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Virulence profile comparison between LEE-negative Shiga toxin-producing *Escherichia coli* (STEC) strains isolated from cattle and humans

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ABSTRACT

For comparison purposes, the prevalence of 8 virulence markers was investigated, by PCR, in 153 cattle and 47 human Locus for Enterocyte Effacement (LEE)-negative Shiga toxin-producing *Escherichia coli* (STEC) strains isolated in Argentina. Also, their correlation with severe disease was established. The virulence markers studied comprises 5 fimbrial and nonfimbrial adhesin-encoding genes (*fimA*, *iha*, *efa1*, $lpfA_{O113}$, and *saa*) and 3 toxin genes (*cdt*-V, *subAB* and *astA*) in addition to the Shiga toxins. The most prevalent virulence marker found was that encoded by the $lpfA_{O113}$ gene (199/200, 99%). Comparatively, the $lpfA_{O113}$, *fimA*, *iha*, *saa*, *subAB*, *cdt*-V and *astA* genes were detected in 100%, 92.8%, 85%, 52.9%, 36%, 11.8% and 9.8% of the cattle strains and in 97.9%, 95.7%, 89.4%, 40.4%, 32%, 17% and 10.6% of the human strains, respectively. All STEC strains were *efa1* negative. The most prevalent profile observed among cattle and human STEC strains was $lpfA_{O113}$ *iha fimA*. These results show that bovine LEE-negative STEC strains possessed genes encoding virulence factors present in human LEE-negative STEC strains that are associated with disease. Despite a great diversity of virulence profiles observed, further studies comparing wild type strains and their allelic mutants are needed to evaluate the role of each factor in the pathogenesis of LEE-negative STEC strains during human infections.

1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) is an important pathogen associated with different clinical manifestations and the life threatening hemolytic uremic syndrome (HUS) (Nataro and Kaper, 1998). Although *E. coli* O157:H7 is the most prevalent serotype associated with HUS, there is growing concern over the emergence of highly virulent STEC non-O157 serotypes that are globally distributed, several of which are associated with outbreaks and/or severe human illness (Coombes et al., 2008). Ruminants, particularly cattle, are recognized as their main natural reservoir and cattle-derived foods have been implicated in many outbreaks (Caprioli et al., 2005).

Initial bacterial colonization may be mediated by appendages such as fimbria and flagella. Type 1 fimbriae, encoded by a *fim* gene cluster, can be expressed by most *E. coli* strains and mediate mannose-sensitive adherence to mammalian epithelial cells (Yamamoto and Echeverria, 1996). Some STEC serotypes harbor a large pathogenicity island, termed the Locus for Enterocyte Effacement (LEE), required for the formation of Attaching and Effacing (A/E) lesions and intestinal colonization (McDaniel and Kaper, 1997). However, the presence of this island is not essential

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for pathogenesis, as a wide number of LEE-negative STEC strains are capable of causing human disease (Bettelheim, 2007).

Additional proteins encoded outside the LEE region, were proposed to be novel adhesive factors, such as Saa, Iha, Efa1/LifA, Lpf and ToxB (Paton et al., 2001; Tarr et al., 2001; Nicholls et al., 2000; Doughty et al., 2002; Tatsuno et al., 2001). However, their roles and the mechanism used by LEE-negative STEC strains to adhere to epithelial cells and to colonize animals are poorly understood.

Moreover, while the production of Shiga toxin by STEC is the primary virulence trait responsible for HUS, the presence of other toxins that may play a role in pathogenesis has also been described. Paton et al. (2004) reported that some STEC strains produce an additional virulence AB_5 cytotoxin termed subtilase cytotoxin (SubAB). Another toxin, CDT-V, a new member of the Cytolethal Distending Toxin (CDT) family, was identified in O157 and particularly in STEC non-O157 serotypes (Janka et al., 2003). The *astA* gene, which encodes EAST1 toxin, has been found in several categories of human diarrheagenic *E. coli* and is considered an additional determinant in the pathogenesis of *E. coli* diarrhea (Veilleux and Dubreuil, 2005).

