ENTEROHEMORRHAGIC *E. coli*: VIRULENCE FACTORS AND INFECTION IN CATTLE

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Abstract: *Enterohemorrhagic E. coli (EHEC) strains constitute a subset of the verotoxigenic E. coli (VTEC) or Shiga toxin (Stx)-producing E. coli (STEC). Within this group, E. coli O157:H7 is the most well-known Stx-producing serotype. EHEC strains produce hemorrhagic colitis and hemolytic uremic syndrome in humans. Like enteropathogenic E. coli (EPEC) strains, they mediate their pathogenesis through "attaching and effacing" (A/E) lesions. Cattle are the main reservoir of E. coli O157:H7 and they are directly linked to most of the human outbreaks. In this review, the virulence factors involved in the pathogenesis of EHEC strains, especially those participating in the colonization of the bovine intestinal mucosa, are analyzed.*

Key Words: EHEC, *E. coli* O157:H7, virulence factors, cattle, colonization

E. coli ENTEROHEMORRÁGICA: FACTORES DE VIRULENCIA E INFECCIÓN EN EL GANADO

Resumen: *Las cepas de E. coli enterohemorrágica (EHEC) constituyen un subgrupo de las E. coli verotoxigénicas (VTEC) o E. coli productoras de toxinas Shiga (Stx) (STEC). Dentro de este grupo, E. coli O157:H7 es el serotipo productor de Stx más conocido. Las cepas de EHEC producen colitis hemorrágica y el síndrome urémico hemolítico en humanos. Al igual que las cepas enteropatogénicas de E. coli (EPEC), estas cepas median su patogénesis a través de lesiones de "adherencia y destrucción" (lesión A/E). El ganado bovino es el principal reservorio de E. coli O157:H7 y se lo asocia directamente a la mayoría de los brotes en humanos. En esta revisión se analizan los factores de virulencia involucrados en la patogénesis de las cepas EHEC, especialmente aquellos que participan en la colonización de la mucosa intestinal de los bovinos.*

Palabras Clave: EHEC, *E. coli* O157:H7, factores de virulencia, ganado bovino, colonización

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INTRODUCTION

Escherichia coli belongs to the family *Enterobacteriaceae*, which contains mostly motile, Gram-negative bacilli. *E. coli* strains that cause diarrhea are classified into six categories: EPEC (enteropathogenic *E. coli*); EHEC (enterohemorrhagic *E. coli*); ETEC (enterotoxigenic *E. coli*); EIEC (enteroinvasive *E. coli*); EAEC (enteroaggregative *E. coli*); and DAEC (diffusely adherent *E. coli*) (25). EPEC strains are an important cause of diarrhea in developing countries and, as part of their pathogenesis, they mediate the production of "attaching and effacing" (A/E) lesions (25). ETEC strains elaborate enterotoxins, which are the main cause of intestinal secretion. EAEC strains do not secrete enterotoxins and they have the ability to attach to each other in aggregates or in a "stacked-brick" configuration on the surface of host cells *in vitro*. In contrast, DAEC strains produce a diffuse pattern of adherence. EIEC strains are closely related to *Shigella spp.* and they cause invasive inflammatory colitis, dysentery and watery diarrhea (25). Finally, EHEC strains are an important cause of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) in humans. Like EPEC strains, they also cause A/E lesions. EHEC strains constitute a subset of the verotoxigenic *E. coli* (VTEC) or Shiga toxin (Stx)-producing *E. coli* (STEC) because of their ability to produce toxins that are cytotoxic to Vero cells (immortalized monkey kidney cells) and because these toxins are variants of the Shiga toxin produced by *Shigella dysenteriae* serotype 1. Not all STEC are pathogenic; however, based on the original definition, all EHEC are considered pathogenic (29). The incidence of EHEC infection in humans is low even when the prevalence of Stx-producing strains in cattle (the main reservoir) is high, which suggests that Stx production alone may not be sufficient for EHEC infection (8, 27, 60). Therefore, the terms STEC or VTEC are used to characterize bacterial strains that express cytotoxin irrespective of their pathogenicity. Additional factors, such as a pathogenicity island (PAI) called "The Locus of Enterocyte Effacement" (LEE) and the large pO157 plasmid (60) appears to be required for full virulence. Within the EHEC group, *E. coli* O157:H7 is the most well-known Stx-producing serotype (40). *E. coli* O157:H7 is responsible for two-thirds of the EHEC infections in the United States, with the other one-third of cases attributed to non-O157 STEC, mainly O26, O111, O103 and O145 serotypes (27, 57).

