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Prevalence, O-genotype and Shiga toxin (Stx) 2 subtype of Stx-producing *Escherichia coli* strains isolated from Argentinean beef cattle

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ABSTRACT. The aims of this study were to investigate prevalence, O-genotype, and virulence gene profile including Shiga toxin (Stx) 2 gene-subtype of Stx-producing *Escherichia coli* (STEC) in beef cattle from the Bahía Blanca in Argentina. Rectal swabs were collected from 283 beef cattle in 2012. *stx* genes were detected in 90 (32%) out of the 283 rectal swabs by *stx* gene-specific PCR assay. The positive cases were 13 with *stx1*, 58 with *stx2,* and 19 with both *stx1* and *stx2*. Among 90 *stx* gene-positive samples, 45 STEC strains were isolated, which included 3 *stx1*, 34 *stx2*, and eight *stx1* and *stx2* genes positive isolates. O-genotyping grouped 45 STEC strains into 19 different O-genotypes such as Og8, Og145, Og171, Og185 (4 from each), Og22, Og153, Og157 (3 from each) and others. Various *stx2* gene-subtypes were identified in 42 STEC strains: 13 positive cases for *stx2a*, 11 for *stx2c*, 3 for *stx2g*, 10 for *stx2a* and *stx2d*, 4 for *stx2a* and *stx2c*, and 1 for *stx2b*, *stx2c* and *stx2g*. *efal* gene, generally prevalent in clinical strains, was detected in relatively high in the STEC strains. These data suggest that *stx2a* and *stx2c* were distributed not only in O145 and O157 but also in minor O-genotypes of STEC in Argentina.

KEY WORDS: Argentina, beef cattle, *Escherichia coli*, enterohemorrhagic *Escherichia coli*, Shiga toxin-producing *Escherichia coli*

A number of sporadic cases and outbreaks of Shiga toxin-producing *Escherichia coli* (STEC) infections have been reported in many countries since 1982 when first STEC food poisoning occurred in the US [27]. STEC can cause acute gastroenteritis, hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) in humans, particularly in infants [27]. In Argentina, around 400 HUS cases continue to be reported annually, with an incidence of 8.4 cases/100,000 children less than five years old and a lethality ranging from 2–5% [1].

The major virulence factors of STEC are Shiga toxin (Stx) and intimin. Stx is divided into two immunologically distinct groups: Stx1 and Stx2, which share approximately 56% amino acid sequence homology. Both Stx1 and Stx2 inhibit protein synthesis in eukaryotic cells, resulting in cell death [39]. Intimin encoded by the *eae* (*E. coli*-attaching and effacing) gene, which is located on the chromosomal pathogenicity island termed locus for enterocyte effacement (LEE). This gene is responsible for attaching and effacing, resulting in pedestal formation on intestinal epithelial cells [27].

In general, STEC producing Stx2 causes more severe disease than that producing Stx1. Stx2 is classified into at least 7 subtypes (Stx2a to Stx2g) based on their amino acid sequence diversity [34]. Among these subtypes, epidemiological data indicate that Stx2a and/or Stx2c producers were more frequently associated with HC and HUS cases [20, 34]. Therefore, Stx2 subtyping is

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important since it may provide valuable information about the virulence of STEC strains. Furthermore, in particular O serogroup has also been associated with the severity of the diseases as well as isolation frequency from patients. For example, O157 has been most frequently isolated from patients with severe diseases. In addition, O26, O45, O103, O111, O121 and O145, known as the "Big Six non-O157" serogroups [2], have also been more frequently isolated from outbreaks and sporadic cases with HC and HUS than other non-O157 serogroups [6]. These STEC serogroups normally contain *eae* gene; however, rare serogroups such as O104 and O113 without *eae* gene have also been isolated from patients with HUS [11, 28].