Although some studies have evaluated the presence of putative adhesins and the newly described toxins in *E. coli* strains, this information in LEE-negative STEC strains isolated from cattle and humans, is scarce (Mihai Szalo et al., 2002; Toma et al., 2004; Tatarczak et al., 2005; Vidal et al., 2007; Cergole-Novella et al., 2007). Knowledge on the virulence profiles presented by LEE-negative STEC strains, would contribute to a better understanding of the virulence mechanism of this versatile group of bacteria. The purpose of this study was to compare the prevalence of putative adhesin genes in LEE-negative STEC strains of bovine and human origin isolated in Argentina, and establish their correlation with other virulence markers and the ability to cause severe disease.

2. Materials and methods

2.1. Bacterial strains

A total of 200 randomly selected LEE-negative STEC strains belonging to different non-O157 serotypes, were studied. A hundred and fifty-three strains isolated from fecal samples and carcasses from healthy beef cattle, during surveys and research programs carried out between 2005 and 2007, were included. For comparison purposes, 47 human strains isolated between 2001 and 2008, during the surveillance of HUS and diarrheal disease, were studied. The human strains were isolated from non-bloody ($n = 17$) and bloody ($n = 2$) diarrheal cases, HUS ($n = 19$) and asymptomatic household contacts of HUS cases ($n = 9$). All strains were previously tested for the presence of *stx*, *eae*, and *ehxA* genes using the techniques described by Leotta et al. (2005), Gannon et al. (1993) and Schmidt et al. (1995), respectively.

The STEC strains were tested for XbaI macrorestriction enzyme digestion patterns by pulsed-field gel electrophoresis using the 24-h CDC protocol (CDC, 2007), with minor modifications, to establish their clonal relatedness.

2.2. Genotypic characterization

The *stx* variants, putative adhesins and additional virulence markers were characterized by PCR. The analysis of stx_1 variants was performed according to Zhang et al. (2002). For differentiation of the stx_2 variants, the genotyping methods RFLP-PCR of Tyler et al. (1991) and Piérard et al. (1998) were performed. The presence of the gene encoding $Stx2d_{activatable}$ was determined by RFLP-PCR, according to Jelasic et al. (2003).

All STEC strains were tested for the presence of the *saa*, *iha*, *efa1*/*lifA*, $lpfA_{O113}$, *subAB*, *cdt*-V and *astA* genes according to the references cited in Table 1. Positive control strains were *E. coli* EDL933 (stx_1 and stx_2, *efa1*); 93-016, serotype O113:H21 (stx_{2d2}, $lpfA_{O113}$, *subAB* and *cdt*-V); 32511, serotype O157:HNM ($stx_{2c(vh-a)}$); EH250, serotype O118:H12 ($stx_{2\text{-}O118}$); 434-1, serotype O2:H8 (*saa*); 226/07, serotype O157:H7 (*iha*); and EIEC C481 strain, for the *astA* gene. The negative control strain was *E. coli* K12 strain JM109 (Promega, Madison, WI).

2.3. Serotyping

The identification of O and H antigens was carried out by following the standard procedures (Ewing, 1986), using currently available O (O1–O181) and H (H1–H56) antisera, prepared at Instituto Aldolfo Lutz, Brazil.

Table 1
Putative virulence factors of Shiga toxin-producing *Escherichia coli* (STEC).

