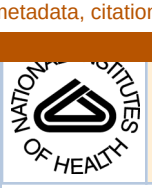

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Identification of the Long Polar Fimbriae gene variants in Locus of Enterocyte Effacement-negative Shiga toxin-producing *Escherichia coli* strains isolated from humans and cattle in Argentina

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Abstract

The Long Polar Fimbriae (Lpf) is one of few adhesive factors of Shiga toxin-producing *Escherichia coli* (STEC) and it is associated with colonization of the intestine. Studies have demonstrated the presence of *lpf* genes in several pathogenic *E. coli* strains and classification of variants based on polymorphisms in the *lpfA1* and *lpfA2* genes has been adopted. Using a collection of Argentinean Locus of Enterocyte Effacement (LEE)-negative STEC strains, we determined that the different *lpfA* types were present in a wide variety of serotypes and no apparent association was observed between the types of *lpfA1* or *lpfA2* genes and the severity of human disease. The *lpfA2-1* was the most prevalent variant identified, which was present in 95.8% of the isolates, and the *lpfA1-3* and *lpfA2-2*, proposed as specific biomarkers of *E. coli* O157:H7, were not found in any of the serotypes studied. The prevalence of *lpf* genes in a large number of strains is useful to understand the genetic diversity of LEE-negative STEC and to define the association of some of these isolates carrying specific *lpf*-variants with disease.

Keywords

LEE-negative; Shiga toxin-producing *Escherichia coli*; *lpf* variant; virulence profile; human strain; cattle strain

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) strains are foodborne enteric pathogens associated with different clinical manifestations such as non-bloody diarrhea, hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (reviewed in Nataro & Kaper, 1998). Although *E. coli* O157:H7 is the most prevalent serotype associated with sporadic cases and large outbreaks of HC and HUS, there is growing concern over the emergence of highly virulent STEC non-O157 serotypes that become globally distributed and associated with outbreaks and/or severe human illness (Coombes *et al.*, 2008). Ruminants, particularly

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cattle, are recognized as the main natural reservoir for non-O157 STEC and cattle-derived food products have been implicated in many outbreaks (Caprioli *et al.*, 2005).

Shiga toxins (Stx) are considered to be the major virulence markers of STEC, but adherence to the intestinal epithelium and colonization of the gut is an essential component of pathogenic process (reviewed in Torres *et al.*, 2005). Some STEC serotypes harbor a large pathogenicity island, termed the Locus of Enterocyte Effacement (LEE), required for the formation of Attaching and Effacing (A/E) lesions (McDaniel & Kaper, 1997). The LEE carries the *eae* gene encoding the adhesin intimin, responsible for the intimate adherence of the bacteria to the enterocyte and linked to cytoskeleton rearrangements. However, the presence of the LEE does not seem to be essential for full virulence, as a wide number of LEE-negative STEC strains have been associated with sporadic cases and small outbreaks of HC and HUS (reviewed in Bettelheim, 2007). Of these LEE-negative organisms, O113:H21 is one of the most commonly isolated STEC serotypes in many regions. Clinical isolates of LEE-negative STEC typically express Stx2 and also harbor a ca. 90-kb plasmid encoding several virulence factors, including EHEC hemolysin (Newton *et al.*, 2009). Some studies have elucidated different mechanisms by which these strains interact with the host intestinal mucosa and induce disease; i.e. it has been demonstrated that STEC O113:H21 can invade tissue cultured cells (Luck *et al.*, 2006). Besides the knowledge that some STEC strains do not carry the LEE, very little is known about other strategies used by these pathogens to adhere and to colonize the host intestine.

Analysis of the genome sequence of *E. coli* O157:H7 showed that several O157-specific islands contain putative fimbrial biosynthesis operons, including two regions encoding the Long Polar Fimbriae (Lpf). The Lpf loci are related at the genetic and protein level to Lpf of *Salmonella enterica* serovar Typhimurium and the latter have been shown to facilitate attachment of the bacteria to intestinal Peyer´s patches (Bäumler *et al.*, 1996). In *E. coli* O157:H7, expression of the *lpf* operon 1 (*lpf1*) in an *E. coli* K-12 strain was linked to increased adherence to tissue-cultured cells and has been associated with the appearance of long fimbriae (Torres *et al.*, 2002, 2004). The *lpf2* operon has also been associated to adherence to epithelial cells and its expression in some pathogenic *E. coli* strains is believed important for the development of severe diarrhea (Doughty *et al.*, 2002; Osek *et al.*, 2003). *E. coli* O157:H7 strains harboring mutations in one or both of the *lpf* loci (named *lpf1* and *lpf2* operons) have diminished intestinal colonization abilities and persistence in several animal models of infection (Jordan *et al.*, 2004; Torres *et al.*, 2007; Newton *et al.* 2004). Further, these *lpf* mutant strains also displayed an altered human intestinal tissue tropism (Fitzhenry *et al.*, 2006).