EHEC-ASSOCIATED HUMAN DISEASE

E. coli O157:H7 is an emerging zoonotic pathogen that causes HC and HUS. In some cases, humans infected with EHEC strains may remain asymptomatic or exhibit mild, non-bloody diarrhea (40, 54). In other cases, HC may be the only manifestation of *E. coli* O157:H7 infection, or this condition can progress to HUS (54). HUS occurs most frequently in children younger than 5 years old, after a bloody diarrheal prodrome (40, 54). Elderly and immunocompromised individuals also have a high risk of developing clinical disease (42, 54).

HUS is characterized by non-immune hemolytic anemia, thrombocytopenia and acute renal failure (42). Complications involving the central nervous system (CNS) can also occur in 30 %-50 % of patients. Thrombotic thrombocytopenia purpura (TTP), which has only been reported in adults, was thought to represent a more extensive form of the clinical spectrum that causes HUS (54). However, TTP is now known to involve a process distinct from STEC-induced damage (56). There is no specific treatment for *E. coli* O157:H7 infections, other than supportive therapy and management of complications (54, 56, 57).

PATHOGENESIS OF EHEC

The infection dose of *E. coli* O157:H7 is low, and the production of Stx is the most important virulence trait responsible for the local and systemic outcomes of infection. Local intestinal damage, which includes microvascular injury, results in bloody diarrhea, whereas HUS results from microvascular injury at extra-intestinal sites, especially in the kidney and brain (40, 56, 60). It has been estimated that the infective dose to cause HUS is <50 organisms. Approximately 10 organisms are able to cause disease in outbreaks associated with non-O157 STEC (27). The incubation period ranges from 1 to 9 days (54, 56, 60) and diarrhea usually lasts for 3 to 7.5 days (54, 56). Clinical symptoms of EHEC infection include severe abdominal cramps, nausea and vomiting. Unlike other bacterial infections causing bloody diarrhea, fever is not always present or it can be very mild (54).

EHEC produces two types of Stx, Stx1 and Stx2. The risk of HUS is higher with *E. coli* O157:H7 isolates containing Stx2 genes, which suggests that this toxin is more virulent than Stx1 (54). There is no evidence of bacteremia in human disease (40). However, Stx production triggers a cascade of coagulative events that results in widespread thrombus formation and systemic disease. It is believed that *E. coli* O157:H7 releases toxins in the bowel and after their absorption into the circulation, they induce vascular endothelial damage (54). The kidney and CNS have high levels of toxin receptor (40, 60). Thus, these organs are particularly targeted during infection. Thrombotic microangiopathy is the main histopathological feature observed in patients with HUS. At the intestinal level, the

most severe abnormalities occur in the cecum and ascending colon. In cases of HC, colonic mucosal edema, erosion and hemorrhages are evident (54).

TRANSMISSION AND EPIDEMIOLOGY

E. coli O157 is a widespread zoonotic, food-borne pathogen, with water-borne, animal-to-person and person-to-person transmission implicated in human outbreaks (42, 59). Infected cattle are the main reservoir of EHEC O157 and other strains (54, 59). Animals colonized by EHEC are difficult to identify since infection of calves, adult cattle and sheep is asymptomatic (53). STEC strains can remain viable in feces for months (8). The presence of *E. coli* O157 in cattle feces represents a serious public health risk due to direct bacterial transmission to people or fecal contamination of food, water and/or the environment (42). Feces of non-bovine species such as sheep, dogs, horses, flies and birds can also contain *E. coli* O157. Vegetables, fruits and inadequately pasteurized milk are other vehicles of infection (59, 60).

Many recent outbreaks of *E. coli* O157:H7 infection have been associated with low pH products (salami, yogurt), highlighting the ability of the bacterium to tolerate acidic pH and survive processes of fermentation and drying (8). Transmission of *E. coli* O157:H7 to humans has been primarily linked to undercooked meat since under these conditions, the bacteria survive and retain pathogenicity. Food can be cross-contaminated by improper handling procedures during manufacturing, storage, marketing and even in the household itself (59).