Factor	Activity/effect	References
Efa1/LifA (enterohemorrhagic *E. coli* factor for adherence)	Chromosomal nonfimbrial adhesin	Nicholls et al. (2000)
Iha (iron regulated gene A homologue similar to *V. cholerae*)	Chromosomal nonfimbrial adhesin	Schmidt et al. (2001)
Saa (Shiga toxin-producing *E. coli* autoagglutinating adhesin)	Plasmid-encoded nonfimbrial adhesin	Paton and Paton (2002)
$LpfA_{O113}$ (long polar fimbriae closely related to Lpf of *S. enterica* serovar Typhimurium)	Chromosomal fimbrial adhesin	Doughty et al. (2002)
Type 1 fimbriae	Fimbrial adhesin	Toma et al. (2007)
CDT-V (cytolethal distending toxin)	ABC toxin; DNaseI activity, interferes with cell cycling	Cergole-Novella et al. (2007)
EAST 1 (enteroaggregative heat-stable enterotoxin)	Heat-stable toxin; activates GC[a] resulting in ion secretion	Yamamoto and Echeverria (1996)
Subtilase cytotoxin	Plasmid-encoded AB_5 potent and lethal cytotoxin	Paton and Paton (2005)
EHEC hemolysin	Plasmid-encoded RTX toxin; release Hb[b]; induces inflammatory cytokines	Schmidt et al. (1995)

[a] GC, guanylate cyclase.
[b] Hb, hemoglobin.

Table 2
Serotypes, source, Shiga toxin genotypes and other virulence markers of the *E. coli* strains studied.

Serotype	Source		*stx* genotype (No. of strains)	No. of strains positive for							
	Cattle	Human		*saa*	*ehx*A	*lpfA*$_{O113}$	*iha*	*astA*	*subAB*	*cdt*-V	*fim*A
O2:H25	1		stx_{2d} (1)			1	1	1			1
O5:HNM		1[a]	stx_1 (1)	1	1	1	1				1
O7:HNM	2		stx_1 (2)			2		2			
O7:H21	3		stx_1/stx_2 (1), stx_2 (1), $stx_{2c\ (vh\text{-}a)}$ (1)	2	2	3	3		2	2	3
O8:H16	3	1[b]	stx_1/stx_2 (2), stx_2 (1), stx_1/stx_{2d} (1)	4	4	4	4		1		4
O8:H19	3	3[b]	stx_{2d} (4), stx_1/stx_2 (1), stx_{2d2} (1)	3	6	6	2		1	1	6
O15:H27	2	2[b,c]	stx_{2d2} (3), stx_{1c}/stx_{2d2} (1)			4	4				4
O20:H19		1[b]	stx_{2d2} (1)			1	1				1
O22:H8	2		stx_{2d2} (2)			2	2				2
O22:H16	3		stx_{2d1} (1), stx_{2d1}/stx_{2d2} (1), $stx_{2\text{-}OX3a}$ (1)			3	3				3
O39:H49	1		stx_2 (1)	1	1	1	1		1		1
O46:H38	2		stx_1/stx_2 (2)	2	2	2	2				2
O58:H40		1[c]	stx_1 (1)			1					1
O59:H19		4[b]	stx_2 (4)			4	4				4
O74:H12	1		stx_1 (1)			1		1			
O74:H28	1		stx_{2d2} (1)	1	1	1	1		1		1
O74:H42	1		stx_1/stx_{2d}/stx_{2d2} (1)	1	1	1	1				1
O79:H19	2		stx_1/stx_{2d2} (2)	2	2	2	2		2		2
O79:H28	1		stx_{2d2} (1)	1	1	1	1				1
O82:H8	1		stx_1/stx_2 (1)	1	1	1	1				1
O91:H16	1		stx_{2d} (1)		1	1					1
O91:H21	1	3[a,c]	stx_1/stx_{2d}/stx_{2d2} (1), stx_2 (1), stx_{2d2} (1), stx_{2d}/stx_{2d2} (1)	4	4	4	4		4	4	4
O113:H21	9	3[b,c]	stx_2 (4), stx_{2d} (3), stx_1/stx_{2d}/stx_{2d2} (2), stx_1/stx_{2d2} (1) stx_{2d}/stx_{2d2} (1), stx_{2d2} (1)	11	11	12	12	2	11	6	12
O116:H21	5		stx_{2d} (2), stx_1/stx_{2d} (2), stx_1/stx_{2d}/stx_{2d2} (1)	5	5	5	5		5	3	5
O124:H19	1		stx_1/stx_2 (1)	1	1	1					1
O130:H11	7	2[c,d]	stx_1/stx_{2d2} (4), stx_1 (3) stx_1/stx_{2d} (1), stx_1/stx_2 (1)	8	8	9	8		8		9
O136:H12	7		stx_1 (7)			7		7			
O141:H49	3		stx_{2d} (2), stx_2 (1)	3	3	3	3		1		3
O143:HNM		1[b]	stx_{2d} (1)			1	1				1
O163:H19	3	1[c]	stx_2 (2), stx_{2d} (2)	4	4	4	4		4	4	4
O171:H2		2[c]	$stx_{2c(vh\text{-}a)}$ (1), stx_{2d2} (1)			2	2				2
O174:H8		1[d]	$stx_{2\text{-}Ox3a}$ (1)			1					1
O174:H21	1	9[b,c,d]	stx_{2d2} (10)	1	1	10	10	4			10
O174:H28	1	2[b]	stx_{2d} (2), stx_1/stx_{2d} (1)	3	3	3	3		3	1	3
O174:HNM		1[c]	stx_{1c}/$stx_{2\text{-}O118}$ (1)	1	1	1	1				1
O178:H19	18		stx_1/stx_2 (7), stx_1/stx_{2d} (4), $stx_{2c(vh\text{-}a)}$ (4), stx_{2d2} (2), stx_{2d}/stx_{2d2} (1)	12	12	18	18		5	3	18
O179:H8	3		stx_{2d} (3)	3	3	3	3		3		3
ONT:H2	21		$stx_{2c(vh\text{-}a)}$ (6), stx_{2d2} (5), stx_{2d1}/stx_{2d2} (5), stx_{2d}/stx_{2d1} (2), stx_1 (1), stx_{2d1} (1), 2UT (1)			21	21				21
ONT:H4		2[b]	stx_2 (2)			1	2	1			
ONT:H7	8	1[c]	stx_{2d2} (7), stx_{2d1} (1), stx_1/stx_2 (1)	1	1	9	7		1		9
ONT:H14	1		stx_{2d2} (1)			1	1				1
ONT:H19	7	1[d]	stx_{2d}/stx_{2d2} (5), stx_2 (1), stx_{2d2} (1), $stx_{2c(vh\text{-}b)}$ (1)	8	8	8	7		8		8
ONT:H21	6		stx_1/stx_2 (3), stx_{2d} (2), stx_{2d2} (1)	5	6	6	5				6
ONT:H25	1		stx_2 (1)	1	1	1	1		1	1	1
ONT:H28	2		stx_{2d} (2)		2	2		1			2
ONT:H46	6	1[d]	stx_{2d} (5), stx_{2d2} (1), stx_{2d}/stx_{2d2} (1)	7	7	7	6		7	1	7
ONT:H49		1[a]	stx_1/stx_{2d} (1)	1	1	1	1		1		1
ONT:HNM	1	3[b,c]	stx_2 (3), stx_1 (1)			4	3	1			3

