{2c(vh-b)}$				*		
E. coli	IGEVET 933	F	ONT:H7	$stx_{2c(vh-b)}$				*		
E. coli	IGEVET 1008	E	O171:H14	$stx_{2c(vh-b)}$				*		
E. coli	IGEVET 1012	E	O21:H21	stx_{1a}				*		
E. coli	IGEVET 1033	F	O91:H21	stx_{2only}				*		
E. coli	IGEVET 1036	E	O91:H21	stx_{2only}				*		
E. coli	IGEVET 1039	H	ONT:NM	$stx_{2c(vh-b)}$				*		
E. coli	IGEVET 1044	F	O113:H21	stx_{2only}				*		
E. coli	IGEVET 61	H	O113:H21	$stx_{2c(vh-a)}$		*	*	*		*
E. coli	IGEVET 1255	F	O60:H16	stx_{1a}/stx_{2only}				*		
E. coli	IGEVET 48	C	O146:H28	stx_{1b}/$stx_{2d-OUNT}$				*		
E. coli	IGEVET 35	H	O103:H2	stx_{1b}				*		
E. coli	IGEVET 1304	U	O26:H11	stx_{1a}/$stx_{2c(vh-a)}$				*		
E. coli	IGEVET 1305	U	O174:H21	stx_{1c}/$stx_{2c(vh-b)}$				*		
E. coli	IGEVET 1306	U	ONT:H19	stx_{1d}				*		
E. coli	IGEVET 1307	U	O117:H7	stx_{2c}				*		
E. coli	IGEVET 1308	U	O8:H2	stx_{2c}				*		
E. coli	IGEVET 1309	U	O139:H1	stx_{2e}				*		
E. coli	IGEVET 1311	U	O8:H31	stx_{2g}				*		
E. coli	IGEVET 1322	F	O130:H11	stx_{1a}/$stx_{2+2c(vh-b)}$				*		
E. coli	IGEVET 1323	F	O91:H21	stx_{2only}				*		
P. aeruginosa	IGEVET 1	U							*	
Y. enterocolitica	IGEVET 2	U							*	
S. enterica	IGEVET 8	F	Meleagridis						*	
S. enterica	IGEVET 11	F	Derby						*	
E. coli	IGEVET 12	U							*	
P. aeruginosa	IGEVET 13	U							*	
E. coli	IGEVET 14	U							*	
S. flexneri	IGEVET 15	U	1						*	
P. mirabilis	IGEVET 16	U							*	
S. flexneri	IGEVET 17	U							*	
E. coli	IGEVET 19	U	O127:H6						*	
S. dysenteriae	IGEVET 20	U	2						*	
E. coli	IGEVET 22	U							*	
E. coli	IGEVET 26	U							*	*
S. aureus	IGEVET 115	U							*	
E. coli	IGEVET 345	C	EPEC						*	
K. pneumoniae	IGEVET 390	U							*	
S. flexneri	IGEVET 392	U							*	
E. hermannii	IGEVET 393	U							*	
H. alvei	IGEVET 662	E							*	
H. alvei	IGEVET 663	E							*	
M. morganii	IGEVET 752	E							*	
C. freundii	IGEVET 849	F							*	

Table 1 (*continued*)

Bacteria	Strain	Source	Serotype	*stx* variant	LOD and R			I	E	MS
					SYBR PCR_1	SYBR PCR_2	RT-PCR			
C. freundii	IGEVET 881	E							*	
S. enterica	IGEVET 957	F	Newport						*	
E. cloacae	IGEVET 983	U							*	
S. marcescens	IGEVET 984	U							*	
Shigella	IGEVET 993	U	Boydii						*	
S. enterica	IGEVET 1051	F	Typhimurium						*	
S. enterica	IGEVET 1092	U	Enteritidis						*	

Abbreviations: IGEVET: Instituto de Genética Veterinaria "Ing. Fernando N. Dulout" (UNLP-CONICET), Facultad de Ciencias Veterinarias; H: human; U: unknown; C: cattle, E: environment; F: food; LOD and R: limit of detection and robustness; I: inclusivity; E: exclusivity; MS: matrix study; *: strain used for each parameter determination.

by the primers; therefore, this subtype was not used for the validation protocol.

STEC DNA extractions were carried out from the serial dilution tubes containing 10^1 to 10^5 CFU mL^{-1}. Each SYBR-PCR assay was performed in triplicate. Standard curves of each PCR were constructed by plotting the C_t values against CFU mL^{-1}. Robustness was determined by different laboratory analysts in alternate days amplifying STEC DNA extractions from tubes containing 10^5 CFU mL^{-1}.

2.4.3. Inclusivity and exclusivity of the SYBR-PCR

For inclusivity and exclusivity assays, strains (Table 1) were grown and serially diluted as described in Section 2.1. DNA extractions were performed from the tubes containing 10^4 and 10^5 CFU mL^{-1} of STEC and non-STEC strains, respectively. Inclusivity was determined using 19 and 44 STEC strains for SYBR-PCR_1 and SYBR-PCR_2, respectively. Thirty non-STEC strains were used for all exclusivity assays, including *Citrobacter* and other *stx*-negative bacteria.