E. coli O157:H7 infection of humans has a peak incidence during warmer months (54). The first outbreaks associated with *E. coli* O157:H7 infection were registered in 1982, in Michigan and Oregon. In both cases EHEC was transmitted by undercooked beef (60). In Europe, serotype O157:H$^-$(non-motile), sorbitol-fermenting STEC strains have emerged as a cause of HUS. In South America, *E. coli* O157:H7 infections are endemic, with Argentina being the country with the highest incidence (more than 300 cases each year) (11).

The case-fatality rate of HUS ranges between 0 % and 30 % and the risk of developing HUS after EHEC infection is 2 % to 7 % (59). About 30 % of HUS patients suffer permanent disabilities, including chronic renal insufficiency, hypertension and neurological disorders (60).

CATTLE AS *E. coli* O157:H7 RESERVOIR

Cattle are considered the main reservoir of *E. coli* O157:H7 strains (8, 42) and they can also harbor non-O157 STEC serogroups (27). Presently, sheep are also considered an important EHEC reservoir (8). It is estimated that 10% to 80% of cattle may carry Stx-producing *E. coli* (60) and because most ground beef is derived from adult cattle, it is critical to reduce *E. coli* O157:H7 in this population (12). In young calves (> 3 weeks old), experimental inoculation of *E. coli* O157:H7 (10^{10} CFU) does not induce significant clinical disease (13) and the presence of A/E lesions is not readily detected, suggesting that *E. coli* O157:H7 localizes within the intestinal content rather than in the mucosal surface (7).

E. coli O157:H7 fecal shedding in healthy cattle is usually transient or intermittent (26, 27) and the prevalence is highly variable. Smith *et al.* (49) determined that the prevalence by pen in feedlot cattle varied from 1 % to 80 %. In other studies, prevalences of 13.4 % in beef cattle and 16.1 % for cull dairy cattle were reported (42). Fegan *et al.* (15) reported no significant differences in prevalence in grain- (15 %) or grass- (10 %) fed cattle. High isolation rates of *E. coli* O157 in mouth and hide samples have been reported, indicating that cattle may have additional sites of bacterial colonization or carriages other than the distal gastrointestinal tract (52). The prevalence of *E. coli* O157:H7 in fecal samples is highest in summer and lowest during winter (27). It is also known that a high percentage of cattle shed the bacterium for a short period of time, which is followed by longer periods of low prevalence within herds (42). The magnitude and duration of fecal shedding of *E. coli* O157:H7 is usually greater in calves than in adults. It is believed that age-related differences in rumen function (concentration of volatile fatty acids [VFA], pH) may contribute to this phenomenon (12). Gastrointestinal disturbances and feeding regimens can also affect the pattern of fecal shedding (42). It has also been established that previous infection does not prevent re-infection against a high-level challenge of *E. coli* O157:H7 (7, 12).

E. coli O157:H7 can be horizontally transmitted among cattle (42). Bretschneider *et al.* (5) showed that experimentally infected adult beef cattle shed *E. coli* O157:H7 for 24 days (range of 8-42 days). Similarly, Besser *et al.* (1) reported that the duration of fecal excretion of *E. coli* O157:H7 in naturally infected cattle was < 1 month. Kahitsa *et al.* (26) found that the length of fecal shedding in feedlot cattle ranged from 1 to 4.5 weeks, and peaked during the epidemic phase, which corresponds to times of higher challenge levels. In contrast to the findings by Fegan *et al.* (16), in a sheep model, Kudva *et al.* (28) demonstrated that a dietary change from alfalfa pellet to grass, by itself or in combination with withholding of feed for 24 hours, triggered the excretion of *E. coli* O157:H7 in experimental infected lambs. It was hypothesized that the

dietary shift induced a selective *E. coli* O157:H7 growth in the intestine. They suggested that feed withholding might induce bacterial shedding in animals by triggering the growth of *E. coli* O157:H7 present in the intestine or by increasing the susceptibility to infection. Furthermore, fasting also causes an increase in rumen pH and a drop in VFA concentration, which favor *E. coli* O157:H7 proliferation (53).

