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Isolation of Shiga Toxin-Producing *Escherichia coli* from Ground Beef Using Multiple Combinations of Enrichment Broths and Selective Agars

Victoria Brusa,[1,3] Pablo E. Piñeyro,[2] Lucía Galli,[3] Luciano H. Linares,[1] Emanuel E. Ortega,[1] Nora L. Padola,[4] and Gerardo A. Leotta[3]

Abstract

Shiga toxin-producing *Escherichia coli* (STEC) are foodborne pathogens, and beef cattle are recognized as the principal reservoir. The aims of this study were (1) to identify the most sensitive combination of selective enrichment broths and agars for STEC isolation in artificially inoculated ground beef samples, and (2) to evaluate the most efficient combination(s) of methods for naturally contaminated ground beef samples. A total of 192 ground beef samples were artificially inoculated with STEC and non-*stx* bacterial strains. A combination of four enrichment broths and three agars were evaluated for sensitivity, specificity, and positive predictive value for STEC isolation from experimentally inoculated samples. Enrichments with either modified tryptic soy broth (mTSB) containing 8 mg/L novobiocin (mTSB-8) or modified *Escherichia coli* (mEC) broth followed by isolation in MacConkey agar were the most sensitive combinations for STEC isolation of artificially inoculated samples. Independently, both enrichments media followed by isolation in MacConkey were used to evaluate ground beef samples from 43 retail stores, yielding 65.1% and 58.1% *stx*-positive samples by RT-PCR, respectively. No difference was observed in the isolate proportions between these two methods (8/25 [32%] and 8/28 [28.6%]). Identical serotypes and *stx* genotypes were observed in STEC strains isolated from the same samples by either method. In this study, no single enrichment protocol was sufficient to detect all STEC in artificially inoculated samples and had considerable variation in detection ability with naturally contaminated samples. Moreover, none of the single or combinations of multiple isolation agars used were capable of identifying all STEC serogroups in either artificially inoculated or naturally occurring STEC-contaminated ground beef. Therefore, it may be prudent to conclude that there is no single method or combination of isolation methods capable of identifying all STEC serogroups.

Introduction

Shiga toxin–producing *Escherichia coli* (STEC) are recognized as food-borne pathogens that can cause a variety of clinical outcomes ranging from diarrhea to hemorrhagic colitis and the life-threatening complication hemolytic uremic syndrome (Melton-Celsa *et al*., 2012).

STEC strains have been isolated from a variety of domestic animals with ruminants, especially beef cattle, recognized as their main natural reservoir (Caprioli *et al*., 2005). During carcass processing, transfer of bacteria from the animal's hide or fecal contamination of the carcass can facilitate the transmission of pathogenic *E. coli* to meat products (Elder *et al*., 2000).

There is no single marker or combination of markers to differentiate STEC from nonpathogenic *E. coli* strains (Brusa *et al*., 2013). Therefore, several methods for the isolation, detection, and characterization of STEC O157:H7 are currently recommended worldwide (ISO, 2001; USDA/FSIS, 2015). Although enrichment protocols are suitable for the most prevalent serogroups identified epidemiologically in clinical infections (O26, O45, O103, O111, O121, O145, and

[1]Laboratorio de Microbiología de Alimentos, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata (UNLP), Buenos Aires, Argentina.
[2]Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, Iowa.
[3]Instituto de Genética Veterinaria ''Ing. Fernando N. Dulout,'' Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) La Plata, Facultad de Ciencias Veterinarias UNLP, Buenos Aires, Argentina.
[4]Centro de Investigación Veterinaria Tandil, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CICPBA), Facultad Ciencias Veterinarias, Universidad Nacional del Centro de la Provincia de Buenos Aires, Buenos Aires, Argentina.

O157) (Gill *et al*., 2014), they are not always effective for the other STEC serogroups. Media with various selective or enrichment compounds have been used to inhibit the competitive flora in food samples (Vimont *et al*., 2006; Wang *et al*., 2013); however, STEC is a heterogeneous bacterial group, and selective enrichment broths can inhibit the development of some serogroups (Drysdale *et al*., 2004; Feng et al., 2011). Buffered peptone water (BPW) at $37 \pm 1°C$ can be used as an alternative enrichment protocol; however, the isolation rate with this enrichment media can be affected due to the high number of background microflora allowed by BPW (Drysdale *et al*., 2004). In addition to the standard methods, other studies have shown experimentally the benefits of using modified *E. coli* broth (mEC) as enrichment media (Brusa *et al*., 2013).