2.4.4. SYBR-PCR screening of artificially contaminated samples

Matrix study was performed with three and seven STEC strains for SYBR-PCR_1 and SYBR-PCR_2, respectively. One non-STEC strain was used to study both SYBR-PCRs. Samples obtained from a commercial retail store were confirmed to be *stx* gene-negative by PCR as described by Karch and Meyer (1989) (MK PCR) with some modifications, including the use of a competitive internal amplification control (Masana et al., 2011). The MK PCR was previously in-house validated for *stx* screening from enriched samples and used as a reference method in this study. Portions of 25-g ground beef were artificially contaminated. Serial dilutions of strains (Table 1) were performed as described in Section 2.1. Each sample was inoculated with strains diluted at the following concentrations ranges: STEC strains, 1–4, 9–15, and 87–145 CFU mL^{-1}; non-STEC strains, 10^8 CFU mL^{-1}. One mL of the appropriate dilution was added to each sample. All inoculations were performed in duplicate. Two samples were mock-inoculated with 1 mL of sterile BPW. All samples were incubated at 42 ± 1 °C in 225 mL mEC for 20 h. All DNA extractions were performed as described in Section 2.3.

2.5. RT-PCR validation

The validation protocol was carried out according to AOAC International (2012) with minimal modifications.

2.5.1. RT-PCR conditions

The thermocycler and the final volume per tube were the same as described in Section 2.4.1. The reaction mixture contained 12.5 µL Kapa Probe Fast ABI (BioSystems, Boston, MA, USA), 0.5 µM of each stx_1 primer, 0.125 µM of each stx_2 primer, 0.125 µM of each stx_1P and stx_2P, 2.5 µL 10× Exogenous Internal Positive Control Mix (Applied Biosystems, Life Technologies, Austin, TX, USA), 0.5 µL 50× Exogenous Internal Positive Control DNA (Applied Biosystems), and 4 µL template DNA. TaqMan® Exogenous Internal Positive Control Reagent (Applied Biosystems) was used as IAC in all reactions. IGEVET strain 40 (Table 1), molecular biology-grade water, and Exogenous Internal Positive Control Block (Applied Biosystems) were included as external positive, no-template, and no amplification controls in each run. Thermal cycling was the following: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of amplification at 95 °C for 15 s and at 59 °C for 1 min. Samples were considered *stx*-positive when cycle threshold (C_t) values ≤30 were obtained.

2.5.2. Dynamic range and efficiency of the RT-PCR

These parameters were determined with IGEVET strain 40 DNA (Table 1). DNA quantification was performed using NanoVue (HealthCare Bio-Sciences AB, Uppsala, Sweden). The DNA copy number was calculated with Life Technologies online tool (https://www.lifetechnologies.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/dna-copy-number-calculator.html). The initial concentration was 9.2×10^7 copies μL^{-1}; seven 10-fold log dilutions were performed. Each dilution was assayed in triplicate. Standard curves were constructed to assess stx_1 and stx_2 primer Eff. Amplification Eff was calculated using the following formula: $Ef = (10^{-1/slope}) - 1$.

2.5.3. RT-PCR limit of detection and robustness

LOD and robustness were assessed as described in Section 2.4.2, except that 12 STEC strains (Table 1) were used to determine both parameters.

2.5.4. RT-PCR inclusivity and exclusivity

Inclusivity and exclusivity were evaluated as described in Section 2.4.3, except that 50 STEC strains (Table 1) were used to determine the inclusivity value.

Before these assays, we checked the correct design of the sets of primers by testing SYBR-PCR inclusivity and exclusivity. RT-PCR incorporates specifically labelled probes for *stx* genes. Therefore, in addition to primers, the probes should recognize a specific DNA sequence to obtain a positive signal, so inclusivity and exclusivity RT-PCR assays must be performed.

2.5.5. RT-PCR screening of artificially contaminated samples

A matrix study was carried out as described in Section 2.4.4, except that nine STEC and one non-STEC strains (Table 1) were used to assess each parameter.

2.6. SYBR-PCR and RT-PCR screening of ground beef samples from retail stores

Ground beef samples (n = 103) from 103 retail stores in Argentina were collected from October 2010 to August 2011. Sampling was randomly performed and all the geographic areas of Berisso city were covered. Three different retailers were weekly sampled until all the city's butcher shops were sampled. Twenty-five g portions of each one kg sample were homogenized and incubated at 42 ± 1 °C in mEC for

20 h. DNA extraction was performed as described in Section 2.3. Each sample was screened by both SYBR-PCR and RT-PCR once they arrived at the laboratory. Positive samples were plated onto MacConkey agar (Becton Dickinson Co., Sparks, MD, USA) for STEC isolation as described by Brusa et al. (2013).