Cumulative evidence indicates that homologues to the *lpf* genes are found in other pathogenic *E. coli*, *Salmonella*, *Shigella* and even in some commensal *E. coli* isolates (Doughty *et al.*, 2002; Szalo *et al.*, 2002; Toma *et al.*, 2004, 2006; Cergole-Novella *et al.*, 2006; Galli *et al.*, 2009). A recent study identified several polymorphisms within *lpfA* (encoding the major fimbrial Lpf subunit) genes, and this finding was used to classify the *lpfA* genes in distinct variants. The *lpfA1* gene was classified in 5 different types (named as alleles *1, 2, 3, 4* and *5*) and the *lpfA2* gene in 3 distinct types (alleles *1, 2* and *3*) (Torres *et al.*, 2009). In the current study, we investigated the presence of these *lpf* variants in a collection of 120 LEE-negative STEC strains, 70 isolated from human infections and 50 from cattle; and explore the relationship between the presence of determined combination of *lpf* variants with other virulence determinants and severity of disease.

Materials and methods

Bacterial strains

A total of 120 randomly selected LEE-negative STEC strains belonging to different non-O157 serotypes were included in this study. Seventy human strains isolated during surveillance of HUS and diarrheal diseases, in a period from 2001 through 2009, and submitted to the Argentinean National Reference Laboratory, were included. The human strains were isolated from diarrheal cases (n=26), HUS (n=28) and asymptomatic household contacts (n=16). For comparison purposes, 50 strains isolated from fecal samples and carcasses from healthy Argentinean beef cattle, obtained during surveys and research programs carried out from 2005 to 2007 were also included. All the strains were previously serotypified and the presence of virulence genes (*stx*, *eae*, *ehxA*, *saa*, *iha*, *fimA*, *efa1*, *astA*, *subAB*, *cdt-V*) also determined (Galli *et al.*, 2009).

Detection of *lpfA* variants

The primers and conditions employed in the PCR assays for identification of *lpfA* gene variants were identical to those reported by Torres *et al.* (2009). The DNA template was prepared boiling isolated single colonies of the strains in 150 μl of 1% Triton X-100 in TE buffer by 15 min. All amplifications began with a five-minute hot start at 94°C followed by 35 cycles of denaturing at 94°C for 30s, annealing for 30s in a range of temperatures ranging from 52°C–72°C (depending of the *lpfA* variant amplified), and extension at 72°C for 30s. *E. coli* strain EDL933 was used as positive control for *lpfA1-3* and *lpfA2-2*; *E. coli* EH41 (O113:H21) for *lpfA2*-1 (kindly provided by Elizabeth Hartland); enteropathogenic *E. coli* (EPEC) 2348/69 (O127:H6) for *lpfA1-1*, and EPEC H30 (O26:H11) for *lpfA1-2*.

Statistical analysis

ANOVA and Pearson`s Chi square test were used to test associations between clinical courses (diarrhea, HUS and asymptomatic carriers) and the presence of the *lpfA* variant genes.

Results

Presence of *lpf1* and *lpf2* gene variants in LEE-negative STEC strains

Using the experimental classification of *lpfA* gene variants described by Torres *et al.* (2009), we found that the *lpfA2-1* was the most commonly found variant in our isolates. As shown in Table 1, 95.8% of the strains carried the *lpfA2-1* variant, while the *lpfA2-3* variant was present in only one strain and 3.3% of the strains were negative for both *lpfA1* and *lpfA2* genes. The frequency of *lpfA1-2* was 56.6% and it seems that this gene is frequently linked to *lpfA2-1*. We did not find *lpfA1* variants *1, 3, 4* and *5*, or *lpfA2* variant *2* in any of the strains studied.

