

Identification of the long polar fimbriae gene variants in the locus of enterocyte effacement-negative Shiga toxin-producing *Escherichia coli* strains isolated from humans and cattle in Argentina

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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 nonbloody 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 about 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 cattle, are recognized as the main natural reservoir for 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

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 with no apparent association 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 *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.

component of the 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 c. 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: for example 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 to and 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 with adherence to epithelial cells and its expression in some pathogenic *E. coli* strains is believed to be important for the development of severe diarrhea (Doughty *et al.*, 2002; Osek *et al.*, 2003). *Escherichia 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; Newton *et al.*, 2004; Torres *et al.*, 2007). Further, these *lpf* mutant strains also displayed an altered human intestinal tissue tropism (Fitzhenry *et al.*, 2006).

Cumulative evidence indicates that homologues of 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, 2008; Cergole-Novella *et al.*, 2007; Galli *et al.*, 2009, 2010). A recent study identified several polymorphisms within *lpfA* (encoding the major fimbrial Lpf subunit) genes, and this result led to the classification of the *lpfA* genes into distinct variants. The *lpfA1* gene was classified as five different types (named as alleles 1–5) and the *lpfA2* gene as three (alleles 1–3) (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. We 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, 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 in 2005–2007, were also included. All the strains were serotyped previously and the presence of virulence genes (*stx*, *eae*, *ehxA*, *saa*, *iha*, *fimA*, *efa1*, *astA*, *subAB*, *cdt-V*) was also determined (Galli *et al.*, 2009, 2010).

Detection of *lpfA* variants

The primers and conditions used in the PCR assays for the identification of *lpfA* gene variants were identical to those reported by Torres *et al.* (2009). The DNA template was prepared by boiling isolated single colonies of the strains in 150 μ L of 1% Triton X-100 in TE buffer for 15 min. All amplifications began with a 5-min hot start at 94 °C, followed by 35 cycles of denaturing at 94 °C for 30 s, annealing for 30 s in a range of temperatures ranging from 52 to 72 °C (depending of the *lpfA* variant amplified) and extension at 72 °C for 30 s. *Escherichia coli* strain EDL933 was used as a 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 χ^2 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 *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, whereas 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, 3–5, or *lpfA2* variant 2 in any of the strains studied.

Table 1. Characteristics of STEC strains in terms of *lpfA* variants, origin, associated disease, *stx* genotype and serotype

Antigen		No. of strains	Origin/associated disease (no. of strains)	<i>stx</i> genotype	<i>lpfA1</i>	<i>lpfA2</i>
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
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	–	–

Table 1. Continued.

Antigen		No. of strains	Origin/associated disease (no. of strains)	<i>stx</i> genotype	<i>lpfA1</i>	<i>lpfA2</i>
O	H					
NT	4	1	HUS	2	2	1
NT	7	2	D(1), C(1)	2	2	1
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, nonmotile strains; NT, nontypeable.

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, unusually, it was nonsorbitol fermenting and carried the putative virulence factors *iha* and *astA*.

In the current study, we could not find a 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 of 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 that carried only *lpfA2-1*. Interestingly, we found that these strains belonging to the same serogroup and, isolated from cattle, shared the same virulence profiles (Table 1). One more isolate from serotype O22:HNT, 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, whereas the other O174 serotypes (O174:H8, O174:H28, O174:NM) carried the *lpfA2-1* gene and no common theme with respect to the virulence profile or the source of isolation was observed.

The most variable serogroup with respect to the *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, whereas 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; whereas 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 serotype 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. One strain was *lpfA1-2* positive, whereas the other was both *lpfA1-2* and *lpfA2-1*-positive.

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

Discussion

The virulence factors of LEE-negative STEC strains are limited not only 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, 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 the development of severe diarrhea and hence its identification is potentially clinically relevant (Doughty *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 the strains possess another *lpf* operon that we could not identify using our detection methods.

The majority of the strains possessed the *lpfA2-1* allele (95.8%). Indeed, 39.1% of the strains were only *lpfA2-1* positive, whereas 56.6% were positive for both *lpfA1-2* and *lpfA2-1* genes. It is interesting to note that the most common variant in bovine isolates was that encoded by the *lpfA2-1* gene, whereas the combination *lpfA1-2* and *lpfA2-1* was the common genotype in human isolates. This finding suggests

Table 2. Comparison of virulence profiles and *lpfA* variants identified in human and cattle LEE-negative STEC strains

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

that the presence of the *lpfA2-1* variant may be linked to those isolates that are cattle colonizers, whereas the strains containing the *lpfA1-2* and *lpfA2-1* genes have the ability to colonize the human intestine and eventually 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. an *lpfA2-3*-positive strain adheres to the HEp-2 cell surface in a localized adherence-like pattern, whereas three cattle *lpfA2-1*-positive strains adhere, but also invade these tissue-cultured cells (Galli et al., 2010).

Although a huge diversity of serotypes and virulence profiles was observed among human and bovine LEE-negative STEC strains, seven of the 18 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 in 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 these 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 with specific *lpfA* gene variants, and the presence of both *lpfA1* and *lpfA2* alleles is also linked to specific pathogenic *E. coli* strains, particularly those belonging to the STEC pathotype group (Torres et al., 2009). However, that study did not include the strains that are LEE negative (intimin-negative), a significant difference from our current study, because, in addition to confirming some of their findings, we now provide 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 may play an important, but not fully characterized role in adherence and/or colonization capabilities of LEE-negative STEC strains.

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