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Shiga toxin-producing *Escherichia coli*
Factors involved in virulence and cattle colonization

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Abbreviations: STEC, Shiga toxin-producing *Escherichia coli*; EHEC, enterohemorrhagic *Escherichia coli*; EPEC, enteropathogenic *Escherichia coli*; HC, hemorrhagic colitis; HUS, hemolytic uremic syndrome; Stx1, Shiga toxin type 1; Stx2, Shiga toxin type 2; LEE, locus of enterocyte effacement; ehxA, enterohemolysin; Saa, autoagglutinating adhesin; katP, catalase-peroxidase; esp, extracellular serine protease; stcE, zinc metalloprotease; subAB, subtilase cytotoxin; RAJ, recto anal junction; ECP, *E. coli* common pili; Eha, enterohemorrhagic *E. coli* autotransporter; Iha, iron-regulated gene A homolog adhesion; efa-1, enterohemorrhagic *E. coli* factor for adherence 1; LifA, lymphostatin; Iha, iron-regulated gene A homolog adhesion; *lpf*, long polar fimbria

Shiga toxin-producing *Escherichia coli* (STEC) cause hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) in humans. Outbreaks are linked to bovine food sources. STEC O157:H7 has been responsible for the most severe outbreaks worldwide. However, non-O157 serotypes have emerged as important enteric pathogens in several countries. The main virulence factor of STEC is the production of Shiga toxins 1 and 2. Additional virulence markers are a plasmid-encoded enterohemolysin (ehxA), an autoagglutinating adhesin (Saa), a catalase-peroxidase (katP), an extracellular serine protease (espP), a zinc metalloprotease (stcE), a subtilase cytotoxin (subAB), among others. Other virulence factors are intimin and adhesins that had a roll in the adherence of STEC to bovine colon. This review focuses on the virulence traits of STEC and especially on those related to the adhesion to bovine colon. The known of the interaction between STEC and the bovine host is crucial to develop strategies to control cattle colonization.

STEC: Clinical Aspects, Transmission and Epidemiology

The term Shiga toxin-producing *E. coli* (STEC) refers to *E. coli* pathotypes capable of producing Shiga toxin type 1 (Stx1), type 2 (Stx2), or both, encoded by *stx1* and stx2 genes, respectively.[1] STEC, also known as "verocytotoxin-producing *E. coli*", are zoonotic pathogens that cause the vascular endothelial damage observed in patients with hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS).[2] HUS is characterized by acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia and is a potentially fatal cause of acute renal failure in children.[3] In 1955, Gasser et al.[4] first described hemolytic uremic syndrome as a self-limited illness associated with a prodrome of diarrhea that resulted in spontaneous recovery. Typically, STEC affects children less than 5 y of age, elderly and immunocompromised patients.[5] The easy transmission and its very low infectious dose, i.e., < 10 cells, remark the significance of this bacterium as a foodborne pathogen that has been associated with both, outbreaks and sporadic cases of human disease.[6]

Outbreaks are commonly attributed to the consumption of contaminated meat, milk and dairy products, in particular those derived from cattle;[7,8] however, the consumption of water, unpasteurized apple drinks and vegetables contaminated with ruminant feces and direct contact with ruminant on farms are frequently implicated in outbreaks.[9-11] A recent example is the large outbreak of the HUS associated with the rare *E. coli* serotype O104:H4 harboring stx2/aggr occurred in Germany in 2011. Of the affected case subjects, 90% were adults, and more than two-thirds of case subjects with the HUS were female. All of the infections were associated with the consumption of contaminated bean and seed sprouts.[12-14] There is a growing concern about some sporadic cases and outbreaks attributable to direct contact with the animal environment.[15-17] In fact, in a study from a dairy farm, the ground and the environment of the rearing calves are the sites with the highest number of STEC-positive samples; however, cattle water troughs and the environment of cows are the places with the greater chance of finding $stx2_{EDL933}$ which is a subtype associated with serious disease in humans.[18] Cattle's feces and hides are considered to be sources of STEC contamination of carcasses during slaughter and it can occur during removal of the hide or the gastrointestinal tract.[19-21] Contamination of meat with STEC during slaughter is the main route by which these pathogens enter at the meat supply chain.[22] Among the numerous retail meat cuts, ground beef possesses more risk than intact muscle because it can be contaminated during the grinding operation. The increased risk is associated with the protection of *E. coli* from heat during the cooking process, since the organism may be protected inside the body of reformed meat.[23] In the conversion of beef carcasses

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to ground beef and retail cuts, microbial contamination is currently unavoidable.[24] The importance of the contamination were studied by Etcheverría et al.[25] who detected an increase STEC contamination when the meat is transported from the slaughters until the sale point.

