Short communication: Characterization of Shiga toxin-producing *Escherichia coli* isolated from newborn, milk-fed, and growing calves in Argentina

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ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) cause foodborne pathogenic disease that is shed in the feces of cattle. The aim of this study was to evaluate how early young calves are colonized by STEC strains, potentially pathogenic for humans, and the prevalence in different calf categories. From 808 rectal swabs analyzed by PCR, 38% were *stx* positive. The prevalence in newborn (<24 h from birth), milk-fed (<2-mo-old), and growing calves (2–8 mo old) were 25, 43, and 58%, respectively. Forty different STEC serotypes were found among isolates from newborn, milk-fed, and growing calves that shed STEC strains potentially pathogenic for humans. The STEC strains could be acquired early from mothers, enabling the infection of other animal categories and confirming the risk to public health.

Key words: Shiga toxin-producing *Escherichia coli*, calf, Shiga toxin, dairy

Short Communication

Shiga toxin-producing *Escherichia coli* (STEC) are considered the main causative agents of hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS; Aidar Ugrinovich et al., 2007). In Argentina, HUS is endemic, with 500 cases per year and an incidence of 17/100,000 children less than 5 yr of age (Rivas et al., 2010). Feces of cattle and direct contact with dairy cattle and the dairy farm environment (Oliver et al., 2005), consumption of undercooked meat, nonpasteurized dairy products and vegetables, and water contaminated with feces are some of the possible routes for STEC human exposure (Hussein and Sakuma, 2005).

In several countries, including Argentina, most outbreaks of HC and HUS have been attributed to the STEC O157:H7 serotype (Rivas et al., 2006) but

infections with some non-O157 STEC serotypes are frequently associated with HC and HUS, mainly in Europe and Latin America (Blanco et al., 2004; Bettelheim, 2007).

Shiga toxin-producing *Escherichia coli* produce 2 potent toxins called Shiga toxins 1 and 2 (**Stx1** and **Stx2**) encoded by *stx1* and *stx2* genes (Paton and Paton, 1998). In addition to toxin production, another virulence factor expressed by STEC is intimin (encoded by the chromosomal gene *eae*), which is responsible for the intimate attachment of STEC to the intestinal epithelial cells, causing attaching and effacing lesions. Other virulence factors of STEC are an enterohemolysin (encoded by the *ehxA* gene) and the autoagglutinating adhesin (**Saa**) encoded by the *saa* gene, which was found in the *eae*-negative STEC (Paton et al., 2001).

Cattle are the main reservoir of STEC but they are not usually associated with disease. In previous studies, we had demonstrated the role of Argentine cattle as main reservoir of non-O157:H7 serotypes and many of them have been involved in HUS and HC outbreaks worldwide (Fernández et al., 2010). Young calves have been considered the category with the highest prevalence of STEC, although Cobbold and Desmarchelier (2002) found higher prevalence in heifers. However, no studies are available on STEC prevalence in newborn calves. Differences in fecal excretion of STEC by cattle could be due to factors such as age, diet, climate conditions, and animal management, or herd factors such as stocking density, waste management, and housing systems (Vicente et al., 2005). The hypothesis of this work is that a very quick and early colonization of STEC strains potentially pathogenic for humans exist in young calves, allowing the detection of high prevalence in other calf categories.

Between April 2006 and February 2007, newborn (<24 h from birth), milk-fed (<2-mo-old), and growing calves (2–8 mo old) from 5 dairy farms in the Pampeana Region of Argentina were sampled by rectal swab. Dairy farms were geographically separated and were named A, B, C, D, and E. Farms had herds in which no outside breeding stock cattle were introduced (closed herds).

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Newborn calves remained with their mothers for 24 h after birth. Milk-fed calves were fed milk (farms A, B, and E) or milk replacer (farms C and D) until 2 mo of age. Growing calves (2 to 8 mo of age) were housed in pens and blocked by age, size, and weight and fed a balanced feed according to the nutritional requirements of the NRC (NRC, 2001). All animals in this study were sanitary, fit, healthy, and without treatment with antibiotics or other drugs.

Sample size (25%) for each category of calves was determined taking into account an occurrence less than 10% and a confidence level of 95%. Fecal samples (808) were distributed into 378 samples from newborn calves, 252 samples from milk-fed calves, and 178 samples from growing calves. Newborn and growing calves were sampled by convenience (nonrandom method) during the spring calving season, and milk-fed calves were randomly sampled.

Rectal swabs were immediately transported to the laboratory and plated to obtain confluent growth according to the protocol described by Fernández et al. (2010). Briefly, an aliquot of confluent growth, previously cultured on MacConkey agar plates, was inoculated into 30 mL of Luria Bertani broth, incubated in an orbital shaking incubator at 37°C for 4 h, and processed for DNA extraction (Padola et al., 2004). The *stx* genes were screened by multiplex PCR (Fernández et al., 2009) and were used as the indicator for STEC. One milliliter from each culture was frozen at -70°C with the addition of 20% glycerol for isolation of individual *stx*-positive colonies.