Table 2 (*Continued*)

Serotype	Source		*stx* genotype (No. of strains)	No. of strains positive for							
	Cattle	Human		*saa*	*ehx*A	*lpfA*$_{O113}$	*iha*	*astA*	*subAB*	*cdt*-V	*fim*A
OR:H2	10		$stx_{2c(vh\text{-}a)}$ (5), stx_{2d1} (1), stx_{2d2} (1), stx_1/stx_2 (1), stx_{2d1}/stx_{2d2} (1), 2UT (1)	1	1	10	9				10
OR:H19	1		stx_1/stx_2 (1)	1	1	1	1				1
Total (% strains)	153 (76.5)	47 (23.5)		100 (50)	107 (53.5)	199 (99.5)	172 (86)	20 (10)	70 (35)	26 (13)	187 (93.5)

[a] Bloody diarrhea.
[b] HUS.
[c] Diarrhea.
[d] Household HUS contact.

2.4. Type I fimbriae agglutination assay

A single colony of each strain was used to inoculate 5 ml Luria–Bertani (LB) broth, cultured in static conditions at 37 °C during 18 h, then subcultured twice in the same medium and conditions. Agglutination was assayed on glass slides by mixing 20 μl bacterial culture with an equal volume of baker's yeast suspension (10 mg ml^{-1}). Mannose inhibition of agglutination was confirmed using 3% α-D-mannose in the yeast suspension.