VIRULENCE FACTORS

LOCUS OF ENTEROCYTE EFFACEMENT (LEE)

PAIs are large regions of chromosomal DNA (10-200 kilobases) that are essential for bacterial pathogenicity. PAIs are usually associated with tRNAs, they have different G+C content and codon usage from the rest of the genome and their borders are marked by repeated sequences or insertion elements (25, 31). The ability to induce A/E lesions is encoded by genes on a 35-kilobase PAI called "The Locus of Enterocyte Effacement" (LEE) (25, 35). LEE 1, 2 and 3 are identical in EHEC and EPEC and they encode products required for type III secretion. LEE 4 encodes the *E. coli* secreted proteins EspA, EspB, EspD and EspF. A further operon contains Tir, its chaperone CesT, and intimin (45). In EHEC, LEE enables bacterial colonization of the intestinal tract (43).

The Ler locus in EHEC and EPEC encodes a regulator of virulence gene expression, which directly modulates genes within the LEE elements in response to environmental signals inside the human gut (21). Therefore, Ler is essential for the formation of A/E lesion.

Quorum sensing (QS) is a mechanism by which bacteria regulate their gene expression in response to cell density, via the production of hormone-like compounds (auto-inducers), which stimulate gene expression (10). QS has been shown to be involved in the regulation of the expression of EPEC and EHEC LEE operons by Ler. However, because of the low infectious dose of EHEC, these strains should detect auto-inducer signals produced by commensal *E. coli* and other bacteria present at high concentration in the large intestine to initiate the expression of virulence factors (25, 40).

THE TYPE III SECRETION SYSTEM (TTSS)

Four pathways of protein secretion have been described in Gram-negative bacteria (types I to IV). A fifth system, involved in conjugal transfer of plasmids, is not well-characterized (15, 18, 23). For some Gram- negative pathogens, the TTSS is an essential virulence determinant (23) required for the delivery of bacterial factors into the host cells (9, 18, 31). The best-studied TTSS is that of *Yersinia spp.* In EHEC, the TTSS is primarily required for the persistence of the bacterium in the terminal portion of the gastrointestinal tract (45).

Most of the bacterial pathogenicity factors interact with eukaryotic host cells (23). Therefore, virulence factors are exposed either at the surface of the bacterial cell or are transported out of the bacteria. The outer membrane in Gram-negative microorganisms led to the evolution of secretion systems which are different from those of Gram-positive bacteria. In EPEC and EHEC, the TTSS is dedicated to the secretion of specific proteins (Tir, EspA, EspB and EspD), which are essential for the subversion of signal transduction pathways and the formation of A/E lesions (9). The TTSS functions by a "contact-mediated" mechanism, *i.e* it is triggered when a pathogen comes in close contact with a host cell (18, 31). The TTSS is composed of about 20 proteins, most of which are located in the inner membrane (15, 18, 23), and are homologous to the flagellar biosynthesis apparatus (18, 23, 31).

Secreted proteins require small cytoplasmic proteins (chaperones), to prevent premature interaction with other components of the secretion system (23, 31). Chaperones physically associate with the effector in the bacterial cytosol and remain at this location following translocation of the effector into the host cell. Then, bacterial proteins are released through a prominent needle-like structure on the outer surface of the bacterial cell (18).

When A/E pathogens enter their host via ingestion, the gastrointestinal conditions activate LEE gene expression, leading to the assembly of the TTSS apparatus. A/E pathogens use calcium concentrations to regulate type III secretion and to control the hierarchy of translocators (EspA, EspB and EspD) and effectors (EspF, EspG, EspH, Tir and Map). The levels of calcium present in the extracellular fluid of the intestinal lumen trigger the secretion of EspA, EspB and EspD, which mediate the assembly of the translocon and the formation of a pore in the host cell membrane. In the bridge that connects the bacterial and host cytoplasm, the calcium concentration is limited. Therefore, low levels of calcium suppress the secretion of translocators and activate the secretion of effectors. Other environmental signals might also play a role in regulating TTSS (14).