The aim of this study was to evaluate multiple combinations of selective enrichment broths and agars for STEC isolation in artificially inoculated ground beef samples and then apply the most efficient methods combination in a survey of naturally contaminated ground beef samples from retail stores. While this study was underway, the International Organization for Standardization (ISO/TS 13136:2012), the United States Department of Agriculture (USDA MLG 5B.03) and the Food and Drug Administration (Feng et al., 2011) all updated techniques; however, they still have different recommended enrichment protocols that combined different selective broths with different incubation temperatures.

Material and Methods

Artificially inoculated samples

Bacterial strains. A total of 12 STEC and 24 non-*stx* bacterial strains were used to artificially inoculate ground beef samples (Table 1). All strains belong to the collection of the Instituto de Genética Veterinaria ''Ing. Fernando N. Dulout''. They were stored at −70°C in brain heart infusion (BHI) broth (Biokar, Zac de Ther, France) with 30% glycerol (ICN Biomedicals, Solon, Ohio).

Table 1. Bacterial Strains Used for the Specificity, Sensitivity, and Positive Predictive Value of Positive Results Calculations of STEC Isolation with Different Enrichment Media–Agar Combinations

				Virulence profile	
Species	*Strain*	*Source*	*Serotype*	stx_1	stx_2
Escherichia coli	IGEVET 33	H	O26:H11	+	−
Escherichia coli	IGEVET 36	H	O111HNM	+	−
Escherichia coli	IGEVET 40	U	O157:H7	+	+
Escherichia coli	IGEVET 59	H	O91:H21	−	+
Escherichia coli	IGEVET 63	H	O145:HNM	−	+
Escherichia coli	IGEVET 67	U	O103:H25	−	+
Escherichia coli	IGEVET 85	C	O8:H2	−	+
Escherichia coli	IGEVET 86	C	O112:H2	−	+
Escherichia coli	IGEVET 103	U	O121:H19	−	+
Escherichia coli	IGEVET 589	E	O130:H21	+	+
Escherichia coli	IGEVET 727	E	O178:H19	−	+
Escherichia coli	IGEVET 1070	H	O113:H21	−	+
Yersinia enterocolitica	IGEVET 2	U			
Edwardsiella tarda	IGEVET 6	A			
Enteroaggregative *E. coli*	IGEVET 12	U			
Enteroinvasive *E. coli*	IGEVET 14	U			
Shigella flexneri	IGEVET 15	U	1		
Proteus mirabilis	IGEVET 16	U			
Proteus vulgaris	IGEVET 18	U			
Enteropathogenic *E. coli*	IGEVET 19	U			
Shigella dysenteriae	IGEVET 20	U	2		
Morganella morgani	IGEVET 21	U			
Escherichia coli	IGEVET 26	U			
Staphylococcus aureus	IGEVET 115	U			
Salmonella enterica	IGEVET 127	A	Choleraesuis		
Salmonella enterica	IGEVET 253	F	Enteritidis		
Salmonella enterica	IGEVET 256	U	Dublin		
Pseudomonas aeruginosa	IGEVET 362	W			
Salmonella enterica	IGEVET 384	A	Typhimurium		
Salmonella enterica	IGEVET 388	A	Newport		
Shigella flexneri	IGEVET 392	U			
Escherichia hermanii	IGEVET 393	U			
Citrobacter freundii	IGEVET 646	F			
Enterobacter cloacae	IGEVET 983	U			
Shigella boydii	IGEVET 993	U			
Enterotoxigenic *E. coli*	IGEVET 996	A			

A, animal; C, cattle; E: environment; F, food; H, human; IGEVET, Instituto de Genética Veterinaria ''Ing. Fernando N. Dulout'' (Universidad Nacional de La Plata (UNLP) - Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) LA PLATA), Facultad de Ciencias Veterinarias; U, unknown; W, water.