STEC O113 without *eae* gene was isolated in 1998 from children with HUS in Australia [28]. A novel autoagglutinating adhesin, so-called Saa (STEC autoagglutinating adhesin), was discovered in this strain [29]. In 2011, a large outbreak of STEC food poisoning occurred in Germany: 855 cases of HUS, 2,987 cases of acute gastroenteritis, and 53 fatalities [33]. In this outbreak, the causative agent was a hybrid strain between STEC and enteroaggregative *E. coli* (EAEC) serotype O104:H4, which carried *stx2a* genes as well as virulence gene for EAEC such as *aggA* and *aggR* genes [11]. Similarly, hybrid strains of EHEC/enterotoxigenic *E. coli* (ETEC) such as serotypes O101:H- and O2:H27 have also been isolated from an infant with HUS in Finland [25]. Furthermore, hybrid strains of EHEC/ETEC such as O15, O136 and O175 have been isolated from water, cattle, and patients with diarrhea, fever and abdominal pain in Germany [31]. These strains contained virulence gene for ETEC such as heat-stable enterotoxin (*est*) in addition to *stx1* or *stx2*. Therefore, O serogrouping and virulence gene profiling are important for the identification of more virulent STEC strains isolated from patients and animals.

Argentina has one of the highest HUS rate globally (7.8–17/100,000 in children under 5 years of age) [36], which is about 10-fold higher than that of any other industrialized countries [36]. Cattle and other ruminants are the primary reservoirs of STEC. Contamination of meat during slaughtering and processing is the pathway most likely associated with human infection. Most of the works regarding isolation and characterization of STEC strains from cattle have been performed in the Buenos Aires city, 600 km to the north from study place [10, 22], but little is known about the prevalence, O serogroup and virulence gene profile including *stx2* subtypes of STEC isolated from beef cattle in the southern region of the country and the work performed in the Bahía Blanca region is pioneer.

In this study, therefore, we investigated prevalence of STEC among beef cattle from rural areas nearby Bahía Blanca city in Argentina and extensively analyzed STEC isolates for O-genotype and virulence gene profile including *stx2* gene-subtype. We hypothesized that the reason why HUS cases are high in this region could be a higher prevalence and/or more pathogenic of STEC strains compared with those isolated from other regions of the world.

MATERIALS AND METHODS

Stool samples

Rectal swabs were collected from 283 beef cattle, selected from 31 different farms, at slaughterhouses located in Bahía Blanca city (Fig. 1), during the period of November to December 2012. The beef cattle were from 3 provinces near Bahía Blanca city (Fig. 1): the south of Buenos Aires (n=193), La Pampa (n=54) and the north of Río Negro (n=36). This region is located in south of the Pampas and the northern part of Patagonia. Five to ten rectal swabs were collected from each farm and were placed in Cary-Blair medium (Beckton, Dickinson and Co., Franklin Lakes, NJ, USA), transported to laboratory at ambient temperature and processed within 4 hr of sample collection.

Detection and isolation of STEC

Rectal swabs were inoculated into 3 ml of tryptic soy broth (TSB) (Becton, Dickinson and Co.) and incubated at 37°C for 14 hr with vigorous shaking. Presence of *stx1* and *stx2* genes was examined by a PCR assay [26]. Briefly, overnight culture was diluted 10-fold in sterile TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and boiled for 10 min. After centrifugation at 12,000 *g* for 3 min, the supernatant was subjected to a PCR assay using a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems Japan, Tokyo, Japan). A total 30 µl of PCR mixture included appropriate concentration of each primer set [26], 0.2 mmol l^{-1} dNTP, 0.75 U of r*Taq* in 1x PCR buffer (Takara Bio Inc., Shiga, Japan). The PCR products were then analyzed by electrophoresis in 1.5% agarose gels (Seakem LE Agarose; Lonza, Basel, Switzerland), stained with 2 µg/ml of ethidium bromide and visualized under UV light with a gel documentation system (Bio-Rad Laboratories, Hercules, CA,