E. coli -SECRETED PROTEINS (ESP)

At least eight Esp have been identified, of which six are encoded by the LEE. Particularly, EspA, EspB and EspD are proteins secreted in significant quantities in a TTSS-dependent manner by EPEC strains. These proteins are required for effector proteins secretion and A/E lesion formation (10).

EspA is a 20-kDa protein (198 amino-acids) (18, 23) which forms filaments that bridge the bacteria and the host cell membrane (46) to carry proteins from the pathogenic bacteria to the host cell (18). EspA filaments are usually 90 nm in length (18) and are found on the bacterial surface at early stages of A/E lesion formation (9). EspA might also act as a bacterial adhesin at the initial stages of infection (9, 15). EspA filaments are required for the delivery of EspB. EspA initiates polymerization from the tip of the needle and assembles a sheath-like structure, which is expandable and its elongation is controlled by the amount of EspA (46). EspA amino and carboxyl termini are conserved between EPEC and EHEC. However, the central region of the protein is variable and may influence the cell-binding specificity of the different strains (51).

The *espB* gene product is a 37-kDa protein (321 amino acids), which is an essential signal transduction molecule (23). EspB is translocated and it is required for the translocation of other proteins (15). EspB becomes resistant to protease digestion upon association with the host cell. It is required for tyrosine phosphorylation of Tir in EPEC and for the accumulation of actin filaments beneath adherent bacteria (23). EspB binds EspA, which suggests that there is a continuous path between the filament and the host cell. Together with EspD, EspB forms a pore in the distal part of the EspA filament (18, 45) which is required to insert Tir into the eukaryotic membrane (23). EspA and EspB are critical for disease and are involved in inflammation and disruption of the mucosal epithelial surface. They are critical for A/E lesion formation, cytoskeletal rearrangement and formation of cup-like structures *in vivo* (15).

EspD is a 39-kDa protein (381 amino acids) (10, 23). It is involved in the formation of the translocon; thus, its primary function would be the delivery of other virulence factors rather than acting as an effector (9).

EspF is a 21-kDa, proline-rich, effector protein translocated by the TTSS (9, 10) but not required for A/E lesion formation (10). It is involved in EPEC-induced host cell death (45) and disruption of the host intestinal tight-junctions through the re-distribution of occludin, a component of the cellular tight-junctions (9, 25). EspF also appears to promote EHEC colonization by modulating the host inflammatory response through inhibition of polymorphonuclear cells accumulation in the colon (43).

Little is known about EspG function. It is a 44 kDa-protein, which appears to be involved in virulence, but which does not seem to function in A/E lesion development (10). However, EspG deletion mutants have reduced levels of intestinal colonization. This protein would cause transient microtubule destruction and actin polymerization (51).

The functions of EspH have not been completely studied. It is a modulator of the host actin cytoskeleton with the effect of repressing the formation of filopodia and enhancing the formation of actin pedestals (58). EspH also promotes EHEC-induced diarrhea and intestinal colonization by increasing the initial intestinal adherence (43).

MITOCHONDRION-ASSOCIATED PROTEIN (MAP)

Map is a multifunctional protein that induces the formation of filopodia via the Rho GTPase Cdc42 and also targets the mitochondria to disrupt their membrane potential (25). Map-induced signaling inhibits pedestal formation. In contrast, Tir, in association with intimin, down-regulates filopodia formation by Map (45), probably as a required step for A/E lesion formation (10). In a yeast model, EPEC Map, together with EspD and EspG, was demonstrated to inhibit growth by depolarization of the actin cytoskeleton (44).

In conclusion, EspH, EspF, EspG and Map are not required for A/E lesion formation *in vitro* or *in vivo*. However, they appear to be important to guarantee maximal intestinal colonization (43).

TRANSLOCATED-INTIMIN RECEPTOR (TIR)

Tir is a 72-kDa protein (549 amino acids), which is secreted by the TTSS into the eukaryotic membrane (23). Tir is the first recognized example of a bacterium inserting its own receptor into the host cell membrane (9). It contains two potential transmembrane domains (15, 17, 23), and functions as a receptor for the bacterial attachment factor, intimin (16, 23, 25). Tir anchors intimin to the host cell actin and other cytoskeleton proteins. Thus, the bacteria can initiate pedestal formation and mediate its pathogenic effect while remaining on the extracellular surface (9). Tir promoter activity is almost undetectable before bacteria-host cell contact. However, it is expressed at higher levels after host cell contact has taken place (45).