Preparation of inoculum, sample inoculation, and enrichment protocols. STEC and non-*stx* strain frozen stocks were grown in BHI broth at 37°C for 18 h overnight, streaked onto BHI agar plates, and incubated 24 h at 37°C to assure purity. For ground beef inoculation, a single colony of each strain was selected and grown overnight in 4 mL of BHI broth at 37°C. A total of 196 ground beef samples (10 g) were prepared from a single batch of ground beef obtained from a retail store which was supplied with meat from a single processing plant. For the purpose of this specific study, we were provided with meat from a single cow that was confirmed to be STEC-negative by stx_1 and stx_2 gene detection with RT-PCR (ISO, 2012).

Final STEC and non-*stx* strains overnight cultures were serially diluted in BPW (Biokar). Eight samples per strain were artificially inoculated with 12 different STEC strains (n = 96), and four samples per strain were artificially inoculated with 24 different non-*stx* bacterial strains (n = 96) by adding 1 mL inoculum of 100 CFU/mL or 1000 CFU/mL, respectively. One milliliter of sterile BPW (Biokar) was inoculated to four negative control samples. The inoculum level was confirmed by plating on plate count agar (Laboratorios Britania, Buenos Aires, Argentina) and incubating overnight at 37°C.

STEC and non-*stx* inoculated samples were divided into four groups of 24 samples each and assigned to four different enrichment protocols as follows: protocol A, modified tryptic soy broth (mTSB) containing 16 mg/L novobiocin (Acumedia Manufacturers, Lansing, MI) at 37 ± 1°C for 20 h (mTSB-16) (ISO-13136:2012); protocol B, mTSB containing 8 mg/L novobiocin plus casamino acids (Acumedia) at 42 ± 1°C for 20 h (mTSB-8) (USDA/FSIS, 2012); protocol C, mEC broth (Acumedia) at 42 ± 1°C for 20 h (mEC); and protocol D, modified BPW with pyruvate at 37 ± 1°C for 5 h followed by acriflavine, cefsulodin, and vancomycin addition (Biokar) at 42 ± 1°C (mBPWp+ACV) (Feng *et al*., 2011). Enrichment consisted in incubating each group of artificially inoculated samples (n = 49) with 90 mL of respective enrichment media. One negative control sample was assigned to each enrichment protocol.

Screening by RT-PCR and STEC isolation. Bacterial DNA extraction was performed directly from 1 mL of each enrichment broth, in 1% Triton X-100 in TE buffer 1× (10 mM Tris: 1 mM Ethylenediaminetetraacetic acid, pH 8), and boiled for 10–15 min (Leotta *et al*., 2005). Detection of *stx* genes by RT-PCR was carried out as previously described (Brusa *et al*., 2013). Samples were considered STEC positive when stx_1 and/or stx_2 genes were detected. One mL of each PCR-positive sample was spun down and the pellet was plated onto three different agars: agar 1, Levine eosin methylene blue (L-EMB) (Biokar); agar 2, MacConkey agar (MC) (Becton Dickinson Co., Sparks, MD); And agar 3, triptone bile X-glucuronide (TBX) (Biokar). All plates were incubated at 37°C for 18 h. Fifty colonies with *E. coli* morphology were selected from each plate and point-inoculated on nutrient agar (Laboratorios Britania). After incubation, selected colonies were pooled in five groups of 10 and were screened for the presence of stx_1 and stx_2 genes by multiplex-PCR (Leotta *et al*., 2005). Positive *stx*-pools were further analyzed by multiplex-PCR in order to identify each individual *stx*-positive colony.