2.5. Determination of fim switch status

The orientation of the invertible DNA element containing the *fimA* promoter was determined using a PCR-based assay. This method exploited a restriction fragment length dimorphism, which arises out of the orientation-dependent location of a unique SnaBI restriction site within the amplified DNA (Toma et al., 2007).

2.6. Statistical analysis

Statistical analysis of the frequency of virulence markers and virulence profiles between human and cattle strains was performed by the two-tailed Fisher's exact test, using InStat version 3.05. A *P*-value of <0.05 was considered statistically significant.

3. Results

3.1. Phenotypic and genotypic strain characterization

Serotypes, source of isolation, Shiga toxin genotypes and additional virulence markers are shown in Table 2. PFGE was able to subtype the 200 isolates generating 143 different PFGE patterns (data not shown).

As inclusion criterium, all strains were intimin-negative and also fermented D-sorbitol. Thirty-seven different O and H serotypes were identified among the strains, comprising 27 O serogroups and 18 H antigens. Sixty-two strains were O non-typeable and 11 were O-rough, 191 strains were H typeable and 9 were non-motile. It is important to point out that 13 serotypes were common to strains isolated from both origins, however some were species-specific. The most prevalent common serotypes according to the frequency were O113:H21, O174:H21, O130:H11, O8:H19, O15:H27, O91:H21 and O163:H19.

Different *stx* genotypes occurred among cattle and human strains. Comparatively, stx_2, stx_1/stx_2 and stx_1 genes were detected in 64%, 26.8% and 9.2% of the cattle strains and in 83%, 10.6% and 6.4% of the human strains, respectively.

3.2. Virulence markers

The distribution of putative adhesin genes is shown in Table 2. The most prevalent genes identified among all strains were those encoded by *lpfA*$_{O113}$ (199, 99.5%) and *fimA* (187, 93.5%). Of note, the only *lpfA*$_{O113}$-negative strain was isolated from an HUS case. The *fimA* gene promoter was present in 142 (92.8%) cattle strains and in 45 (95.7%) human strains (Fig. 1), meanwhile the *iha* gene was present in 130 (85%) and in 42 (89.4%), respectively. Out of the 187 *fimA*-positive strains, 175 expressed the fimbriae by the agglutination assay. There was also a correlation between the phenotype and the PCR digestion products. The *fimA*-negative strains isolated from both origins belonged to serotypes O136:H12 (7 strains), O7:HNM (2 strains), O74:H12 (1 strain) and 3 were non-typeable. The *iha*-negative ones belonged to serotypes O136:H12 (7 strains), O8:H19 (4 strains), O7:HNM (2 strains), O58:H40, O74:H12, O91:H16, O124:H19, O130:H11, O174:H8 (one strain each) and 9 were non-typeable. Interestingly, all *fimA*-negative strains were also *iha*-negative, except the two strains of serotype ONT:H4 (Table 2).

The *ehxA* gene was carried by 107 (53.5%) of the studied strains, independently of the source. From those *ehxA*-positive strains, 100 were also *saa*-positive. The *ehxA* gene was found in 87 (56.9%) of the cattle strains and in 20 (42.5%) of the human STEC strains. Moreover, the *saa* gene was detected in 81 (52.9%) and 19 (40.4%), respectively (Fig. 1). Interestingly, none *ehxA*-negative strain harbored the *saa* gene. All strains were *efa1* negative and this finding is consistent with previous studies that described *efa1* more frequently in intimin-positive strains (Toma et al., 2004; Cergole-Novella et al., 2007).