2.7. Statistical analysis

To measure the level of agreement between results obtained by SYBR-PCR and RT-PCR for both genes (stx_1 and stx_2), we performed a *kappa* statistic (Cohen, 1960) that expresses the level of agreement beyond what would have been expected by chance (Dohoo et al., 2003). Non-parametric tests were used with Infostat software (Universidad Nacional de Córdoba).

2.8. STEC strain characterization

Serotyping of O and H antigens, as well as *eae* gene (Intimin production) detection, was carried out according to Brusa et al. (2013). A molecular approach on genes encoding virulence characteristics additional to the presence of *stx* genes, such as *aggR* and *aaiC* was proposed (EFSA, 2013). The *agg*R (plasmid-encoded regulator) and *aai*C genes (secreted protein of EAEC) were determined using primers described by the EU Reference Laboratory for *E. coli*, Department of Veterinary Public Health and Food Safety Unit of Foodborne Zoonoses. Istituto Superiore di Sanità (2013). Enteroaggregative *E. coli* strain IGEVET 12 and ATCC 25922 were used as positive and negative controls, respectively. The stx_1 variant was determined with primers stx1a-F1, stx1a-R1, stx1c-F1, stx1c-R1, stx1d-F1, and stx1d-R1 (Scheutz et al., 2012). *E. coli* strains IGEVET 1304, IGEVET 1305, IGEVET 1306, and ATCC 25922 were used as stx_{1a}, stx_{1b}, and stx_{1c} positive and negative controls, respectively. The stx_2 variant was determined with primers VT2-c, VT2-d, VT2v-1, and VT2v-2, and subsequent restriction endonuclease digestion with *Hae*III, *Rsa*I, and *Nci*I (Fermentas, Lithuania) (Tyler et al., 1991). *E. coli* strains EDL933, E32511, IGEVET 98, and ATCC 25922 were used as sxt_{2only}, stx_{2a}, and stx_{2b} positive and negative controls, respectively.

3. Results

3.1. SYBR green PCR validation

SYBR-PCR LOD was 1×10^2 CFU mL^{-1}, and all strains positive at 1×10^3 CFU mL^{-1} were used. Inclusivity detection was successful in all STEC strains analysed by each PCR for the gene carried by each strain, obtaining an inclusivity value equal to 100.0% for both stx_1 and stx_2 SYBR-PCR. None of the non-STEC strains used to determine exclusivity showed amplification signals, obtaining an exclusivity value equal to 100.0%. The same results were obtained with the robustness study, with different laboratory analysts performing the PCRs in alternate days. In the matrix study, samples artificially contaminated with STEC strains were positive, and the sample contaminated with non-STEC strain was negative.

3.2. Multiplex RT-PCR validation

Positive signals were obtained over a range of concentrations (3.7×10^2 to 3.7×10^8 copies per reaction) for all DR repetitions. The Eff of every set of primers and probes was calculated based on the DR of each reaction, obtaining 106.4% and 100.6% for stx_1 and stx_2, respectively (slopes equal to -3.178 and -3.308) (Fig. 1). The LOD was 1×10^2 CFU mL^{-1}, and all strains positive at 1×10^3 CFU mL^{-1} were used. Inclusivity was successfully detected in STEC strains analysed for the stx_1 and/or stx_2 carried by each strain, obtaining an inclusivity value equal to 100.0%. None of the non-STEC strains used to determine exclusivity showed amplification signals, obtaining an exclusivity value equal to 100.0%. During the robustness study, the same PCR results were obtained with different laboratory analysts in alternate days. In the matrix study, the samples artificially contaminated with STEC strains were positive, and the sample contaminated with the non-STEC strain was negative. IAC showed a positive signal in all reactions tubes, excepting those containing no amplification control.