Experimental design

Phase one: Experimentally inoculated samples. The isolation proportions obtained with a combination of different methods in experimentally inoculated sample were evaluated. The first combination was based on the enrichment media–single agar [E-SA] method (Fig. 1A). The performance of each enrichment media (protocols A, B, C, and D) was evaluated by isolation in three different agars (agars 1, 2, and 3). Thus, the isolation on E-SA method was expressed as the number of isolates obtained from a single enrichment media followed by a single agar (A1 = mTSB-16 + L-EMB, A2 = mTSB-16 + MC, A3 = mTSB-16 + TBX; B1 = mTSB-8 + L-EMB, B2 = mTSB-8 + MC, A3 = mTSB-8 + TBX; C1 = mEC + L-EMB, C2 = mEC + MC, C3 = mEC + TBX; D1 = mBPW + L-EMB, D2 = mBPW + MC, D3 = mBPW + TBX) (Fig. 1A). The second combination, the enrichment media–triple agar method [E-TA], computed the number of isolates obtained from three different agars after each enrichment protocol (Fig. 1B). Thus, the isolation proportion on E-TA methods was expressed as single enrichment media followed by adding the number of isolates from three agars (A123 = mTSB-16 + L-EMB + MC + TBX; B123 = mTSB-8 + L-EMB + MC + TBX; C123 = mEC + L-EMB + MC + TBX; D123 = mBPW + L-EMB + MC +TBX) (Fig. 1B).

Phase two: Naturally contaminated samples. Based on the results obtained with experimentally inoculated samples, we selected the best two E-SA methods for detection of STEC in naturally contaminated samples. From February to September 2013, 43 ground beef samples (25 g) from 43 retail stores were collected in duplicate and enriched in 225 mL of enrichment protocols B and C. Then, samples were screened for the presence of *stx* genes as previously described for artificially inoculated samples. Isolation of STEC-positive samples was carried out in MC agar. Serotyping of O and H antigens was performed by plate micro-agglutination and tube agglutination, respectively, using antisera kit (O1-O186) and 56 H antisera produced by the Laboratorio de Referencia de *E. coli* (Lugo, Spain), as described by Blanco *et al*. (1997). The *eae* gene was determined using primers SK1-SK2 as described by Gannon *et al.* (1993). *E. coli* strains 2348/69 and ATCC 25922 were used as positive and negative controls, respectively.

Statistical analysis

Isolation sensitivity, specificity, and positive predictive values of positive results (PPV^+), as well as 95% confidence intervals (CI) were computed for the enrichment E-SA method and for the enrichment E-TA method. Sensitivity and specificity and PPV^+ were calculated as follows: sensitivity, a/(a+b), where a = *stx* PCR-positive and positive isolate, b = *stx* PCR-positive and negative isolate; specificity d/(c+d), where b = *stx* PCR-negative and positive isolate, c = *stx* PCR-negative and negative isolate; and PPV^+, a/(a+c), where a = *stx* PCR-positive and positive isolate, c = *stx* PCR-negative and negative isolate. Fishers' exact test and chi-square test with Yates' correction were used to compare the proportions of STEC positive results obtained from E-SA and E-TA. The difference in frequency of isolations obtained from each E-TA method with the different enrichment media was determined with Pearson's chi-square test. *P*-values less