Tir and its chaperone, CesT (16, 45), are encoded by LEE, immediately upstream of the *eae* intimin gene (16). In EPEC, CesT is critical for Tir secretion by the recruitment of Tir to the inner membrane (45). The affinity of Tir for intimin is higher in its free state than in its chaperone-bound form (CesT-Tir). In addition to binding Tir, *E. coli* - CesT binds Map (18), having a central role in its secretion.

Once transferred into eukaryotic cells, Tir is phosphorylated on C-terminal tyrosines and

this phosphorylation appears to be required for the function of Tir as an actin nucleator (23, 25, 45) and as a signal transduction molecule in epithelial cells. Tir orchestrates pedestal formation and directs the recruitment of several cytoskeletal proteins to the site of EHEC attachment. In contrast to EPEC, in EHEC, cytoskeletal protein recruitment is independent of the tyrosine phosphorylation by Tir (45). Furthermore, the Tir protein of EHEC O157:H7 is not functionally identical to that of EPEC because EHEC pedestal formation is initiated without Tir binding to the adaptor protein Nck (25), which is implicated in the initiation of actin signaling (10). Thus, in EHEC, additional bacterial factors are translocated to trigger actin signaling (25).

Recently, studies demonstrated that Tir is essential for *E. coli* O157:H7 intestinal colonization in calves (47), mainly because it mediates bacterial attachment to the intestinal mucosa via intimin binding.

INTIMIN

Intimin is a 94-kDa outer membrane protein (23), which resembles eukaryotic adhesion molecules (16). It is not secreted by the TTSS, but it is encoded within the same gene cluster that encodes this bacterial system (23). The *eae* gene is present in EPEC and EHEC and there is an 86% sequence homology (60). Expression of intimin is essential for A/E lesion formation and is required for full virulence (15, 23, 47). Intimin binding to Tir elicits cytoskeletal rearrangements within the host cell, leading to the formation of actin-rich pedestals beneath the adherent bacteria (15). Intimin also binds host-encoded receptors, such as β1 integrin (35). A role for intimin in the stimulation of mucosal Th1 responses and intestinal crypt hyperplasia has also been reported (25). At an early stage of A/E lesion formation, intimin would have an additional, Tir-independent function, involving the remodeling of the mammalian cell surface (9, 17).

At least 17 types of intimin (α, β, γ, δ, ε and others) have been identified, according to amino-acid variations at the C-terminal domain. Intimin α is found in EPEC strains, whereas intimin γ is specifically associated with EHEC O157:H7. The existence of different intimin types suggests that the protein is responsible for the differential tissue targeting exhibited by EHEC and EPEC (8, 9, 17). Despite the importance of intimin in intestinal colonization of cattle, the *eae* gene is not present in all Stx-producing *E. coli* strains isolated from cattle, suggesting that other factors may influence the persistence of the bacteria in the bovine gastrointestinal tract (53).

ATTACHING AND EFFACING (A/E) LESION

EPEC is a well-studied pathogen that causes A/E lesions in intestinal cells. Other pathogens include *Hafnia alvei, Citrobacter rodentium* and EHEC (35). A/E bacteria can affect the small or the large intestine, and they cause diarrhea (60), likely because of a reduction in the absorptive capacity of the intestinal mucosa and the consequent electrolyte imbalance (10). Whereas EPEC strains colonize both the small and large bowel, EHEC strains are only localized in the large intestine (33). The mucosa of the terminal rectum (3-5 cm proximal to the recto-anal junction) is the principal site of colonization of EHEC O157:H7 (32). After initial adherence, EPEC strains attach intimately to the epithelial cell surface, leading to the effacement of the microvilli beneath the bacteria. This characteristic clustered pattern of adherence produces the histopathologic phenotype known as the A/E lesion. Both intimin and EPEC TTSS are required for intimate attachment (23) and lesion formation.

Subjacent to adherent bacteria, the host cellular cytoskeleton is re-organized into a lesion in which the surface microvilli are disrupted and F-actin is recruited into a pedestal structure (15). As long as the host cell remains healthy, the bacteria remain attached and multiply on the surface of the cell (45).