The four *lpfA*-negative STEC strains identified in our study were of human origin (serotypes O8:H16, O117:H7, ONT:H4 and ONT:HNM). Two of them, serotypes O8:H16 and ONT:H4, were isolated from HUS cases and the only putative virulence factor currently identified in these strains is encoded by the *iha* gene. The ONT:HNM strain was isolated from a patient suffering from diarrhea and the only virulence factor found in this serotype is encoded by the stx_1 toxin gene. Finally, the O117:H7 strain was isolated from an asymptomatic carrier with prolonged shedding and had the particularity to be non-sorbitol fermenting and carrying the putative virulence factors *iha* and *astA*.

In the current study, we could not find an statistical association between the presence of a particular *lpfA* variant and the severity of the disease. However, we observed that most

serotypes maintained the same combination of *lpf* variants independent from the source of isolation. Therefore, we observed a good association between the *lpfA* variant and the serotype of the strain, i.e. we identified two strains from serotype O22:H8 that carried *lpfA1-2* and *lpfA2-1*, and two strains from serotype O22:H16 which carried only *lpfA2-1*. Interestingly, we found that these strains belonging to the same serogroup and which were isolated from cattle, shared the same virulence profiles (Table 1). One more isolate from serotype O22:HNT and which was isolated from a human diarrheal case, carried the *lpfA1-2* and *lpfA2-1* genes. Similar results occurred in the O174 serogroup, where all the O174:H21 isolates carried the *lpfA1-2* and *lpfA2-1* gene variants, while the other O174 serotypes (O174:H8, O174:H28, O174:NM) carried the *lpfA2-1* gene and not a common theme with respect to the virulence profile or the source of isolation was observed.

The most variable serogroup with respect to *lpfA* gene variant content was O8 from which we identified three O8:H16 and four O8:H19 isolates. In the case of the O8:H16 isolates, two were *lpfA1-2* and *lpfA2-1*-positive and carried the same virulence profile, while a third isolate was *lpfA*-negative and *iha* was the only putative virulence marker. Another difference in these strains, apart from the source of isolation, was the *stx* genotype, while the *lpfA*-negative strain was stx_2-positive, the others were stx_1/stx_2-positive. In the case of the O8:H19 isolates, two carried the *lpfA1-2* and *lpfA2-1* genes, and two strains carried only the *lpfA2-1* gene. Further, all the strains of this sertotype carried different virulence gene profiles.

Another serotype identified in our study was O178:H19, which included two strains sharing the same origin, and carrying the same *stx* gene and a common virulence profile, but differing in the type of *lpfA* variant present. While one strain was *lpfA1-2*-positive, the other was *lpfA1-2* and *lpfA2-1*-positive.

The virulence profiles found in the LEE-negative STEC strains are presented in Table 2, and differences were observed between human and cattle isolates. Sixteen different virulence profiles were identified among the 70 human strains, being the combination of *iha fimA* genes the most prevalent (19 of 70 strains, 27.1%). Within this group, the presence of *lpfA1-2* and *lpfA2-1*(12 of 19 strains, 63%), only *lpfA2-1* (6 of 19 strains, 32%) and only *lpfA2-3* (1 of 19 strains, 5%) was detected. Among the 50 bovine strains, 9 different virulence profiles were observed, being the combination of *iha fimA saa ehxA subAB* the most prevalent (14 of 50 strains, 28%). From these, 8 of 14 strains (57%) carried the *lpfA1-2* and *lpfA2-1* variants, while 6 of the 14 strains (43%) contained the *lpfA2-1* variant.

Discussion

The virulence factors of LEE-negative STEC strains can not only be limited to the production of Stx toxin variants, but also to the presence of adhesins that mediate binding to the intestinal epithelium and eventually, contribute to the colonization of the gut. Some studies have suggested that LEE-negative STEC are invasive and that a particular flagellin type may contribute to cell invasion and gut colonization (Luck *et al.*, 2005, 2006). Besides those observations, still little is known about other adhesins associated with colonization of the intestine and other mechanisms of pathogenesis. Recently, Torres *et al.* (2009) identified several polymorphisms within the *lpfA* genes, which were used to classify the major fimbrial subunit genes in distinct variants. The expression of Lpf in LEE-negative STEC strains is believed to be important for development of severe diarrhea and hence its identification is potentially clinically relevant (Dougthy *et al.*, 2002; Osek *et al.*, 2003).