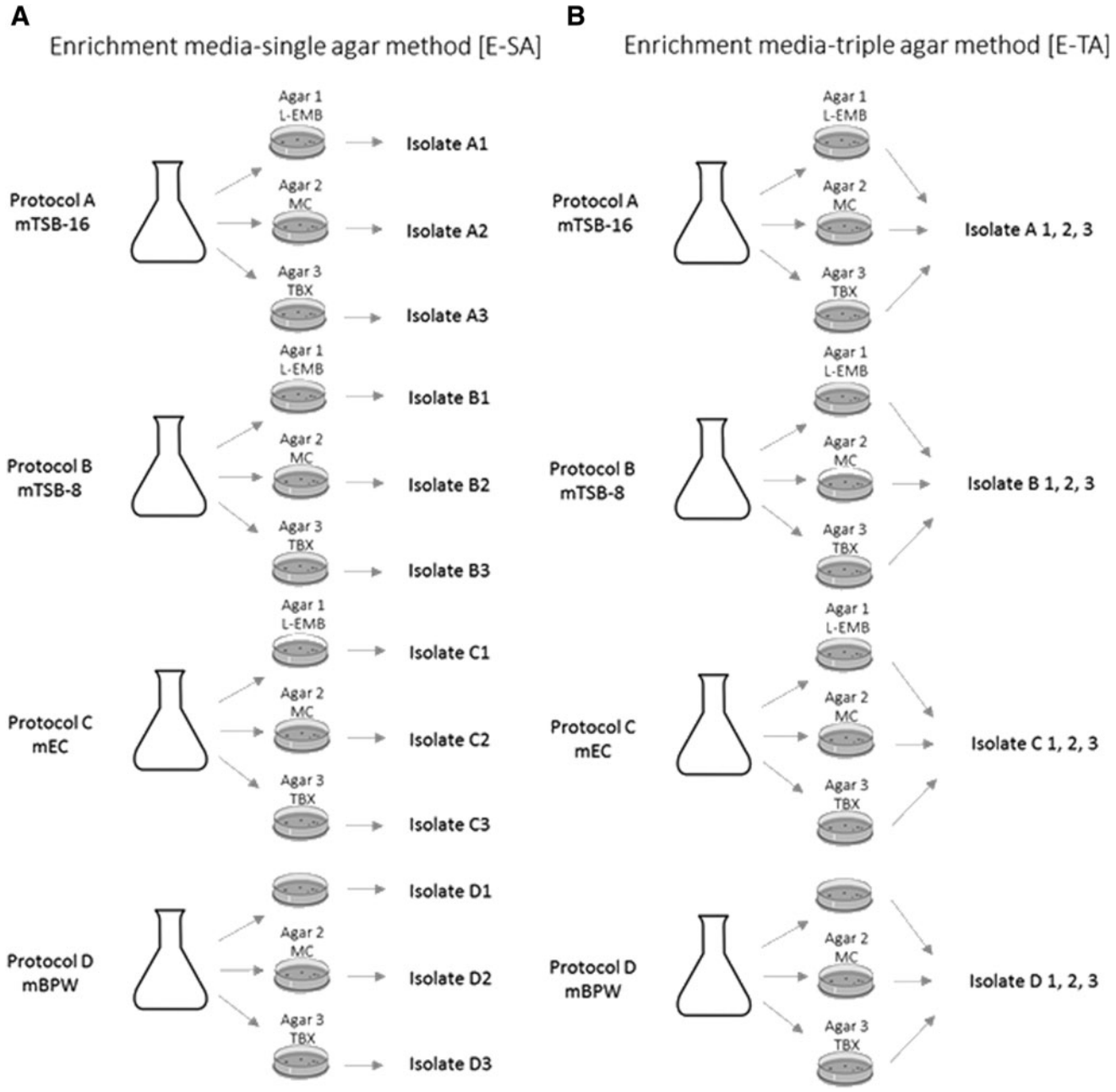

FIG. 1. Protocols for artificially inoculated samples. **(A)** Isolates were obtained from each enrichment medium followed independently by three different agars (enrichment media–single agar method). **(B)** The total number of isolates was calculated by combining the number of isolates obtained from three different agars after each enrichment protocol (enrichment media–triple agar method). Protocol A: modified tryptic soy broth (mTSB) containing 16 mg/L novobiocin (ISO/TS 13136:2012). Protocol B: mTSB containing 8 mg/L novobiocin plus casamino acids (USDA MLG 5B.03). Protocol C: modified *Escherichia coli* broth (mEC) broth. Protocol D: modified buffered peptone water (mBPW) with pyruvate followed by acriflavine, cefsulodin, and vancomycin addition. Numbers indicate different agars: 1, eosine methylene blue (L-EMB); 2, MacConkey (MC); 3, triptone bile X-glucuronide (TBX).

than 0.05 were considered statistically significant and all tests were two-tailed. To determine agreement between E-SA and E-TA, weighted Cohen's kappa coefficient (k) with a 95% CI was calculated and interpreted as follows: poor, <0.20; fair, 0.20–0.39; moderate, 0.40–0.59; good, 0.60–0.79; and excellent, ≥0.80. Agreement between tests was significant if $p < 0.05$ by Bowker's test of symmetry. Statistical analyses were performed using JMP 11.