Typical Virulence Factors

The main virulence factor of STEC is the production of Shiga toxins encoded by *stx1* and *stx2* genes carried by lysogenic phages.[26,27] Shiga toxins belong to the family of AB5 protein toxins that contain an enzymatically active A subunit, and 5 B subunits responsible for binding to the Gb3, the cellular receptor that is present in several organs as kidney, brain, liver and pancreas. When Stx bonds its receptor, is internalized and inhibits the protein synthesis in the cytosol by removing an adenine base from the 28S ribonucleic acid (RNA) of the 60S ribosomal subunit.[28] Both Shiga toxin genes present variants although Stx2 is the most heterogeneous group. In contrast to Stx1, that possess stx1c, stx1d and $stx1_{EDL933}$ variants several subtypes of Stx2 have been identified, consisting of Stx2c, Stx2d, $Stx2d_{act}$, Stx2e, Stx2f, and Stx2g.[29,30] The strains *stx2* positive (mainly harboring $stx2_{EDL933}$ subtype) are potentially more virulent and are more frequently related to HUS than those harboring only *stx1* or even those carrying both.[31-33]

Another typical virulence factor is intimin, which is required for intimate bacterial adhesion to epithelial cells inducing a characteristic histopathological lesion defined as "attaching and effacing" (A/E). This lesion is governed by a large pathogenicity island named the locus of enterocyte effacement (LEE). The products of LEE are a type III secretion system, intimin and its translocated intimin receptor, and other secreted proteins. The secretion system is a molecular syringe for which secreted proteins are transferred into host cell cytoplasm. Intimin is encoded by *eae* gene that presents heterogeneity in their 3' end, involved in binding to the enterocytes. Actually, there are 17 types of intimin α1, α2, β1, ξR/β2B, δ/κ/β2O, γ1, θ/γ2, ε1, νR/ε2, ζ, η, ι1, μR/ι2, λ, μB, νB, and ξB. The *eae* gene was detected in 24% of 186 STEC isolates obtained from cattle and food with a lower frequency in strains isolated from food than from cattle. In relation to *stx* genes, *eae* was detected more frequently in stx_1-positive than stx_2-positive STEC strains, and both *eae* and stx_1 were more frequent in calves than adult cattle. Subtypes γ and β were the predominant *eae* variants, while ε was present at a lower rate and α was absent. As expected, isolates belonging to the same serotype presented the same *eae* variant.[34,35]

Additional virulence-associated markers, among many others, are a plasmid-encoded enterohemolysin[33] and, in strains lacking *eae*, an autoagglutinating adhesin (Saa) which could be involved in the adhesion of strains.[36] This plasmid is present in STEC O157 and non-O157 strains although with a considerable variability among them. Furthermore, strains that belonged to identical pulsed-field gel electrophoresis types could be further discriminated by the detection of plasmid-encoded genes as hemolysin (*ehxA*), a catalase-peroxidase (*katP*), an extracellular serine protease (*espP*), a zinc metalloprotease (*stcE*, also called *tagA*), a subtilase cytotoxin (*subAB*), among others. KatP may help *E. coli* O157:H7 to colonize host intestines by reducing oxidative stress, EspP is known to cleave pepsin A and human coagulation factor V which could contribute to mucosal hemorrhage observed in HC and influences the intestinal colonization and adherence in bovines, while StcE contributes to intimate adherence of this bacterium to host cells. SubAB has been described in certain highly virulent STEC strains which are negative for the LEE and shown to be cytotoxic to Vero cells and lethal for mice. However, in this set of strains the authors identified that *espP* was the most prevalent gene, meanwhile *katP* was present only in serotypes O145:H- and O157:H7, and *stcE* only in O157:H7 strains.[18,37]

STEC O157 and Non-O157

In several countries, including Argentina, most outbreaks of HC and HUS have been attributed to STEC O157:H7 serotype[38] but infections with some non-O157 STEC serotypes are frequently associated with HC and HUS mainly in Europe and Latin America. However, the potential of the non-O157 serotypes as human pathogens should not be underestimated, because as long as these serotypes are not regularly sought in clinical laboratories, they will neither be found, nor reported.[39,40]