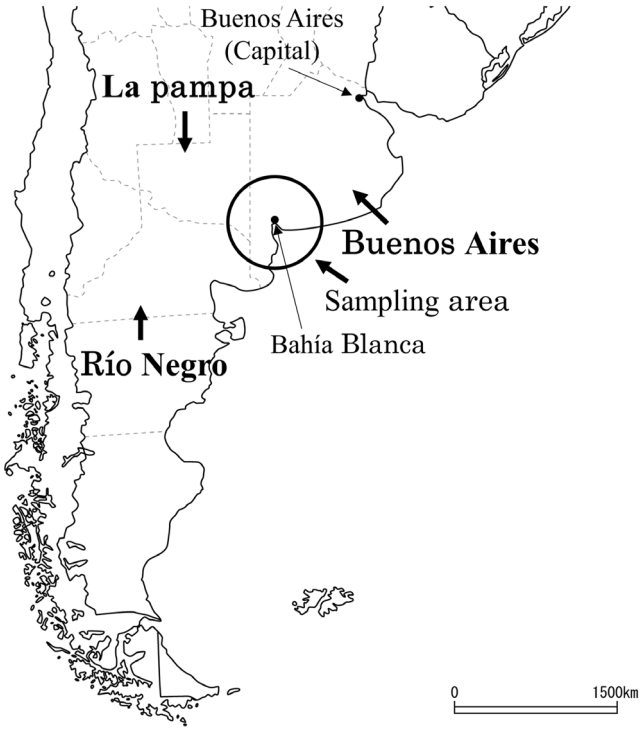

Fig. 1. Map of sampling site in Argentina. Arrow indicates Buenos Aires city (capital of Argentina) and Bahía Blanca city, while bold arrow indicates Buenos Aires, La Pampa and Río Negro provinces, respectively. A circle indicates the sampling area within 400 km from Bahía Blanca city.

USA). STEC O157 strain Sakai and *E. coli* strain C600 were used as a positive and negative control for all experiments in this study, respectively.

Broth cultures, which gave *stx1* and/or *stx2* gene-specific amplicons, were serially diluted in sterile PBS (pH 7.0) and 100 µl of each dilution was spread on MacConkey agar (Becton, Dickinson and Co.) and incubated at 37°C for 14 hr. Obtained colonies were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) by a replica blotting method, and the colony hybridization assay was conducted by using ^{32}P-labeled *stx1* and *stx2* gene-probes [38]. The *stx1* and *stx2* gene-probes were PCR-amplified by using primers [26] and a genomic DNA of STEC O157 strain Sakai (*stx1*$^{+}$/*stx2*$^{+}$). The amplicons were purified by QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany) and labeled by Multiprime DNA Labeling System (GE Healthcare Life Sciences, Buckinghamshire, UK) and [α-^{32}P] d-CTP (111 TBq mmol^{-1}, Perkin Elmer Inc., Waltham, MA, USA). The *stx* gene-positive bacteria were confirmed to be *E. coli* by biochemical tests including lysine indole motility medium, Simmons citrate agar, methylred-Voges Proskauer medium, sulfide indole motility medium and triple iron agar (Eiken Chemical Co., Ltd., Tokyo, Japan). The tests were performed as described elsewhere [8].

O serogrouping, O-genotyping and virulence gene profiling

O serogroups were determined by tube agglutination method using somatic antisera (O2, O8, O91, O145, O153, and O157) [8], which were prepared at the Osaka Prefectural Institute of Public Health, Osaka, Japan and/or as O-genotype by *E. coli* O-genotyping PCR method as described previously [17].

Presence of virulence genes, with exception of *astA*, was analyzed by colony hybridization assays as described previously [38]. Enteropathogenic *E. coli* strain GB1371 (*eae*), STEC strains D-129 (*saa*, *lpfA*) and Sakai (*efa*, *iha*, *eha*), cytolethal distending toxin (CDT)-producing *E. coli* strains AH-2 (*α-hlyA*, *cnf1*, *Eccdt-I*), AH-6 (*cnf2*, *Eccdt-III*), AH-12 (*Eccdt-IV*) and AH-11 (*Eccdt-V*), EAEC strain 042 (*Eagg*, *astA*), enteroinvasive *E. coli* (EIEC) strain 3 (*invE*), ETEC strains 12566 (*elt*) and 12671 (*est*), and *E. albertii* strain AH-5 (*Eacdt*) were used as positive controls [13, 14, 26]. Each gene was amplified using primers and conditions as summarized in Supplementary Table 1 [13, 14, 26]. Each PCR product was radiolabeled as described for the radiolabeling of the *stx* genes. Presence of *astA* gene was determined by PCR [26].