Results

Phase one: Experimentally inoculated samples

Both sensitivity and positive predictive values of E-SA and E-TA isolation of artificially inoculated samples are presented in Table 2. The specificity of all E-SA and E-TA methods was 100% (95% CI 85.62–100%). No statistical differences in the proportion of isolates obtained with E-SA

Table 2. Sensitivity and Predictive Positive Value for *E. coli* (STEC) PCR and Isolation After Different Enrichment Protocols for Artificially Inoculated Samples

Protocols[a]	*Sensitivity (%)*	*95% CI*	*PPV*$^+$ *(%)*	*95% CI*
A.1	75	53.28–90.16	44	32–55
A.2	75	53.28–90.16	44	32–55
A.3	83.3	62.60–95.16	46	35–57
B.1	91.6	72.96–98.73	49	38–59
B.2	95.8	78.81–99.30	50	39–60
B.3	91.6	72.96–98.73	49	38–59
C.1	83.3	62.60–95.16	46	35–57
C.2	91.6	72.96–98.73	49	38–59
C.3	91.6	72.96–98.73	49	37–60
D.1	45.8	25.58–67.16	32	18–46
D.2	45.8	25.58–67.16	32	18–46
D.3	37.5	18.84–59.40	28	13–43
A.123	91.6	72.96–98.73	49	38–59
B.123	95.8	78.81–99.30	50	39–60
C.123	95.8	78.81–99.30	50	39–60
D.123	58.3	36.66–77.86	38	22–53

[a]Letters in protocol names indicate enrichment selective media: complete version: A: modified tryptic soy broth containing 16 mg/L novobiocin (ISO/TS 13136:2012); B: mTSB containing 8 mg/L novobiocin plus casamino acids (USDA MLG 5.03); C: modified Escherichia coli broth; D: modified buffered peptone water with pyruvate followed by acriflavine, cefsulodin, and vancomycin addition (Feng *et al.,* 2011). Numbers indicate agars used: 1, Levine eosin methylene blue; 2: MacConkey; 3: triptone bile X-glucuronide.

95% CI, 95% confidence interval; PPR+, positive predictive value of positive results.

and E-TA were observed ($p < 0.05$), except for the following combinations: A3 versus A123, B2 versus B123 and D123 versus D1, D2, and D3 (Table 3). The proportion of positive E-TAs after different enrichment media was statistically significant (chi-squared = 19.06; DF = 3; $p = 0.001$). The correlation between E-SA and E-TA is shown in Table 3. The *k* coefficient showed good agreement between A3 and A123, C2, C3, and C123, and excellent agreement between B2 and B123, and D1 and D2 with D123 (Table 3). The proportion of isolates obtained with the E-TA, showed good agreement among methods A123, B123, and C123 (*k*-value 0.647, 95% CI, 0.0131.0) and fair agreement between methods A123 and C123 (*k*-value 0.032, 95% CI 0.22–0.291).

Phase two: naturally contaminated samples

Screening of ground beef samples obtained from retail outlets showed that 25/43 (58.1%) and 28/43 (65.1%) samples enriched in protocols B and C, respectively, were *stx*-positive. No significant differences (chi-squared = 0.124, DF = 1, $p = 0.72$) were observed in the isolation proportion between methods B2 (8/25; 32.0%) and C2 (8/28; 28.6%). STEC strains isolated with both methods showed identical serotypes and *stx* genotypes. Eight different O:H serotypes and three different *stx*-genotypes were found: O26:H11 (stx_1), O64:H20 (stx_2), O91:H21 (stx_2), O113:H21 (stx_2), O141:H49 (stx_2), O178:H19 (stx_1/stx_2), OUT:H19 (stx_2), and OUT:HUT (stx_2). Only O26:H11 (stx_1) was positive for the *eae* gene.

Discussion

The current study evaluates the performance of four enrichment protocols in combination with three agars for the isolation of all STEC strains in artificially inoculated samples and in naturally infected ground beef from 43 retail stores.

ISO Technical Specification (TS) 13136:2012 refers to a horizontal method for STEC detection and determination of serogroups O157, O111, O26, O103, and O145 (ISO, 2012). Due to the emergence of atypical *stx*- positive strains associated with severe disease outbreaks (Frank *et al*., 2011), the ISO determined that all STEC should be considered pathogenic and in ISO/TS 13136:2012 therefore proposed three different enrichment broths followed by isolation in TBX based upon the sample matrix to be evaluated. Accordingly, enrichment protocol A was used because of the sample characteristics of this study. The sensitivity of the ISO/TS 13136:2012 method A3 (mTSB-16+ TBX) was significantly higher for E-SA as compared with method A1 (mTSB-16 + EMB) and A2 (mTSB-16+ MC). However, the isolation proportion for E-TA increased significantly (91.6%) in artificially inoculated samples. It can be speculated that the increment in isolation proportion observed in the E-TA