EspA filaments are excluded from the region of intimate contact once A/E lesions are produced. Therefore, fully developed A/E lesions are devoid of EspA filaments (15).

A three-stage model was described for the interaction of EPEC with epithelial cells and A/E lesion formation: (1) localized adherence (initial, non-intimate attachement); (2) signal transduction; and (3) intimate adherence. In EPEC, localized adherence is mediated by the plasmid-encoded bundle forming pili (BFP) (9, 35). Although initial adherence is not essential for A/E lesion development (9, 35), BFP helps to bring the bacteria in close contact with the host cells (35). In contrast to EPEC, EHEC does not carry BFP (60, 51). In association with the constant peristaltic flushing that this microorganism must surmount, the specific tropism of *E. coli* O157:H7 for the terminal rectum (32) would indicate that an initial adherence factor is needed to facilitate the final colonization by a highly complex A/E mechanism. In this regard, it was recently established that H7 flagella act as an adhesin to the bovine intestine in the crucial initiating step of *E. coli* O157:H7 colonization (30). During the intimate adherence, EspA filaments are lost from the bacterial cell surface (10) and intimin/Tir binding activates additional signaling cascades that induce cytoskeletal rearrangements to form the pedestals upon which A/E bacteria reside

(9, 15, 35). Actin is the major component of the brush border cytoskeleton and the site-specific concentration of cytoskeletal actin is characteristic of A/E histopathology. Actin pedestals are up to 10 um in length (9). Other cytoskeletal proteins (α-actinin, talin, erzin, villin, myosin light chain, VASP, WASP and arp2/3 complex) are also present in A/E lesions, and they accumulate under the adherent bacteria within 3 hours of EPEC infection (9). An elevation in the intracellular levels of calcium can contribute to the depolymerization of actin and, consequently, to the breakdown of host cytoskeleton during A/E lesion formation. Interestingly, EPEC does not have an effect on the Rho family of small GTP-binding proteins that are normally involved in cytoskeletal rearrangement. Therefore, EPEC would use a non-traditional mechanism for actin rearrangement.

SHIGA TOXIN (STX) FAMILY

The toxin known as "Shiga toxin" is the exotoxin of *Shigella dysenteriae* type I. The terms Shiga toxin and Verotoxin are synonymous. Vero cells are killed within a few hours by low doses of Stx (57). Stx is considered the key virulence factor of EHEC (54). As previously mentioned, the Stx family contains two subgroups, Stx1 and Stx2, which share approximately 55% amino-acid homology (25, 40). Stx1 shows little sequence variation; however, several variants of Stx2 have been described (8).

Most Stxs are encoded by lambda-like bacteriophages (25, 54), and they are transcribed from a promoter that also controls the expression of late lambda phage lysis genes, thereby linking toxin expression with a lytic function responsible for the release of the toxin (25).

All Stxs consist of an A subunit (toxic moiety) and five B subunits (receptor binding) (57, 59). Stx1 differs by only one amino acid on subunit A with respect to Stx2 and they share the same cell receptor and intracellular mechanism of action *in vitro* (54).

To enter into de cell, the B chains bind to the Gb3 receptor, also known as CD77 or PK (57). Once the holotoxin is internalized into an endosome, the A subunit is cleaved. The free A chain has N-glycosidase activity (57, 59); adenine is cleaved from the ribosomal RNA at a point where the aminoacyl transfer RNA is assembled, thus, arresting protein synthesis (51, 57). Stx can also induce direct apoptosis of renal (59) and epithelial cells (25) and it can trigger monocytes to produce and release cytokines such as TNF-α, IL1-β, IL-6 and IL-8 (40), likely using a receptor different from Gb3 (57). In addition, Stx is also able to bind to platelets and trigger their direct activation (40).

Stx2 increases the severity and duration of EHEC-induced diarrhea and modulates the host inflammatory response in the infant rabbit model. Gut inflammation would facilitate the severe manifestations of EHEC infection by allowing increased uptake of Stx from the lumen into the systemic circulation (60). In cattle, Stx down-modulates the mucosal inflammatory responses (51).