STEC O157:H7 was described in 1977 by Konowalchuk[41] and has also been responsible for the most severe STEC outbreaks reported worldwide. However, over the past 15 y, non-O157 serotypes have emerged as important enteric pathogens and numerous outbreaks in countries such as Japan, Argentina, Chile, Germany, Australia, the United States and Ireland have been attributed to non-O157 infections.[40,42-44] The serotypes more commonly associated with human infections are: O26:H11/H-, O91:H21/H-, O103:H2, O111:H-, O113:H21, O118:H16, O121:H19, O128:H2/H-, O130:H11, O141:H19, O145:H28/H-, O146:H21, O163:H19, O172:H-, and O178:H19.[45] In Argentina, the country with the highest incidence worldwide of HUS, isolates obtained from 4824 samples from cattle, foods (hamburger and minced meat), and environment of farms were analyzed to detect STEC. From those, 545 isolates were characterized by multiplex PCR to detect *stx1*, *stx2*, *eae*, *ehxA*, and *saa* and then were serotyped. The prevalent serotypes were O8:H19, O26:H11, O91:H21, O113:H21, O117:H7, O130:H11, O145:H-, O157:H7, O171:H2, and O178:H19, corresponding to 61% of typeable strains. There were serotypes shared between cattle and foods, between cattle and the environment and among cattle, foods and environment. Ninety eight serotypes (18%) were non-typeable[46] (**Table 1**).

The predominant virulence profiles, which comprised 78% of the isolates, were *stx2*, *stx2*/*ehxA*/*saa*, *stx1*/*stx2*/*ehxA*/*saa*, and *stx2*/*eae*/*ehxA*, arranged in decreasing order. Among calves, the profiles *stx1*/*eae*/*ehxA* and *stx2*/*eae*/*ehxA* were the most frequent, followed by *stx2*, and these three profiles also predominated among environmental STEC isolates. The profile *stx2* was the most frequent among grown calves, adult cattle and food isolates, followed by *stx2*/*ehxA*/*saa* and *stx1*/*stx2*/*ehxA*/*saa*.[47] The most prevalent serotypes from cattle, foods, and environment in

Table 1. Serotypes shared between cattle and foods, between cattle and the environment and among cattle, foods and environment of strains collection from Argentina

Serotype	Percentage of strains (%)				Virulence profiles
	(n = 447)	Cattle	Foods	Environment	
O178:H19	13	95	5	0	*stx2* *stx2 saa ehxA* *stx1 stx2 saa ehxA*
O130:H11	9	93	7	0	*stx1 stx2 saa ehxA* *stx1 saa ehxA*
O113:H21	8	86	14	0	*stx2 saa ehxA* *stx1 stx2 saa ehxA* *stx1 eae ehxA* *stx2*
O26:H11	5	91	0	9	*stx1 eae ehxA* *stx2 eae ehxA*
O91:H21	5	95	5	0	*stx2 saa ehxA*
O171:H2	5	86	14	0	*stx2*
O117:H7	3	50	50	0	*stx2*
O145:H-	3	93	0	7	*stx1 eae ehxA* *stx2 eae ehxA* *stx1 eae* *stx2 eae*
O157:H7	3	93	7	0	*stx2 eae ehxA*
O8:H19	2	45	45	10	*stx1 stx2 ehxA* *stx1 ehxA* *stx2*

Argentina have been also isolated from human cases in several countries including Argentina, and carry virulence profiles that reflect the pathogenic potential of the strains.[46]

Cattle as Reservoir of STEC

As mentioned above, several studies have demonstrated that cattle are the main reservoir of STEC[44,48-52] with variable prevalence ranged from 22% to 67% in different categories of cattle, and 44% in cattle pre-slaughter.[44,51-53]

The population transmission dynamics of STEC are thought to be a combination of the transmission dynamics of mobile virulence factors, such as the *stx* genes, within the animals and the transmission dynamics of STEC between animals. The two transmission mechanisms result in acquisition and loss of the virulence markers, although they act on different time scales. Competition and dominance of certain STEC strains could be complementary explanations for the observed shedding of predominant *E. coli* isolates over time.[54-57]