The presence of gene sequences related to *subAB*, *cdt*-V and *astA* toxins, occurred in 70 (35%), 26 (13%) and 20 (10%) of the strains, respectively. No statistical difference was observed in the frequency of *subAB*, *cdt*-V and *astA*, between cattle and human strains. The *subAB* gene was present in 55 (36%) of the bovine strains and in 15 (32%) of the human strains; *cdt*-V in 18 (11.8%) and in 8 (17%); and *astA* in 15 (9.8%) and in 5 (10.6%), respectively (Fig. 1). The

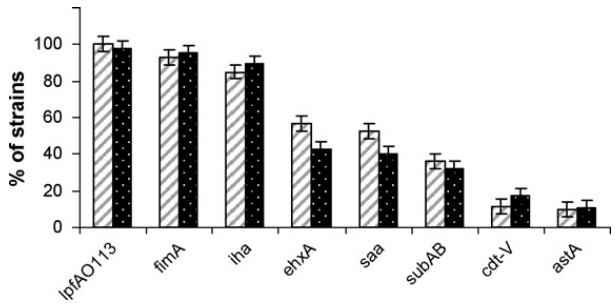

Fig. 1. Distribution of virulence marker genes in STEC strains isolated from cattle and humans. Cattle strains are represented in grey striped bars and human strains in black dot bars.

subAB-positive strains belonged to several serotypes, but the most prevalent were O113:H21 (11 strains), O130:H11 (8 strains), O91:H21 (4 strains), O178:H19 and O116:H21 (5 strains each). The *cdt*-V-positive strains belonged to serotypes O113:H21 (6 strains), O91:H21 and O163:H19 (4 strains each). All *cdt*-V-positive strains were *subAB*-positive. The *astA*-positive strains belonged to serotypes O136:H12 (7 strains), O174:H21 (4 strains), O7:HNM and O113:H21 (2 strains each), O2:H25 and O74:H12 (one strain each), and 3 were non-typeable.

Distinct virulence profiles were found in the LEE-negative STEC strains analyzed, and differences were observed between cattle and human strains (Table 3). Eleven different virulence profiles were identified among the 153 bovine strains, being the combination of $lpfA_{O113}$ *iha fimA* the most prevalent (49, 32%), followed by $lpfA_{O113}$ *iha saa ehxA subAB fimA* (32, 21%) and $lpfA_{O113}$ *iha saa ehxA fimA* (29, 19%). Among the 47 human STEC strains studied, 11 distinct virulence profiles were also detected. The most frequent profile identified was identical to the prevalent detected among bovine strains $lpfA_{O113}$ *iha fimA* (19, 40.4%). The second most frequent profile observed was $lpfA_{O113}$ *iha saa ehxA subAB cdt-V fimA*, occurring in 8 (17.2%) strains.

The association between the virulence profile, the human disease, and serotypes is shown in Table 4. Out of 11 profiles identified in human strains, 9 were associated with HUS. The most prevalent profile, $lpfA_{O113}$ *iha fimA*, was identified in strains of different serotypes isolated from 9 HUS, 8 diarrhea cases and 2 asymptomatic contacts. Of those HUS cases, 2 patients died during the acute phase of the disease and the strains belonged to O143:HNM and ONT:HNM serotypes. Moreover, 2 other HUS patients died and the isolated strains harbored the $lpfA_{O113}$ *iha saa ehxA subAB cdt-V fimA* genes and belonged to O113:H21 and O174:H28 serotypes.

4. Discussion

Understanding the factors that govern the colonization of the animal hosts, and the development of severe disease in human beings would provide the insights for more effective intervention on both these aspects. Moreover, defining the combination of virulence genes and the mechanisms that make a STEC strain fully pathogenic will be pivotal to improve the efficacy of both the diagnostics of human infections and the surveillance of animal reservoirs and the assessment of public health risks.

The epidemiology of STEC infections has remarkably changed during the past 10 years. Cases of human disease caused by non-O157 STEC increased globally by 60.5% between 2000 and 2005, while at the same time, cases caused by STEC O157 increased by only 13% (Coombes et al., 2008). In view of the increasing number of reports of LEE-negative STEC infections, there is now a need for comprehensive data on the molecular basis for their virulence; hence prevalence of adhesins and other virulence markers in these type of strains should be studied. In this work, we investigated the prevalence of 5 putative adhesins and 3 toxin genes among LEE-negative STEC strains isolated from cattle and human infections from a wide range of serotypes.