From each *stx*-positive sample, about 10 to 200 colonies were analyzed by PCR to detect *stx1* and *stx2* for isolation of STEC non-O157. For isolation of STEC O157:H7, each *stx*-positive sample was tested for the presence of *eae-γ1* and then subjected to immunomagnetic separation with polystyrene magnetic beads coated with antibodies against the O157 antigen (Dynal Biotech ASA, Oslo, Norway) following the manufacturer's instructions. Then, the concentrated samples were inoculated on sorbitol MacConkey plates. All negative colonies for sorbitol fermentation were confirmed as *E. coli* O157 with an agglutination test (Oxoid Ltd., Basingstoke, UK; Padola et al., 2004).

Each STEC isolate was also tested for the presence of *stx1*, *stx2*, *eae*, *saa*, and *ehxA* by multiplex PCR according to experimental conditions for *stx1*, *stx2*, *eae*, *eae-γ*, *saa*, and *ehxA* amplification described by Fernández et al. (2010). The O and H antigens were determined by a microagglutination technique in plates and by a tube agglutination technique, respectively, with an antisera kit (O1-O186) and 56 H antisera produced by Labora-

Table 1. Shiga toxin-producing *Escherichia coli* (STEC)-positive calves in 5 dairy farms

Farm	No. of STEC-positive animals (%)		
	Newborn ¹ (n = 378)	Milk-fed ² (n = 252)	Growing ³ (n = 178)
A	22 (37)	28 (56)	14 (56)
B	16 (30)	20 (54)	25 (75)
C	22 (22)	13 (24)	22 (49)
D	21 (20)	17 (31)	15 (56)
E	12 (20)	30 (54)	27 (53)
Total	93 (25)	108 (43)	103 (58)

¹Calves <24 h old.

²Calves <2 mo old.

³Calves 2 to 8 mo old.

torio de Referencia de *E. coli* (LREC, Lugo, Spain), as described by Blanco et al. (1996).

From 808 samples, 38% were STEC positive. Differences in STEC prevalence were detected: 25, 43, and 58% in newborn, milk-fed, and growing calves, respectively (Table 1). These differences could be explained by certain factors and management practices, such as contact between animals, feeding, handling of feces and sewage, and the presence of other domestic animals and insects that could influence the excretion of STEC (Wilson et al., 1993). The present study is the first, to our knowledge, that describes the presence of STEC in newborn calves less than 24 h old, suggesting that they are exposed to this bacterium quickly after birth and play an important role in vertical transmission of STEC.

Calves fed milk replacer (farms C and D) had lower STEC prevalence than calves fed colostrum or milk (farms A, B, and E; Table 1), suggesting that fecal contamination of milk may occur during milking (Doyle, 1991). The high prevalence of STEC in growing calves was observed in all dairy farms, in agreement with Cobbold and Desmarchelier (2000; 42%) and Moreira et al. (2003; 49%). Fernández et al. (2009) found a lower STEC prevalence in dairy cows (37.5%) from the same farms of this study. The different prevalence among dairy cows, milk-fed, and growing calves agrees with the results obtained by other authors (Cobbold and Desmarchelier, 2000; Vicente et al., 2005). This high prevalence in calves may be due to several factors that allow better colonization of the gastrointestinal tract, such as factors derived from weaning stress, minor features of the immune system, changes in diet and intestinal pH, and anatomical and physiological changes in the gastrointestinal tract, among other factors.

From the 304 *stx*-positive samples, 148 STEC isolates were obtained: 26 from newborn, 46 from milk-fed, and 76 from growing dairy calves. Multiplex PCR showed

that 77% of STEC isolates from newborn calves carried *stx2*, 25% carried *stx1*, 81% carried *ehxA*, 65% carried *eae*, and 11% carried *saa*.

In milk-fed calves, 46% of STEC isolates carried *stx2*, 37% carried *stx1*, and 17% carried *stx1* + *stx2*. The *ehxA*, *eae*, and *saa* genes were detected in 87, 72, and 15% STEC isolates, respectively, and only 17% of strains positive for the *ehxA* gene showed *saa*.

In growing calves, 81% of the STEC isolates carried *stx2*, 8% carried *stx1*, and 10% carried *stx1* + *stx2*. The *ehxA*, *eae*, and *saa* genes were detected in 56, 13, and 29% of the isolates, respectively. A high percentage of STEC strains isolated from newborn and milk-fed calves carried *eae*, whereas isolates from growing calves had a low proportion of *eae*, similar to cows from the same farms (Fernández et al., 2010). In newborns and milk-fed calves, *stx2*, *eae*, and *ehxA* made up the main virulence profile, whereas *stx2* and *stx2 saa ehxA* were the predominant virulence profiles found in growing calves (Table 2).