Table 3. Agreement Between Different Enrichment Protocols Using Single Agar Versus Combination of Multiple Agars for *E. coli* (STEC) Isolation After Different Enrichment Protocols

Protocols[a]	p-*Value*[b]	*Cohen kappa coefficient*	*95% CI*	*Bowker's test* p-*value*
A.1 vs. A123	0.054	0.428	0.009–0.848	<0.05
A.2 vs. A123	0.054	0.428	0.009–0.848	<0.05
A.3 vs. A123	<0.05	0.625	0.163–1.00	0.150
B.1 vs. B123	0.083	0.647	0.013–1.00	0.310
B.2 vs. B123	<0.05	1.000	1.000	1.000
B.3 vs. B123	0.083	0.647	0.013–1.000	0.310
C.1 vs. C123	0.166	0.357	–0.164–0.878	0.080
C.2 vs. C123	0.083	0.647	0.013–1.000	0.310
C.3 vs. C123	0.083	0.647	0.013–1.000	0.310
D.1 vs. D123	<0.001	0.753	0.500–1.000	0.080
D.2 vs. D123	<0.001	0.753	0.500–1.000	0.080
D.3 vs. D123	<0.01	0.600	0.314–0.885	<0.05

[a]In protocol names, letters indicate enrichment selective media: A, ISO/TS 13136:2012; B, USDA MLG 5.03; C, modified *Escherichia coli* broth; D, Feng *et al.,* 2011. Numbers indicate agars: 1, Levine eosin methylene blue; 2, MacConkey; 3, triptone bile X-glucoronide.
[b]*p* values refer to Fisher's exact test.

compared with each E-SA method in samples enriched with protocol A, is due to an increment in the number of plates evaluated but not due to better growth condition provided by the agars used for isolation.

The USDA *Microbiology Laboratory Guidebook* (MLG) 5B.03 (effective date November 6, 2012) describes the methodology necessary to detect and isolate non-O157 STEC serogroups including O26, O45, O103, O111, O121, and O145 from meat products (USDA MLG 5B.03). USDA guidelines also require isolation by immunomagnetic separation followed by culture in Rainbow Agar. Among all three E-SA method using the enrichment condition recommended by USDA MLG 5B.03 (enrichment protocol B), the isolation sensitivity in artificially inoculated samples was higher (95.8%) with MC agar compared with EMB and TBX agars. In addition, we did not observe significant improvements in sensitivity with enrichment protocol B regardless of isolation with E-SA or E-TA methods.

The FDA proposes a method for diarrheagenic *E. coli*, including STEC O157:H7 and non-O157 implicated in foodborne illnesses (Feng *et al.*, 2011). Although this method was not designed for meat products, it can be used to analyze ready-to-eat food containing meat or ground beef. In our screening of artificially inoculated samples, enrichment protocol D was the less effective of all isolation methods (D1, D2, and D3). Despite the significant improvements in isolation sensitivity observed with E-TA method (58.3%), this method remained significantly below the sensitivity values obtained with other methods evaluated in this study. Several authors have described the use of protocol D to enrich non-O157:H7 STEC from different foodstuffs (Baranzoni *et al*., 2014; Kase *et al*., 2015); however, results obtained in the present study showed that enrichment protocol D with either E-SA or E-TA methods was less sensitive than the other enrichments media for non-O157 STEC used to evaluated ground beef.

Previous reports have shown the potential use of enrichment protocol C for the detection of non-O157 STEC in bovine carcasses in slaughterhouses (Masana *et al*., 2011), ground beef samples, and environmental samples from retail stores (Brusa *et al*., 2013). In this study the sensitivity of two of the E-SA methods, enrichment protocol C followed by isolation either in MC or TBX, was comparable with the values observed with standard methods (91.6%). Moreover, the sensitivity with enrichment protocol C increased significantly (95.8%) with the E-TA method.