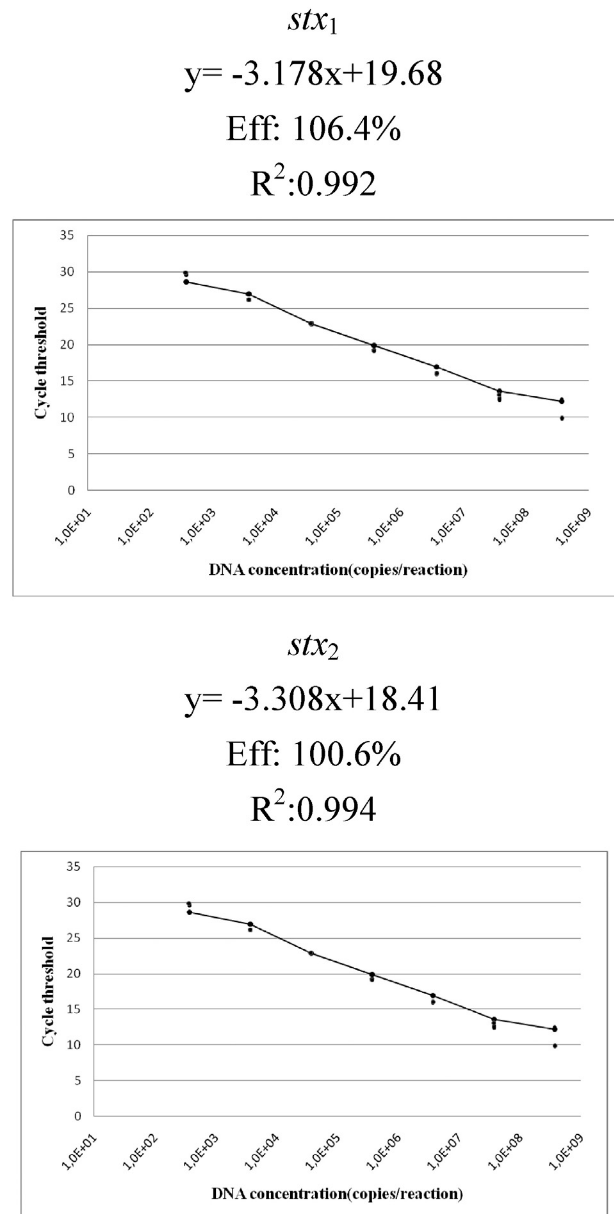

Fig. 1. RT-PCR dynamic range for sxt_1 and stx_2. Standard curves of 3 individual replicates for each concentration (3.7×10^8 to 3.7×10^2 copies per reaction).

3.3. Screening of ground beef samples from retail stores by SYBR-PCR and RT-PCR

From the 103 ground beef samples collected from retails stores, 55 (53.4%) and 46 (44.7%) were *stx*-positive by SYBR-PCR and RT-PCR, respectively. For stx_1 gene detection, the level of agreement (*kappa* = 0.758) between the results obtained by SYBR-PCR and RT-PCR was substantial and statistically significant ($P < 0.001$), even better than expected due to chance (Table 2). In the case of stx_2 gene detection, the level of agreement (*kappa* = 0.801) was almost perfect and statistically significant ($P < 0.001$), as well as better than expected due to chance (Table 2).

Table 2
Screening results of ground beef samples from retail stores by SYBR-PCR$_1$ versus RT-PCR and SYBR-PCR$_2$ versus RT-PCR.

		RT-PCR *stx*$_1$		Total
		Negative	Positive	
SYBR-PCR$_1$	Negative	63	9	72
	Positive	2	29	31
Total		65	38	103
		RT-PCR *stx*$_2$		Total
		Negative	Positive	
SYBR-PCR$_2$	Negative	48	1	49
	Positive	9	45	54
Total		57	46	103

Thirty-five ground beef samples were identically detected by both SYBRs and the RT-PCR performed. Seven of these samples were *stx*$_1$-negative and *stx*$_2$-positive, whereas 28 samples were *stx*$_1$ and *stx*$_2$ positive, simultaneously detected by SYBR and RT-PCR. From these, 11 STEC strains were isolated. The serotype and genotypic characterization (*stx*, *ehxA*, *eae* and *saa*) of nine out of 11 isolates has been partially published (Brusa et al., 2013). Nine different serotypes and ten different genotypes were found: O8:H19 *stx*$_{2only}$, O8:H19 *stx*$_{1a}$/*stx*$_{2+2c(vh-b)}$, O8/O60:H19 *stx*$_{1a}$/*stx*$_{2only}$, O41:H14 *stx*$_{2c(vh-b)}$ ($n = 2$), O113:H21 *stx*$_{1a}$/*stx*$_{2only}$, O174:H21 *stx*$_{2c(vh-b)}$, O174:H8 *stx*$_{1a+1c}$, O178:H19 *stx*$_{2c(vh-b)}$, O181:H49 *stx*$_{1a}$/*stx*$_{2+2c(vh-b)}$, and ONT:H7 *stx*$_{2c(vh-b)}$. All strains were negative for *eae*, *aai*C, and *agg*R genes.

4. Discussion

The One Health concept involves human health, animal health and the environment (http://www.cdc.gov/onehealth/). It is thus necessary to work on the entire food production chain to ensure final product quality. Protocols to monitor food safety during processing demand the use of rapid analytical screening. Although many unofficial PCR assays have been used, the reference methods are subject to extensive validation processes to ensure the results. At present, several official protocols are used to find STEC in meat products, and they only include some serogroups, such as O26, O45, O103, O104, O111, O121, O145, and O157 (International Organization for Standardization. ISO/TS 13136, 2012; United State Department of Agriculture. USDA MLG 5B.05; United State Department of Agriculture. USDA MLG 5.09). Consequently, commercial RT-PCRs are based on the detection of some *stx* and *eae* genes, and these STEC serogroups. The *stx* zero tolerance criteria implemented in the European Union, prompted us to develop and validate the two SYBR-PCR and the multiplex RT-PCR assays to detect *stx*$_1$ and *stx*$_2$ genes in meat samples, and further compare them using ground beef samples from retail stores. However, we considered it necessary to compare the official protocols and commercial kits with the SYBR-PCR and RT- PCR developed and validated in this study.