In this study, the prevalence of $lpfA_{O113}$ and *iha* occurred in 99.5% and 86% of the STEC strains, respectively. Although similar results were described by Cergole-Novella et al. (2007), who found $lpfA_{O113}$ (89.2%) and *iha* (87.6%) as the most prevalent adhesins; Toma et al. (2004) found *iha* as the most prevalent one (91%), followed by $lpfA_{O113}$ (73%).

Table 3
Comparison of virulence profiles identified in cattle and human LEE-negative STEC strains.

Virulence profile carried by	No. of strains (%)		*P*-value
	Cattle	Human	
$lpfA_{O113}$ *iha fimA*	49 (32)	19 (40.4)	NS
$lpfA_{O113}$ *iha saa ehxA subA fimA*	32 (21)	6 (12.8)	NS
$lpfA_{O113}$ *iha saa ehxA fimA*	29 (19)	3 (6.4)	<0.05
$lpfA_{O113}$ *iha saa ehxA subA cdt*-V *fimA*	17 (11)	8 (17.2)	NS
$lpfA_{O113}$ *astA*	11 (7.2)	0 (0)	<0.05
$lpfA_{O113}$ *ehxA fimA*	5 (3.5)	1 (2.1)	NS
$lpfA_{O113}$ *fimA*	4 (2.6)	2 (4.3)	NS
$lpfA_{O113}$ *iha astA fimA*	2 (1.3)	4 (8.5)	<0.05
$lpfA_{O113}$ *saa ehxA fimA*	2 (1.3)	1 (2.1)	NS
$lpfA_{O113}$ *iha saa ehxA subA cdt-V astA fimA*	1 (0.6)	0 (0)	NS
$lpfA_{O113}$ *ehxA astA fimA*	1 (0.6)	0 (0)	NS
$lpfA_{O113}$ *iha astA*	0 (0)	1 (2.1)	NS
$lpfA_{O113}$ *saa ehxA subA fimA*	0 (0)	1 (2.1)	NS
iha	0 (0)	1 (2.1)	NS
Total	153	47	

NS, not significant ($p > 0.05$).

Table 4
Association between virulence profile, human disease and STEC serotype.

Profile	Human disease[a] (No. of cases)	Serotype (No. of strains)
lpfA$_{O113}$ *fimA iha*	HUS (9)	O15:H27 (1), O20:H19 (1), O59:H19 (4), O143:NM (1)[b], ONT:NM (2)[b]
	D (8)	O15:H27 (1), O171:H2 (2), O174:H21 (3), ONT:H7 (1), ONT:NM (1)
	AC (2)	O174:H21 (2)
lpfA$_{O113}$ *fimA iha saa ehxA*	HUS (1)	O8:H16 (1)
	D (1)	O174:NM (1)
	AC (1)	O5:NM (1)
lpfA$_{O113}$ *fimA iha saa ehxA subA*	HUS (1)	O174:H28 (1)
	D (2)	O113:H21 (1), O130:H11 (1)
	BD (1)	ONT:H49 (1)
	AC (2)	O130:H11 (1), ONT:H46 (1)
lpfA$_{O113}$ *fimA iha saa ehxA subA cdt-V*	HUS (3)	O113:H21 (2)[b], O174:H28 (1)[b]
	D (4)	O91:H21 (2), O8:H19 (1), O163:H19 (1)
	BD (1)	O91:H21 (1)
lpfA$_{O113}$ *fimA iha astA*	HUS (1), D (1), AC (2)	O174:H21 (4)
lpfA$_{O113}$ *iha astA*	HUS (1)	ONT:H4 (1)
lpfA$_{O113}$ *fimA*	D (1), AC (1)	O58:H40 (1), O174:H8 (1)
lpfA$_{O113}$ *fimA ehxA*	HUS (1)	O8:H19 (1)
lpfA$_{O113}$ *fimA ehxA saa*	HUS (1)	O8:H19 (1)
lpfA$_{O113}$ *fimA ehxA saa subA*	AC (1)	ONT:H19 (1)
iha	HUS (1)	ONT:H4 (1)

[a] HUS, hemolytic uremic syndrome. D, diahrrea. BD, bloody diarrhea. AC, asymptomatic household contact.
[b] Four HUS patients died during the acute.