The efficacy of MC for STEC isolation has been previously reported (Drysdale *et al*., 2004; Auvray *et al*., 2009; Masana *et al*., 2011; Brusa *et al*., 2013). Before the existence of official guidelines for non-O157 STEC isolation, MC was used to evaluate and compare the enrichment efficiency of mEC and mTSB (Kanki *et al*., 2009). In our study, MC agar showed to be the most efficient in E-SA methods for enrichment protocols B, C, and D, with a sensitivity of 95.8%, 91.6%, and 45.8%, respectively. However, isolation in MC agar was significantly less sensitive (75%) with E-SA method A3 (mTSB-16 + TBX) (83.3%).

Based on the results obtained with experimentally inoculated samples, we selected two enrichment broths (mTSB-8 and mEC) to evaluate naturally contaminated samples, followed by isolation on MC agar. Based on PCR results, 25/43 (58.1%) and 28/43 (65.1%) of ground beef samples were *stx*-positive with enrichment protocols B and C respectively. The proportion of STEC-positive isolates obtained from methods B2 and C2 was 8/25 (32.0%) and 8/28 (28.5%), respectively. We isolated six STEC serotypes and two untypeable STEC from ground beef using methods B2 and C2. In this study one O26:H11 (stx_1^+/eae^+) strain was isolated by both methods used to evaluate naturally contaminated samples. Serogroup O26:H11 is included in the most prevalent STEC serogroups in clinical cases and human outbreaks (Possé *et al*., 2008; EFSA, 2013). In addition, amongst the isolates detected in naturally contaminated samples we detect O91:H21 (stx_2^+/eae^-), O113:H21 (stx_2^+/eae^-) and O178:H19 ($stx_1^+/stx_2^+/eae^-$), which are sporadically detected in human diseases (EFSA, 2013). Based on these results we support the necessity to reinforce the isolation steps to confirm the presence of the most prevalent serogroups to humans. The current effort to isolate all STEC serotypes could be detrimental in the confirmation of serotypes clinically importance to humans.

The proportion of isolates (32.0% and 28.5%) obtained for both methods used for naturally contaminated samples is similar to previously reported. The success to confirm STEC presence by culture isolation in ground beef was 29.8% in the United States (Bosilevac *et al*., 2011) and 34.0% in France (Pradel *et al*., 2000); in boneless beef trim used for ground beef it was 20% in the Unite dStates, Australia, and New Zealand and 56% in Uruguay (Bosilevac *et al*., 2007). Numerous reports have demonstrated poor correlation between the number of positive samples during *stx* screening and the number of samples that can be confirmed by isolation (Pradel *et al*., 2000; Bosilevac *et al*., 2007; Bosilevac *et al*., 2011). Factors such as low detection limit of the screening technique and high levels of background bacteria could impair the accurate detection of STEC in contaminated meat samples (Auvray *et al*., 2009; Ju *et al*., 2012). In addition, other variables such as volume of samples plated, amount of plates necessary to achieve STEC isolates, number of colonies selected per plate, and laboratory personnel experience might affect STEC detection in meat samples.

Conclusions

Currently, there are multiple STEC isolation methods available to identify contaminated food products. In addition, s*tx*-gene PCR is still being used as a screening for STEC detection. However, *stx*-PCR is also used as a confirmatory for enrichment broths or *stx*-positive samples. Thus, the mere detection of *stx*-genes as a definitive or confirmatory test for STEC presence might be excessive and STEC could be overdiagnosed. In numerous occasions, food products are disqualified due to the presence of *stx*-positive genes, regardless the bacterial strain carrying these genes. On the other hand, as we observed in this study, all possible isolation methods available were not sufficient to detect artificially contaminated samples and performed poorly in naturally infected samples. Therefore, we can conclude that there is no single method or combination of isolation methods capable of identifying all STEC serogroups. It would be necessary not only to combine multiple bacteriological tools but also adapt the most adequate set of techniques based on the regional prevalence of specific STEC that affect human health in order to maximize the use of the available tools for the detection and isolation of STEC strains.

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No competing financial interests exist.

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Address correspondence to:
Dr. Gerardo A. Leotta, PhD
Instituto de Genética Veterinaria
''Ing. Fernando N. Dulout''
Facultad de Ciencias Veterinarias
Universidad Nacional de La Plata
Av. 60 y 118, CC: 296
La Plata, Buenos Aires 1900
Argentina

E-mail: gerardo.leotta@gmail.com