In the current study, the results obtained using pure strains and artificially contaminated samples for SYBR-PCR and RT-PCR validation reflect an ideal situation for *stx* gene detection. The low LOD obtained and the correct recognition of *stx* genes from all ground beef contaminated with a minimum up to 1 CFU of STEC are among such main points. These observations are in agreement with the requirements of food industry regulations for screening techniques (Anklam et al., 2012; Conrad et al., 2014). Kagkli et al. (2011) described DR, Eff, and robustness as essential parameters to establish good PCR performance standards, and demonstrated it through the assays they performed. The use of STEC strains carrying several *stx*$_1$ and *stx*$_2$ variants and belonging to 39 different serotypes, including O26, O103, O111, O121, O145, and O157, showed the ability of PCR to identify a wide spectrum of STEC. The phylogenetic relationship between STEC and non-STEC strains or the use of non-STEC strains that could probably be present in ground beef samples proved the excellent performance of the exclusivity assay. The results obtained by each individual PCR developed in the present study indicate that they are all suitable for the detection of *stx* genes in ground beef samples. However, RT-PCR presented some advantages over SYBR-PCR. Due to the multiplex detection system of RT-PCR, it is possible to obtain in one reaction tube the same quantity of results as when performing both SYBR-PCRs. Further, IAC incorporation is another important issue to consider RT-PCR as a more appropriate screening technique, since it ensures the identification of false negative results (International Organization for Standardization. ISO 22119, 2011; Lin et al., 2011; Verstraete et al., 2014).

The SYBR-PCR and RT-PCR assays are considered non-independent techniques because both use the same pair of primers; this would explain the high level of agreement obtained with them all in ground beef samples (*stx*$_1$ $kappa = 0.758$; *stx*$_2$ $kappa = 0.801$). However, some results did not coincide when comparing both PCR-based methods. Whereas the detection of *stx*$_2$ was higher with SYBR-PCR, that of *stx*$_1$ was higher with RT-PCR. The lower number of positive samples detected by SYBR-PCR$_1$ could be attributed to amplification failures, which were not detected due to the lack of IAC (International Organization for Standardization. ISO 22119, 2011; Kagkli et al., 2011). In addition, the larger number of positive samples detected by SYBR-PCR$_2$ were possibly due to false positive results, as 1) the positive IAC signal in all RT-PCR performed ensured the absence of PCR inhibitions (Derzelle et al., 2011; Lin et al., 2011; Suo et al., 2010), and 2) the use of hydrolysis probes decreased the number of non-specific amplifications by RT-PCR (Anklam et al., 2012; Suo et al., 2010). Therefore, RT-PCR should be considered as a first option to obtain safe results when screening for *stx* genes in the meat production chain.

Since several *E. coli* serogroups could not harbour *stx* genes and other bacteria could be harbouring them, the isolation step is necessary to confirm samples with PCR-positive results. However, STEC isolation is a challenge due to the scarce phenotypical differentiation of *E. coli* strains. In addition, the presence of stressed strains, low initial charges of STEC, and high levels of background bacteria should be considered (Auvray et al., 2009; Ju et al., 2012). We attempted to isolate STEC from all PCR-positive samples. Nine different STEC serotypes, all negative for *eae*, *aaiC*, and *aggR* genes, were isolated in this study. Five (O8:H19, O113:H21, O174:H21, O178:H19, and O181:H49) have been associated with sporadic cases of human diseases (Beutin et al., 2004; Friesema et al., 2015; Käppeli et al., 2011; Miko et al., 2014). However, during the course of the study with samples from retail stores, no human disease case was reported in the same sampling region (Mattarollo pers. Comm.), probably because the strains isolated from ground beef were not enterohaemorrhagic *E. coli* (EHEC), i.e., without an adequate system to adhere to the intestines, and therefore unable to cause disease in the host (Galli et al., 2010). It would be therefore interesting to consider the USDA criteria aimed at identifying only samples containing select STEC serogroups associated with enterohaemorrhagic disease.

5. Conclusion

The availability of molecular techniques to detect STEC from meat and foodstuffs that include ground beef is presently necessary. We developed and validated two PCR strategies for *stx* detection using 39 serotypes and 7 *stx* variants, obtaining excellent performance with artificially contaminated ground beef samples. However, our efforts to isolate STEC from retail store samples were not enough. Only 11 (31.0%) STEC strains were isolated from 35 *stx*-positive ground beef samples identically detected by all PCR. Probably, the isolation chances could have increased using 325 g (USDA MLG 5B.05). New technologies combining molecular approaches, that allow the identification of a gene profile including numerous virulent genes, rapid microbiological methods and traditional bacteriology, that includes a combination of

media, incubation temperature and amount of samples, should be considered to improve isolation.