Previous studies have indicated a relationship between the age of cattle and the *stx* gene carried, with *stx1* being more predominant in calves and *stx2* more common in adult cattle (Cho et al., 2006). However, *stx2* was the predominant *stx* type in all categories of calves, according to previous studies in cattle in Argentina (Fernández et al., 2010). These results are important because Stx2 is more cytotoxic than Stx1 and experimental and epidemiological data have demonstrated that Stx2 may be more important in the development of HUS than Stx1 (Fremaux et al., 2006).

Serotypes O157 and non-O157 of STEC isolates are shown in Table 3. Shiga toxin-producing *E. coli* O157:H7 was detected in 0.8 and 2.9% of milk-fed calves and

Table 3. Shiga toxin-producing *Escherichia coli* serotypes isolated from dairy calves

Source (calves)		
Newborn ¹	Milk-fed ²	Growing ³
O8:H25	O5:H–	O2:H25
O11:H21	O8:H16	O8:H19
O15:H27	O15:H–	O26:H11
O26:H11	O26:H11, H44	O27:H21
O91:H21	O55:H–	O64:H–
O101:H? ⁴	O103:H8, H21	O91:H21, H28
O103:H–, ⁵ H2, H21	O111:H–	O103:H–
O111:H–	O113:H2, H21	O111:H–
O145:H–	O141:H42	O113:H2, H21
O171:H25	O145:H–	O153:H25
ONT ⁶ :H10, H21	O153:H25	O157:H7
	O157:H7	O163:H19
	O165:H42	O171:H?, H2
	O172:H–	O172:H–
		O174:H21
		O178:H–, H19, H25
		ONT:H8

¹Calves <24 h old.
²Calves <2 mo old.
³Calves 2 to 8 mo old.
⁴H? = an untypeable H antigen.
⁵H– = a nonmotile *E. coli* isolated.
⁶ONT = a nontypeable O antigen.

growing calves, respectively, and all carried *stx2*, *eae*, and *ehxA*. Shiga toxin-producing *E. coli* O157:H7 is a serotype detected with a low prevalence in cattle from Argentina, but it is the predominant serotype isolated in human patients in this country (Rivas et al., 2010). This could be due to the use of the selective methods to detect STEC O157 in routine clinical diagnosis. In our study, non-O157 serotypes were isolated in all categories of calves and many of them have been previously isolated from human patients with HUS, diarrhea, or HC in Argentina and other countries (Bettelheim, 2007; Rivas et al., 2010).

The occurrence of O91:H21, O113:H21, O178:H19, and ONT:H21 serotypes were previously reported in dairy cows in the same farms from Argentina (Fernández et al., 2010), indicating the possible transmission of strains among cattle. In newborn and milk-fed calves, STEC O145:H– was also detected, which was first isolated in Argentina in feedlot cattle by Padola et al. (2002) and has previously been involved in outbreaks of HUS in Japan (Kudoh et al., 1994). Currently, this serotype is the second, after O157:H7, most frequently isolated in cases of HUS in Argentina.

Most STEC serotypes isolated in this work carried genes linked to severe forms of human disease, and have been isolated previously in beef cattle, food, and humans (Padola et al., 2004; Rivas et al., 2010). Newborn calves are able to show STEC strains potentially patho-

Table 2. Virulence profile in newborn, milk-fed, and growing calves

Virulence profile ¹	No. of strains (%)		
	Newborn ²	Milk-fed ³	Growing ⁴
<i>stx2</i>	3 (11)	5 (11)	33 (43)
<i>stx2, saa, ehxA</i>	2 (8)	6 (13)	14 (18)
<i>stx2, eae, ehxA</i>	15 (58)	10 (35)	6 (8)
<i>stx1, eae, ehxA</i>	3 (11)	17 (37)	4 (5)
<i>stx1, ehxA</i>	2 (8)	—	2 (3)
<i>stx2, ehxA</i>	—	—	9 (12)
<i>stx1, saa, ehxA</i>	1 (4)	—	—
<i>stx1, stx2</i>	—	1 (2)	—
<i>stx1, stx2, saa, ehxA</i>	—	3 (6)	8 (10)
<i>stx1, stx2, eae, ehxA</i>	—	4 (9)	—
Total	26	55	76

¹Virulence profiles included Shiga toxin 1 (*stx1*), Shiga toxin 2 (*stx2*), intimin (*eae*), enterohemolysin (*ehxA*), and autoagglutinating adhesin Saa (*saa*) genes.
²Calves <24 h old.
³Calves <2 mo old.
⁴Calves 2 to 8 mo old.

genic for humans from the first day of life, allowing the infection of other animal categories, suggesting a vertical transmission and confirming the risk to public health.

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