Stx2 subtyping

The subtypes of *stx2* were essentially determined by PCR as previously described [34] with minor modifications. DNA templates were extracted by a boiling method as described for the amplification of the *stx* genes, and r*Taq* (Takara Bio Inc.) was used for amplification. Since *stx2* subtypes such as *stx2a*, *stx2c* and *stx2d* could not be differentiated by the subtype-specific PCR using the 35 cycle-protocol [34], the subtype-specific PCR was optimized by reducing the PCR cycle to 29. PCR was performed by simplex and the method as described in the chapter of detection and isolation of STEC.

RESULTS

STEC isolation, O serogrouping and O-genotyping

stx genes were detected in 90 (32%) out of 283 bovine rectal swabs. Among 90 *stx* gene-positive samples, 13 were positive for *stx1* (14%), 58 for *stx2* (64%), and 19 for *stx1* and *stx2* (21%) and total of 45 *stx* gene-positive bacteria were isolated and confirmed to be *E. coli*. Forty-five STEC strains were classified into 19 O-genotypes and untypable (Table 1). For example, Og145 and Og157 were identified as major O-genotypes in 4 and 3 isolates, respectively. While the minor O-genotypes, Og8, Og171 and Og185 were identified as most frequently isolated serogroups.

Virulence gene profiling

Virulence profiles, embracing *eae*, *saa*, *lpfA*, *iha*, *eha*, *efa* and *Eagg* genes for adhesins and *astA, elt*, *est, cdt*, *cnf* and *α-hlyA* genes for toxins, were investigated by the colony hybridization and PCR assay. Table 1 shows the virulence profile and prevalence of *iha*, *lpfA*, *eha*, *efa*, *saa*, *eae* and *α-hlyA* genes in the 45 STEC strains, *iha*, *lpfA*, *eha*, *efa*, *saa*, *eae* and *α-hlyA* genes were detected in 37 (82%), 35 (78%), 35 (78%), 24 (53%), 10 (22%), 7 (16%) and 1 (2%) strain, respectively (Table 1).

Stx2 subtyping

The genes *stx2a*, *stx2c* and *stx2g* were detected by the modified subtype-specific PCR in 13, 11 and 3 strains, correspondingly (Table 2). Furthermore, 4 strains were positive for both *stx2a* and *stx2c*, and 10 for *stx2a* and *stx2d* genes. One strain carried the 3 *stx2* genes such as *stx2b*, *stx2c* and *stx2g*, evidencing the reliability of the subtype-specific PCR.

DISCUSSION

In this study, prevalence, O-genotype and Stx2 subtypes of STEC strains isolated from Argentinean beef cattle near Bahía Blanca, southern part of Argentina were for the first time examined. Sample size was calculated after Peck *et al*. [30] taking into account two spatial settings with similar environmental (semiarid) and farming (extensive) characteristics, for the delivery of livestock to Bahía Blanca city, according to information provided by local slaughter houses. In the Bahía Blanca rural department, the area used for cattle raising is about 180,000 ha, with 266 livestock establishments, having areas ranging between 200 and 800 ha [18]. In 2012, cattle stock was 68,000 animals [1]. For this population, a sample size of 272 or 382 animals would be needed

Table 1. O-genotype and virulence gene profile of *stx* gene-positive *Escherichia coli* cattle strains