The persistence of STEC O157:H7 in cattle may be due to the ability of the bacteria to colonize a particular location within the gastrointestinal tract (GIT). Several authors have reported that STEC O157:H7 shows tissue tropisms for the colon, lymphoid follicle-dense mucosa at the terminal rectum, and the rectoanal junction.[58,59] Naylor et al.[59] reported that STEC O157:H7 exhibits tropism for the terminal rectum in a region within 3–5 cm proximal to the rectoanal junction (RAJ) of bovine host. They hypothesized that a subset of cattle (super shedders) may shed STEC O157:H7 at high levels (10^4 CFU/g of feces) and that colonization at the RAJ was necessary for the high level shedding. Subsequent studies have described an association between RAJ colonization and super shedding status.[60,61] STEC O157:H7 intimately attaches to a variety of cell types and tissues, and a few studies have demonstrated that it can form attaching and effacing lesions on explants of bovine intestinal tissues.[62,63]

Enterohemorrhagic *Escherichia coli* (EHEC), a STEC strains subgroup isolated from human cases and actually named as synonymous, adapts an oral-fecal lifestyle in cattle and other ruminants. After being ingested, EHEC enters the rumen of cattle. In order to reach the RAJ for colonization, EHEC must first breach the acidic barrier of the stomachs. EHEC has an intricate acid resistance system that enables it to survive through the acidic environment of the stomach, as exemplified by its low infectious dose of 10 to 100 colony-forming units.[64]

In recent years, several non-LEE encoded effectors EspJ, NleB, NleE, NleF, and NleH also have been shown to influence EHEC survival and colonization. NleE plays a key role inmodulating the innate immune response during EPEC and EHEC infection while NleH is a translocated antagonist of pro-apoptotic effects by EPEC/EHEC. The NleH effectors therefore promote overall cell survival and inhibit enterocyte loss to promote sustained colonization by EPEC/EHEC.[65]

NleE plays a key role in modulating the innate immune response during EPEC and EHEC infection causing a decrease in the expression and production of IL-8,[66] while NleB interfere with inflammatory signaling pathways.[67]

Although EspJ is not required for A/E lesion formation in HEp-2 cells or human intestinal explants, in vivo studies in mice show that EspJ aids in the passage of EHEC through the host's intestinal tract, suggesting a role for EspJ in host survival and pathogen transmission.[68]

Other Factors Involved in Colonization

The ability of bacterial pathogens to bind to the host mucosa is a critical step in the pathogenesis of many bacterial infections, but this characteristic is also present in commensal bacteria, since they have to adhere and colonize specific niches. Both commensal and pathogenic bacteria have several putative adhesins that might participate in the adherence process. In the case of the bovine intestine or other sites where the bacteria are known to persist (vegetables such as lettuce and spinach), the data indicate that STEC O157 and non-O157 strains expressed a wide variety of fimbrial and afimbrial adhesins that might play a key role in persistence in the ruminant reservoir or in the formation of biofilms in other cell surfaces.[69]

One of the best-studied adhesins is the type 1 fimbriae that mediate binding to the intestinal cell surface.[70] The major component of these fibers was a 21-kDa protein encoded by the *yagZ* gene, widely present among pathogenic and commensal *E. coli*, a situation leading to the designation of these pili as *E. coli* common pili or ECP.[71-73]

In studies that used bovine terminal rectal primary epithelial cells it was demonstrated that H7 flagellum acts as an adhesin to bovine intestinal epithelium and supports its involvement in the initiating step for colonization of the cattle reservoir.[74] A putative fimbrial operon was identified in mutagenesis studies designed to find the adhesion factors involved in STEC colonization in cattle.[75,76] Its expression in the *E. coli* K-12 strain resulted in the production of visible fimbriae, of about 1 to 2 μm in length, extending from the bacteria and able to form longer bundles different from flagella.[77] The fimbria, designated F9, was found to be involved in the adherence to bovine epithelial cells and to the bovine extracellular matrix protein fibronectin, but not to bovine gastrointestinal tissue explants.[77]

Enterohemorrhagic *E. coli* autotransporter (Eha) have been identified in several STEC strains of different origins, some of them (EhaA, EhaB, and EhaJ), implicated in attachment to biotic and abiotic surfaces.[18,78-80] EhaA and EhaB are the most prevalent between STEC strains, and EhaA promotes the adhesion to primary epithelial cells of the bovine terminal rectum.[80] Tarr et al.[81] described a novel Iha (iron-regulated gene A homolog adhesion) bacterial adherence-conferring protein. Iha is homologous to a variety of bacterial iron acquisition proteins in the database but not to other known adhesins. It is possible that *iha* does not encode an adhesin but instead encodes a protein that increases the expression of a cryptic adhesin in laboratory *E. coli*.