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References

Adamu, M.T., Shamsul, B.M.T., Desa, M.N., Khairani-Bejo, S., 2014. A review on *Escherichia coli* O157:H7—the super pathogen. Health Environ. J. 5, 78–93.

Anklam, K.S., Kanankege, K.S.T., Gonzales, T.K., Kaspar, C.W., Döpfer, D., 2012. Rapid and reliable detection of Shiga toxin-producing *Escherichia coli* by real-time multiplex PCR. J. Food Prot. 75, 643–650.

AOAC International, 2012. Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces.

Atkinson, R.M., Besser, J.M., Bopp, C.A., Carlson, C., Crandall, C., George, K., Gerner-Smidt, P., Gladbach, S., Hannah Gould, L., Hartley, C.S., Maguire, H., Monson, T.A., Rutledge, D., Shea, S., Somsel, P.A., Strockbine, N.A., 2012. Guidance for Public Health Laboratories on the Isolation and Characterization of Shiga Toxin-Producing *Escherichia coli* (STEC) from Clinical Specimens.

Auvray, F., Lecureuil, C., Dilasser, F., Taché, J., Derzelle, S., 2009. Development of a real-time PCR assay with an internal amplification control for the screening of Shiga toxin-producing *Escherichia coli* in foods. Lett. Appl. Microbiol. 48, 554–559.

Beutin, L., Krause, G., Zimmermann, S., Kaulfuss, S., Gleier, K., 2004. Characterization of Shiga toxin-producing *Escherichia coli* strains isolated from human patients in Germany over a 3-year period. J. Clin. Microbiol. 42, 1099–1108.

Bosilevac, J.M., Koohmaraie, M., 2011. Prevalence and characterization of non-O157 Shiga toxin producing *Escherichia coli* isolated from commercial ground beef in the United States. Appl. Environ. Microbiol. http://dx.doi.org/10.1128/AEM.02833-10.

Brusa, V., Aliverti, V., Aliverti, F., Ortega, E.E., de la Torre, J.H., Linares, L.H., Sanz, M., Etcheverría, A.I., Padola, N.L., Galli, L., Peral García, P., Copes, J.A., Leotta, G.A., 2013. Shiga toxin-producing *Escherichia coli* in beef retail markets from Argentina. Front. Cell. Infect. Microbiol. 2, 171.

Calderwood, S.B., Mekalanos, J.J., 1987. Iron regulation of Shiga-like toxin expression in *Escherichia coli* is mediated by the fur locus. J. Bacteriol. 169, 4759–4764.

Cohen, J., 1960. A coefficient of agreement for nominal scales. Educ. Psychol. Meas. 20, 37–46.

Conrad, C.C., Stanford, K., McAllister, T.A., Thomas, J., Reuter, T., 2014. Further development of sample preparation and detection methods for O157 and the top 6 non-O157 STEC serogroups in cattle feces. J. Microbiol. Methods 105, 22–30.

Derzelle, S., Grine, A., Madic, J., Peytavin de Garam, C., Vingadassalon, N., Dilasser, F., Jamet, E., Auvray, F., 2011. A quantitative PCR assay for the detection and quantification of Shiga toxin-producing *Escherichia coli* (STEC) in minced beef and dairy products. Int. J. Food Microbiol. 151, 44–51.

Dohoo, I., Martin, W., Stryhn, H., 2003. Veterinary Epidemiology Research. Ed. AV Inc., Charlottetown, Prince Edward Island (Canada) 0-919013-41-4, p. 706.

EFSA and ECDC (European Food Safety Authority and European Centre for Disease Prevention and Control), 2015t. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013. EFSA J. 13, 3991. http://dx.doi.org/10.2903/j.efsa.2015.3991 162pp.

EFSA Panel on Biological Hazards (BIOHAZ), 2013. Scientific opinion on VTEC-seropathotype and scientific criteria regarding pathogenicity assessment. EFSA J 11, 3138. http://dx.doi.org/10.2903/j.efsa.2013.3138 106 pp.

Etcheverría, A.I., Padola, N.L., Sanz, M.E., Polifroni, R., Krüger, A., Passucci, J., Rodriguez, E.M., Taraborelli, A.L., Ballerio, M., Parma, A.E., 2010. Occurrence of Shiga toxin-producing *E. coli* (STEC) on carcasses and retail beef cuts in the marketing chain of beef in Argentina. Meat Sci. 86, 418–421.

EU Reference Laboratory for E. coli, Department of Veterinary Public Health and Food Safety Unit of Foodborne Zoonoses. Istituto Superiore di Sanità, 2013P. Detection of Enteroaggregative *Escherichia coli* in food by Real Time PCR amplification of *agg*R and *aai*C genes. http://www.iss.it/binary/vtec/cont/EU_RL_VTEC_Method_05_Rev_1.pdf.