In an attempt to characterize some fimbrial adhesins in these pathogens, we investigated the distribution of *lpfA* gene variants in a wide range of LEE-negative STEC strains isolated in

Argentina from human infections and healthy cattle. We found that the *lpfA1* and *lpfA2* genes are present in 56.6% and 96.6% of the STEC strains studied, respectively, and only 3.3% of the human strains were *lpfA*-negative. These data confirmed that the presence of *lpf* genes in LEE-negative STEC strains seems to be a common characteristic, particularly the presence of the *lpfA2-1* variant. It is plausible to speculate that the four *lpfA*-negative strains identified in this study either contain novel and unidentified adherence factors required for colonization or perhaps the strains possess another *lpf* operon that we could not identified with our detection approach.

The majority of the strains possessed the *lpfA2-1* allele (95.8%). Indeed, 39.1% of the strains were only *lpfA2-1*-positive and 56.6% were positive for both *lpfA1-2* and *lpfA2-1* genes. It is interesting to mention that the most common variant in bovine isolates was that encoded by the *lpfA2-1* gene, while the combination *lpfA1-2* and *lpfA2-1* was the common genotype in human isolates. This finding suggests that, perhaps the presence of the *lpfA2-1* variant might be linked to those isolates that are cattle colonizers, while the strains containing the *lpfA1-2* and *lpfA2-1* genes had an advantage to colonize the human intestine and eventually to cause disease. It is evident that additional experiments are required to confirm Lpf expression in these strains and to establish the association of those strains expressing specific variants of Lpf with human disease. Interestingly, some of our prior studies evaluating adherence of the strains to HEp-2 cell showed different adhesive profiles between those strains possessing different *lpfA* variants, i.e. a *lpfA2-3* positive strain adhere to the HEp-2 cell surface in a localized adherence like pattern, while three cattle *lpfA2-1*-positive strains adhere but also invade these tissue cultured cells (Galli *et al.*, 2009).

Although a great diversity of serotypes and virulence profiles were observed among human and bovine LEE-negative STEC strains, seven of the eighteen profiles were common in both groups. This observation reinforces the idea that cattle are the main natural reservoir of LEE-negative STEC strains, and potentially, the principal source of infection to humans. We also confirmed that LEE-negative STEC strains are not a clonal group of pathogens, as we observed differences in their virulence profiles, including strains from the same serotype. Some of these determinants are not considered essential factors for human infection, although their presence could facilitate survival and persistence of the strains in different environments.

In agreement with previous data described by Torres *et al.* (2009), none of the strains analyzed in this study carried the *lpfA1-3* or the *lpfA2-2* gene variants, either alone or in combination. Therefore, our study strongly supports their observation that those two gene variants are specific for the O157:H7 lineage and are not present in any other STEC isolates, regardless of the source or their association with disease. Interestingly, the only virulence factor that has been associated with the presence of specific *lpf* genes is the adhesin intimin (Torres *et al.*, 2009). That study indicated that different intimin alleles are associated to specific *lpfA* gene variants, and the presence of both *lpfA1* and *lpfA2* alleles is also linked to specific pathogenic *E. coli* strains, particularly with those belonging to the STEC pathotype group (Torres *et al.*, 2009). However, that study did not include those strains that are LEE-negative (intimin-negative), which adds a significant value to our current study, because in addition to confirm some of their findings, we now provided cumulative evidence regarding the distribution of *lpfA* gene variants in other STEC strains that are significant human pathogens. Finally, the presence of the *lpfA* genes in a large number of strains supports the idea that Lpf might play an important but not fully characterized role in adherence and/or colonization capabilities of LEE-negative STEC strains.

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Table 1

Characteristics of STEC strains regarding *lpfA* variants, origin, associated disease, *stx* genotype and serotype.

Antigen		No. of strains	Origin/associated disease (No. of strains)	*stx* genotype	*lpfA1*	*lpfA2*
O	H					
2	6	1	A	2	*2*	*1*
2	25	1	C	2		*1*
5	NM	1	A	1		*1*
7	NM	2	C	1		*1*
7	21	1	C	2		*1*
7	21	1	C	1+2		*1*
8	16	1	HUS	1+2	*2*	*1*
8	16	1	HUS	2	-	-
8	16	1	C	1+2	*2*	*1*
8	19	1	HUS	2	*2*	*1*
8	19	2	HUS	2		*1*
8	19	1	D	2	*2*	*1*
15	27	2	HUS(1), C(1)	2		*1*
15	27	1	D	1+2		*1*
20	19	1	HUS	2		*1*
22	8	2	C	2	*2*	*1*
22	16	2	C	2		*1*
22	NT	1	D	2	*2*	*1*
39	49	1	C	2		*1*
46	38	2	C	1+2		*1*
58	40	1	D	1		*1*
59	19	4	HUS	2	*2*	*1*
74	12	1	C	1		*1*
74	28	1	C	2	*2*	*1*
74	42	1	C	1+2	*2*	*1*
79	19	2	C	1+2		*1*
82	8	1	C	1+2	*2*	*1*
91	16	1	C	2	*2*	*1*