Further, a recent study described the presence of different *lpfA* variants in STEC and EPEC strains from different sources, suggesting that specific Long Polar Fimbriae might be associated with virulent strains (Torres et al., 2009). Therefore, differences in frequency of the adhesins identified could have a profound impact in the subset of strains characterized in each study.

None of the LEE-negative STEC strains studied carried the *efa1* gene, establishing a close correlation between *efa1* and intimin. This is the first study in which the *fimA* gene was widely studied, occurring in 93.5% of the LEE-negative STEC strains, suggesting that the presence of these fimbriae could contribute in the first steps of adhesion to epithelial cells.

The production of other toxins besides the Stx by STEC isolates, including CDT-V and SubAB has also been described, and their role in the pathogenesis of STEC infections has been proposed (Bielaszewsaka et al., 2005; Talbot et al., 2005). The presence of *cdt*-V occurred in 12% and 17% of the cattle and human strains, and *subAB* in 36% and 32%, respectively. The frequency of *cdt*-V detection was higher in human than in cattle strains. Although Cergole-Novella et al. (2007) observed these new gene toxins only in intimin-negative strains isolated from cattle, it is important to mention that in the present study, *cdt*-V and *subAB* were found in STEC strains isolated from severe human disease. Thus, the apparent clinical relevance should not be ignored, especially as it was identified in STEC serotypes such as O113:H21, O91:H21, O130:H11 and O163:H19 (http://www.microbionet.com.au/vtectable), which have been related to HUS cases around the world. In addition to previous studies, new serogroups carrying the *subAB* gene were identified such as O7, O39, O74, O116 and O130. Moreover, the simultaneous presence of *cdt-V* and *subAB* were identified in a vast group of serotypes such as O7:H21, O8:H19, O91:H21, O116:H21, O163:H19, O174:H28, O178:H19, ONT:H25 and ONT:H46; some of which cause human illness. The *astA* gene sequence occurred in 9.8% and 10.6% of the cattle and human strains. It is important to point out that there was a correlation with specific genotype among *astA*-positive human strains being all of them *stx*$_{2d2}$, except one that was *stx*$_2$. However, the cattle strains were mainly *stx*$_1$.

The most prevalent profile observed among the strains of both origins studied coincidentally was *lpfA*$_{O113}$ *iha fimA*, which was present in a great number of serotypes. Although HUS is multifactorial in etiology, involving complex interactions between bacterial and host factors, out of 19 HUS cases studied in 15, the isolated strains carried the above mentioned genes. A great diversity of serotypes and profiles was observed among LEE-negative STEC strains from bovine and human origin, however 8 of the 11 profiles coincided in both groups. This evidence, reinforce the idea of cattle as the main natural reservoir and the principal source of infection.

In conclusion, this study showed that bovine LEE-negative STEC strains possess genes encoding for putative adhesins and toxins present in human LEE-negative STEC strains, some of which cause severe diseases. Despite the diversity of virulence profiles observed among the strains studied, characteristic associations between virulence profiles, serotypes, and their source of isolation could be identified. We also confirmed that LEE-negative STEC strains are not a clonal group of pathogens, as we could observe differences in the virulence profiles, including strains from the same serotype. Although some determinants may not be considered essential virulence factors for human infection, they could facilitate survival and persistence in different environments.

Despite a great effort in elucidating the functions of the virulence markers identified in the present study in the last decade have been done, further studies comparing wild

type strains and their allelic mutants are needed to determine the exact role of each factor in the pathogenesis of LEE-negative STEC-mediated diseases. It is vitally important for public health and clinical laboratories to recognize LEE-negative STEC strains as a cause of human infection and include them in the surveillance system.

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