Frank, C., Faber, M.S., Askar, M., Bernard, H., Fruth, A., Gilsdorf, A., Hohle, M., Karch, H., Krause, G., Prager, R., Spode, A., Stark, K., Werber, D., 2011. Large and ongoing outbreak of haemolytic uraemic syndrome, Germany, May 2011. J. Eurosurveillance 16 (21), Article 1.

Fratamico, P.M., Wasilenko, J.L., Garman, B., DeMarco, D.R., Varkley, S., Jensen, M., Rhoden, K., Tice, G., 2014. Evaluation of a multiplex real-time PCR method for detecting Shiga toxin-producing *Escherichia coli* in beef and comparison to the U.S. Department of Agriculture Food Safety and Inspection Service Microbiology Laboratory Guidebook method. J. Food Prot. 77, 180–188.

Friesema, I.H.M., Keijzer-Veen, M.G., Koppejan, M., Schipper, H.S., van Griethuysen, A.J., Heck, M.E.O.C., van Pelt, W., 2015. Hemolytic uremic syndrome associated with *Escherichia coli* O8:H19 and Shiga toxin 2f gene. Emerg. Infect. Dis. 21, 168–169.

Galli, L., Miliwebsky, E., Irino, K., Leotta, G.A., Rivas, M., 2010. Virulence profile comparison between LEE-negative Shiga toxin-producing *Escherichia coli* strains isolated from humans and cattle. Vet. Microbiol. 143, 307–313.

Hussein, H.S., 2007. Prevalence and pathogenicity of Shiga toxin-producing *Escherichia coli* in beef cattle and their products. J. Anim. Sci. 85, 63–72.

International Organization for Standardization. ISO 22119, 2011. Microbiology of Food and Animal Feeding Stuffs – Real-time Polymerase Chain Reaction (PCR) for the Detection of Food-borne Pathogens – General Requirements and Definitions.

International Organization for Standardization. ISO/TS 13136, 2012. Microbiology of Food and Animal Feed – Real-time Polymerase Chain Reaction (PCR)-based Method for the Detection of Food-borne Pathogens – Horizontal Method for the Detection of Shiga Toxin-producing *Escherichia coli* (STEC) and the Determination of O157, O111, O26, O103 and O145 Serogroups.

Jothikumar, N., Griffiths, M.W., 2002. Rapid detection of *Escherichia coli* O157:H7 with multiplex real-time PCR assays. Appl. Environ. Microbiol. 68, 3169–3171.

Ju, W., Shen, J., Li, Y., Toro, M.A., Zhao, S., Ayers, S., Najjar, M.B., Meng, J., 2012. Non-O157 Shiga toxin-producing *Escherichia coli* in retail ground beef and pork in the Washington D.C. area. Food Microbiol. 32, 371–377.

Kagkli, D.M., Weber, T.P., Van den Bulcke, M., Folloni, S., Tozzoli, R., Morabito, S., Ermolli, M., Gribaldo, L., Van den Eede, G., 2011. Application of the modular approach to an in-house validation study of real-time PCR methods for the detection and serogroup determination of verocytotoxigenic *Escherichia coli*. Appl. Environ. Microbiol. 77, 6954–6963.

Kagkli, D.M., Folloni, S., Barbau-Piednoir, E., Van den Eede, G., Van den Bulcke, M., 2012. Towards a pathogenic *Escherichia coli* detection platform using multiplex SYBR®-Green Real-time PCR methods and high resolution melting analysis. PLoS ONE 7 (6), e39287. http://dx.doi.org/10.1371/journal.pone.0039287.

Karch, H., Meyer, T., 1989. Single primer pair for amplifying segments of distinct Shiga-liketoxin genes by polymerase chain reaction. J. Clin. Microbiol. 27, 2751–2757.

Karmali, M.A., Gannon, V., Sargeant, J.M., 2010. Verocytotoxin-producing *Escherichia coli* (VTEC). Vet. Microbiol. 140, 360–370.

Käppeli, U., Hächler, H., Giezendanner, N., Beutin, L., Stephan, R., 2011. Human infections with non-O157 Shiga Toxin-producing *Escherichia coli*, Switzerland, 2000–2009. Emerg. Infect. Dis. 17, 180–185.

Krüger, A., Lucchesi, P.M.A., 2015. Shiga toxins and stx-phages: highly diverse entities. Microbiology 161, 451–462.

Leotta, G.A., Chinen, I., Epszteyn, S., Miliwebsky, E., Melamed, I.C., Motter, M., Ferrer, M., Marey, E., Rivas, M., 2005. Validación de una técnica de PCR múltiple para la detección de *Escherichia coli* productor de toxina Shiga. Rev. Argent. Microbiol. 37, 1–10.

Leotta, G.A., Miliwebsky, E.S., Chinen, I., Espinosa, E.M., Azzopardi, K., Tennant, S.M., Robins-Browne, R.M., Rivas, M., 2008. Characterization of Shiga toxin-producing *Escherichia coli* O157 strains isolated from humans in Argentina, Australia and New Zealand. BMC Microbiol. http://dx.doi.org/10.1186/1471-2180-8-46.