In STEC strains it was described a family of serin protease autotransporters that include EspP of STEC O157:H7 that contributes to the adherence to bovine primary rectal cells and colonization of the bovine intestines.[82]

The analysis of a clinical isolate of STEC strain serotype O111:H-, highly adherent, led to identification of an enterohemorrhagic *E. coli* factor for adherence 1 or *efa-1* and found to be present in enteropathogenic *Escherichia coli* (EPEC) and in non-O157 STEC strains.[83] In EPEC, Efa-1 was reported to be 97.4% identical to the LifA protein (also called lymphostatin), which inhibits the proliferation of mitogen activated lymphocytes and the synthesis of proinflammatory cytokines, such as IL-2, IL-4, IL-5, and gamma interferon (IFN-γ).[84,85] STEC O157:H7 has a truncated version of the *efa-1* gene in the chromosome, and some researchers have suggested that the truncated Efa-1 protein might share some properties with the full-length Efa-1.[86] A homolog of the *efa-1/lifA* gene is also present on the pO157 large plasmid, and the gene has been designated *toxB*. The ToxB protein exhibits 28% amino acid identity to the Efa-1/LifA protein and contributes to the adherence to cultured epithelial intestinal cells, which has been linked to the ToxB-induced production and/or secretion of type III secreted proteins.[87]

Interesting, in a collection of 538 STEC isolates obtained from cattle and foods, *efa-1* was detected in cattle isolates but no in food isolates, while *iha* was detected in isolates from cattle and food, further demonstrating differences between serotypes (data not published).

In addition to many colonization factors described above, Torres et al.[88] have described in STEC O157:H7 two chromosomal gene clusters closely related to the long polar fimbrial (*lpf*) operon of *Salmonella enterica* serovar Typhimurium. These operons named *lpf1* and *lpf2* have been associated with the appearance of long fimbriae that possess colonization abilities in animals. Different *lpfA* types have been detected in a collection of LEE-negative STEC strains demonstrating no association between the types of *lpfA1* and *lpfA2* and the severity of human disease.[35]

Options for Control Cattle Colonization

Considerable effort has been expended to identify herd management practices and environmental factors that inhibit or facilitate infection of animals with STEC O157:H7.[89] EHEC transmits readily between ruminants in the farm environment[90] and wild animals may represent important vectors. For many years, the cattle industry and researchers have focused on improving the safety of meat products after slaughter. Postslaughter antimicrobial treatments and HACCP policies in slaughter plants have been shown to significantly reduce carcass contamination.[19]

However, illnesses caused by contaminated meat products still occur. Therefore, greater emphasis has recently been placed on the development of intervention strategies that target the pathogenic microbial population of the live animal before slaughter.[91] Because of the widespread distribution of EHEC serotypes, O157, and non-O157, in cattle population, its control will require interventions at the farm level.[92] One strategy has been the development of vaccines to prevent or diminish

shedding of the bacteria in the animal reservoir. Some vaccines had been developed, one based on Type III secreted proteins decreased the shedding of STEC from 23 to 9% and other using EspA, intimin, and Tir, involved in STEC adherence significantly reduced shedding of EHEC O157 from experimentally infected cattle.[35,93] Another option for the control of foodborne pathogens in livestock is the feeding of beneficial bacteria, often referred to as probiotics.[94] Probiotics can interfere with pathogenic strains by producing metabolites that are inhibitory to STEC O157:H7. Some strains of *E. coli* can produce colicins that are inhibitory in vitro to diarrheagenic *E. coli* strains, including O157:H7.[85] Several authors have identified bacteria with potential ability to exclude STEC O157:H7 from the GIT of cattle.[95,96] In a previous study, we isolated strains of colicinogenic *E. coli* from bovine colon which have the ability to inhibit the growth of STEC O157:H7 in vitro.[97] Probiotics can inhibit the attachment of STEC O157:H7 to Hep-2 cells and to bovine colon which is the primary site of colonization. In another study we demonstrated that colicinogenic *E. coli* was able to reduce the adherence of STEC O157:H7 when both strains were inoculated on cell cultures and on bovine colonic explants.[98]

Conclusions

STEC are widely distributed among cattle, foods, and the environment. Multiple colonization factors and cellular processes have been involved in the mechanism of STEC adhesion to bovine intestinal epithelial. Many researchers have made high quality papers regarding adhesins and other factors involved in the mechanism of adhesion. More studies are needed to fully understand the interaction between the pathogen and the host in order to evaluate some strategies to control cattle colonization in an attempt to reduce the transmission to human.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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