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Antigen		No. of strains	Origin/associated disease (No. of strains)	*stx* genotype	*lpfA1*	*lpfA2*
O	**H**					
91	21	5	D(3), HUS(2)	2	*2*	*1*
91	21	1	C	1+2	*2*	*1*
91	NT	1	A	1	*2*	*1*
91	NM	1	HUS	1+2	*2*	*1*
113	21	4	HUS(2)*, D(1), C(1)	2		*1*
113	NM	1	D	2	*2*	*1*
116	21	2	C	1+2		*1*
116	21	1	C	2		*1*
117	7	1	A	1	-	-
130	11	2	D(1), C(1)	1+2	*2*	*1*
130	11	1	C	1	*2*	*1*
136	12	2	C	1		*1*
141	49	2	C	2	*2*	*1*
143	NM	1	HUS*	2	-	*3*
163	19	2	D(1), C(1)	2	*2*	*1*
171	2	2	D	2		*1*
174	8	1	A	2		*1*
174	21	11	HUS(1), D(5), A(4), C(1)	2	*2*	*1*
174	28	2	D(1), HUS(1)*	2	*1*	
174	28	1	C	1+2		*1*
174	NM	1	D	1+2		*1*
174	NM	1	HUS	2	*2*	*1*
178	19	1	C	2		*1*
178	19	1	C	2	*2*	*1*
178	19	2	HUS(1), C(1)	1+2	*2*	*1*
179	8	2	C	2	*2*	*1*
NT	2	2	C	2		*1*
NT	4	1	HUS	2	-	-
NT	4	1	HUS	2	*2*	*1*
NT	7	2	D(1), C(1)	2	*2*	*1*

Antigen		No. of strains	Origin/associated disease (No. of strains)	*stx* genotype	*lpfA1*	*lpfA2*
O	**H**					
OR	11	1	A	2	-	*1*
NT	19	2	A(1), C(1)	2		*1*
NT	19	1	HUS	2	*2*	*1*
NT	21	1	C	2		*1*
NT	21	1	C	2	*2*	*1*
NT	28	2	C	2	*2*	*1*
NT	46	2	A(1), C(1)	2	*2*	*1*
NT	49	1	D	1+2		*1*
NT	NM	5	D(2), HUS(3)	2	*2*	*1*
NT	NM	1	D	1	-	-
NT	NM	1	A	1+2	-	*1*
NT	NM	1	C	1		*1*
OR	NM	1	A	2	*2*	*1*
NT	NT	1	A	1	*2*	*1*
NT	NT	2	D(1), HUS(1)	2	*2*	*1*
NT	NT	1	HUS	2	*2*	*1*
NT	NT	1	A	1	*2*	*1*
Total		**120**				

*HUS patient died; C, cattle; A, asymptomatic patient; D, diarrheal patient; NM, non motile strains; NT, non-typeable.

Table 2

Comparison of virulence profiles and *lpfA* variants identified in human and cattle LEE-negative STEC strains

Virulence profile	No. of strains						
	Human				Cattle		Total
	lpfA1-2 lpfA2-1	*lpfA2-1*	*lpfA2-3*	*lpfA-* negative	*lpfA1-2 lpfA2-1*	*lpfA2-1*	
iha fimA	20	6	1		3	6	36
iha fimA saa ehxA subAB	3	4			8	6	21
iha fimA saa ehxA subAB cdt-V	6	3			2	5	16
iha fimA saa ehxA	2	2			6	2	12
iha fimA astA	5				1		6
iha astA	2			1	1		4
fimA ehxA		1			2	1	4
fimA		2					2
fimA astA	1						1
fimA saa ehxA		1					1
fimA saa ehxA subAB		1					1
iha fimA saa	1						1
iha fimA ehxA	1						1
iha fimA saa ehxA cdt-V	3						3
iha saa ehxA	1						1
Iha				2			2
astA						6	6
fimA ehxA astA					1		1
All negative				1			1
Total	45	20	1	4	24	26	120