Lin, A., Sultan, O., Lau, H.K., Wong, E., Hartman, G., Lauzon, C.R., 2011. O serogroup specific real time PCR assays for the detection and identification of nine clinically relevant non-O157 STECs. Food Microbiol. 28, 478–483.

Llorente, P., Barnech, L., Irino, K., Rumi, M.V., Bentancor, A., 2014. Characterization of Shiga toxin-producing *Escherichia coli* isolated from ground beef collected in different socioeconomic strata markets in Buenos Aires, Argentina. BioMed Res. Int. http://dx.doi.org/10.1155/2014/795,104.

Martin, A., Beutin, L., 2011. Characteristics of Shiga toxin-producing *Escherichia coli* from meat and milk products of different origins and association with food producing animals as main contamination sources. Int. J. Food Microbiol. 46, 99–104.

Masana, M.O., Leotta, G.A., Del Castillo, L.L., D'Astek, B.A., Palladino, P.M., Galli, L., Vilacoba, E., Carbonari, C., Rodríguez, H.R., Rivas, M., 2010. Prevalence, characterization, and genotypic analysis of *Escherichia coli* O157:H7/NM from selected beef exporting abattoirs of Argentina. J. Food Prot. 73, 649–656.

Masana, M.O., D'Astek, B.A., Palladino, P.M., Galli, L., Del Castillo, L.L., Carbonari, C., Leotta, G.A., Vilacoba, E., Irino, K., Rivas, M., 2011. Genotypic characterization of non-O157 Shiga toxin-producing *Escherichia coli* in beef abattoirs of Argentina. J. Food Prot. 74, 2008–2017.

Meichtri, L., Miliwebsky, E., Gioffré, A., Chinen, I., Baschkier, A., Chillemi, G., Guth, B.E.C., Masana, M.O., Cataldi, A., Rodríguez, H.R., Rivas, M., 2004. Shiga toxin-producing *Escherichia coli* in healthy young beef steers from Argentina: prevalence and virulence properties. Int. J. Food Microbiol. 96, 189–198.

Miko, A., Rivas, M., Bentancor, A., Delannoy, S., Fach, P., Beutin, L., 2014. Emerging types of Shiga toxin-producing *E. coli* (STEC) O178 present in cattle, deer, and humans from Argentina and Germany. Front. Cell. Infect. Microbiol. http://dx.doi.org/10.3389/fcimb.2014.00078.

Scheutz, F., Teel, L.D., Beutin, L., Piérard, D., Buvens, G., Karch, H., Mellmann, A., Caprioli, A., Tozzoli, R., Morabito, S., Strockbine, N.A., Melton-Celsa, A.R., Sanchez, M., Persson, S., O'Brien, A.D., 2012. Multicenter evaluation of a sequence-based protocol for subtyping Shiga toxins and standardizing Stx nomenclature. J. Clin. Microbiol. 50, 2951–2963.

Suo, B., He, Y., Tu, S.I., Shi, X., 2010. A multiplex real-time polymerase chain reaction for simultaneous detection of *Salmonella* spp., *Escherichia coli* O157, and *Listeria monocytogenes* in meat products. Foodborne Pathog. Dis. 6, 619–628.

The Rapid Alert System for Food and Feed. RASFF, 2015. https://webgate.ec.europa.eu/rasff-window/portal/?event=notificationsList&StartRow=1.

Tyler, S.D., Johnson, W.M., Lior, H., Wang, G., Rozee, K.R., 1991. Identification of verotoxin type 2 variant B subunit genes in *Escherichia coli* by the polymerase chain reaction and restriction fragment length polymorphism analysis. J. Clin. Microbiol. 29, 1339–1343.

United State Department of Agriculture. USDA MLG 5B.05 Detection and Isolation of non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC) from Meat Products and Carcass and Environmental Sponges.

United State Department of Agriculture. USDA MLG 5.09 Detection, Isolation and Identification of *Escherichia coli* O157:H7 from Meat Products and Carcass and Environmental Sponges.

Varela, G., Chinen, I., Gadea, P., Miliwebsky, E., Mota, M.I., González, S., González, G., Gugliada, M.J., Carbonari, C.C., Aalgorta, G., Bernadá, M., Sabelli, R., Pardo, L., Rivas, M., Schelotto, F., 2008. Detección y caracterización de *Escherichia coli* productor de toxina Shiga a partir de casos clínicos y de alimentos en Uruguay. Rev. Argent. Microbiol. 40, 93–100.

Verstraete, K., Van Coillie, E., Werbrouck, H., Van Weyenberg, S., Herman, L., Del-Favero, J., De Rijk, P., De Zutter, L., Joris, M.A., Heyndrickx, M., De Reu, K., 2014. a qpcr assay to detect and quantify Shiga toxin-producing *e. coli* (stec) in cattle and on farms: a potential predictive tool for STEC culture-positive farms. Toxins 6, 1201–1221.