	O-genotype	Total	No.	Virulence gene profile								
				stx1	*stx2*	*α-hlyA*	*eae*	*saa*	*lpfA*	*iha*	*eha*	*efa*
Major O-genotypes	Og145	4	4	-	+	-	+	-	-	+	+	+
	Og157	3	3	-	+	-	+	-	-	+	+	+
Minor O genotypes	Og8	4	1	-	+	-	-	-	+	-	+	-
			1	-	+	-	-	-	+	+	+	-
			1	+	+	-	-	+	+	+	+	-
			1	-	+	-	-	-	+	-	-	-
	Og171	4	3	-	+	-	-	-	+	+	+	+
			1	+	+	-	-	-	+	+	+	+
	Og185	4	3	-	+	-	-	-	+	+	+	+
			1	-	+	-	-	-	+	+	-	+
	Og22	3	3	-	+	-	-	-	+	+	+	-
	Og153	3	1	-	+	-	-	-	+	-	+	-
			1	+	+	-	-	+	+	+	+	+
			1	-	+	-	-	-	+	+	+	+
	Og2	2	1	-	+	-	-	-	+	+	-	-
			1	-	+	-	-	+	+	+	-	+
	Og91	2	2	+	+	-	-	+	+	+	+	-
	Og116	2	2	-	+	-	-	-	-	-	+	-
	Og130	2	1	+	+	-	-	+	+	+	+	-
			1	+	+	-	-	+	+	+	-	+
	Og178	2	2	-	+	-	-	-	+	+	+	+
	Og3	1	1	+	-	-	-	-	+	-	+	-
	Og15	1	1	-	+	-	-	-	-	-	-	-
	Og82	1	1	-	+	-	-	+	+	+	+	-
	Og88	1	1	+	-	+	-	-	+	+	-	-
	Og163	1	1	+	+	-	-	+	+	+	+	+
	Og168	1	1	-	+	-	-	-	+	+	-	-
	Og179	1	1	-	+	-	-	+	+	+	+	+
	OgUT	3	1	+	-	-	-	-	+	+	-	-
			1	-	+	-	-	-	+	+	+	+
			1	-	+	-	-	-	+	-	-	-
	Total (%)		45	11 (24)	42 (93)	1 (2.2)	7 (16)	10 (22)	35 (78)	37 (82)	35 (78)	24 (53)

+ and - indicate positive and negative, respectively by PCR or colony hybridization assay. All the tested strains were negative for *Eagg, invE, astA, est, elt, cdt, cnf* genes. OgUT: untypable. The number in parenthesis indicates the percentage.

to have confidence levels of 90% or 95%, respectively, that the real value is within ± 5% of the measured one. In order to set the obtained data in a larger geographical context we also considered three neighboring sectors of the provinces Buenos Aires, La Pampa and Río Negro, where livestock is estimated to be around 600,000 animals. This population would require sample sizes of 273 or 384 for confidence levels of 90% or 95%, respectively. Therefore, prevalence and characteristics of STEC were investigated in 283 beef cattle (mostly Aberdeen Angus) from 3 provinces (Buenos Aires, La Pampa and Río Negro) supplied as livestock to Bahía Blanca city (Fig. 1).

In this study, *stx* genes were detected in 90 (32%) out of 283 bovine rectal swabs. In the previous studies, STEC contamination rates in Argentinean cattle, which were mostly from Buenos Aires city and its surroundings, were reported to be from 28 to 78% in cattle pre-slaughter [7, 22]. In other countries, STEC prevalence in cattle was also varying from around 15% in Spain [4], 24% in Japan [19] and 55% in the US [24]. These data indicate that high HUS incidence in the Bahía Blanca region does not seem to be due to a high contamination rate of STEC in beef cattle.

O157 is the most predominant O serogroup associated with outbreaks and severe diseases such as HC and HUS [27]. However, incidence and severe cases of non-O157 have also been increasingly recognized in the world, indicating that STEC are diversifying due to stx2-phage conversion. O26, O45, O103, O111, O121 and O145 have been recognized as the "Big Six non-O157" serogroups, generally associated with severe diseases such as HUS [2]. Therefore, we determined O serogroup by a PCR-based O-genotyping method [17]. Forty-five STEC strains were classified into 19 O-genotypes and untypable (Table 1). In Argentina, O145, O121, O26 and O174 were reported as the four most prevalent serogroups in human diseases among non-O157 STEC strains [32]. Indeed, Og145, frequently isolated from patients in Argentina, was the most predominant O-genotype together with Og8, Og171 and Og185. None of the Og26 and Og121 strains, members of "Big Six non-O157", were isolated in this study. Masana *et al.* [21] reported that O178 (9.7%) was predominant O serogroup, followed by O8 (7.9%), O130 (7.0%), O113 (5.3%), O103 (4.0%) and O174 (3.1%) of STEC isolated from bovine feces in Argentina. Fernández *et al.* [10] also reported that O130 (22%)

Table 2. Association between *stx2* subtype and O-genotype of Shiga toxin-producing *Escherichia coli* (STEC) isolates from beef cattle

stx2 subtype	O-genotype	No. of isolate	Total no. (n=42) (%)
stx2a	Og145	4	13 (31)
	Og22	3	
	Og2	1	
	Og8	1	
	Og82	1	
	Og130	1	
	Og179	1	
	OgUT	1	
stx2c	Og185	4	11 (26)
	Og171	3	
	Og178	2	
	Og8	1	
	Og153	1	
stx2g	Og2	1	3 (7.1)
	Og15	1	
	Og168	1	
stx2a & *stx2c*	Og157	3	4 (9.5)
	Og130	1	
stx2a & *stx2d*	Og8	2	10 (24)
	Og91	2	
	Og116	2	
	Og153	2	
	Og163	1	
	OgUT	1	
stx2b, stx2c & *stx2g*	Og171	1	1 (2.4)

OgUT indicates untypable.

and O178 (21%) were predominant O serogroup, followed by O113 (12%), and O91 (8.8%) of STEC isolated from dairy cattle in Argentina. O serogroups such as O8, O22, O88, O91, O116, O163, O168, O171, O178 isolated from patients with diarrhea, bloody diarrhea and HUS [2, 23], were also detected in Argentinean beef cattle in this study, indicating that these O serogroups in cattle isolates cannot be ignored. In particular, STEC O178 has been known to an emerging type of STEC associated with HUS and diarrhea in Argentina and Germany [23].

Virulence gene profiles were investigated by the colony hybridization and/or PCR assay. Wu *et al* [37] reported that *saa* (91%/3.3% in cattle/human) and *lpfA* (99%/18%) genes were more prevalent in cattle STEC strains, while *eae* (1.2%/95%) and *efa1* (1.2%/95%) genes were more prevalent in clinical STEC strains. Virulence gene profile of Argentinean beef cattle STEC strains is similar to that of Japanese cattle strains except for *saa* and *efaI* genes (Table 1). The STEC strains isolated from Argentinean beef cattle contained 53% of *efa1* genes. It should be emphasized that *efaI* gene (18%/90%) was more prevalent in clinical STEC strains and also detected in all 4 HUS associated strains in Brazil [5]. These data indictae that *efaI* gene might be associated with human infection.

The result of *stx2*-gene subtyping indicates *stx2a* and *stx2c* are prevalent in Argentinean beef cattle strains. It is interesting to note that STEC Og8 and Og171 cattle isolates [3, 9, 12, 35] as well as clinical isolates [12, 15, 16] contain *stx2* (a or c) but not *eae* genes. STEC O8 was isolated from HUS and bloody diarrheal patients in Argentina [12] and Japan [15], respectively. Taken together, these data may indicate that beef cattle in southern region of Argentina carry not only major O serogroups of STEC but also potentially virulent minor O serogroups. Since number of samples and strains analyzed in this study was limited, further studies are required to confirm these findings.

Nonetheless, here we present, for the first time, prevalence of O-genotype and virulence gene profile including *stx2* gene-subtype in the regions near Bahía Blanca including Buenos Aires (southern part), La Pampa and Río Negro provinces, where highest HUS incidences are observed. All settings, extensive to semi-extensive cattle farming, are in rural areas and cattle are slaughtered in urban areas, the comparison between urban and rural settings is impossible in the Bahía Blanca region.

In conclusion, our data indicate that not only *stx2a* and/or *stx2c* gene-positive O145 and O157, which are major O serogroups of STEC but also *stx2a* and/or *stx2c* gene-positive minor O serogroups such as O8, O171 and O185 were most frequently isolated from Argentinean beef cattle.

CONFLICT OF INTEREST. The authors declare that there are no conflicts of interest.

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