Stx1 is more frequently identified in cattle isolates, whereas Stx2 is more prevalent in human isolates (27, 57) and it has been suggested that Stx2 is more virulent in humans than Stx1 (40). It is believed that the distribution of the Gb3 receptor is responsible for the devastating effects of human infections by EHEC and for the absence of clinical symptoms in cattle. Unlike humans, Gb3 is not present on bovine endothelial cells. Therefore, Stx expression does not produce the severe vascular injury and hemorrhage observed in humans (51). The production of Stx distinguishes EHEC from EPEC. STEC serotypes do not normally invade the host, but the immediate proximity of colonizing bacteria to the epithelium might promote the delivery of Stx directly to the mucosa (25, 57).

pO157 PLASMID

The pO157 plasmid of EHEC encodes 35 proteins (8), some of which are accessory toxins (25). This 90-Kb plasmid (8, 60) is present in almost all EHEC isolates that encode an EHEC hemolysin (60). The pO157 plasmid also encodes an EHEC *Kat*P catalase-peroxidase and an extracellular serine-protease, EspP. This protease cleaves pepsin A and human coagulation factor V, suggesting that it may participate in the induction of intestinal hemorrhage (8, 60). Another virulence gene, *tox*B, which appears to be present in all EHEC O157 isolates, is also encoded within this plasmid. *tox*B may influence the expression of LEE-encoded type III secreted proteins and it may inhibit lymphocyte activation (8). Intrarectal inoculation of 5 to 8-month-old cattle with isogenic strains of *E. coli* O157:H7 identified a significant role for the pO157 plasmid in the colonization of the terminal rectum (47).

EHEC HEMOLYSIN

*ehx*A is the structural gene for the enterohemolysin of EHEC, which is a pore-forming toxin (55). It might contribute to EHEC pathogenesis because the hemoglobin released during hemolysis provides a source of iron to the bacteria. This would be especially important for human isolates but not for isolates from cattle. Together with intimin and Stx genes, the enterohemolysin appears to be a marker of virulence for most of the EHEC O157 and non-O157 strains (60).

LIPOPOLYSACCHARIDE (LPS)

LPS is an endotoxin that consist of a lipid A, responsible for the toxic action; an O-antigenic polysaccharide (O-PL), which is structurally non-conserved among related bacterial species; and a core oligosaccharide that links O-PL with lipid A (98). Endotoxemia is observed in patients with HUS caused by *Shigella dysenteriae* type I, but not in EHEC infection (56). However, it has been suggested a synergistic effect of Stx and LPS in the pathogenesis (40).

E. coli LPS has five distinct core oligosaccharides structures, designated K-12 and R1 to R4. R3 is frequently found in STEC isolates from cattle and humans (18). Compared to healthy volunteers, serum from convalescent patients has significantly higher levels of IgA against O157 LPS and R3. Because of its conserved structure, the core oligosaccharide is responsible for cross-reactive antibodies. The R3 core LPS is the most common core type in the STEC group (19).

In calves and adult cattle, the serum antibody response to STEC O157 LPS lasts more than 5 months (24). However, this immune response is unable to prevent re-infection with the homologous STEC strain.

FIMBRIAE

Fimbriae are thread-like structures that extend out from the bacterial surface and provide multiple functions, including adherence to host cells. Although EHEC contains 10 putative fimbrial loci, little is known about their relative contribution to virulence (53). Stx-producing *E. coli* do not produce type I fimbriae, the most common *E. coli* adhesin. Absence of fimbriae expression in EHEC O157 is due to the presence of a 16 base pair deletion within the regulatory region of *fim*A (60).

NON FIMBRIAL ADHESINS

Adhesins are responsible for pathogen binding to intestinal epithelial cells (40). In addition to intimin, other adhesins would support the EHEC carrier state in the bovine intestine. LEE-negative EHEC isolates from ruminants encode an auto-agglutinating adhesin (8, 53) and a novel type IV pilus (51). In non-O157 EHEC strains, a gene known as *efa*1, which encodes a factor of adherence has been identified (51, 53).

E. coli are peritrichous flagellated bacteria that assemble about ten flagella over their cell surface (36).The flagellum consists of three morphologically distinct structures: a basal body, a hook and a helical filament (2, 41, 94). The basal body is embedded in the bacterial cell envelope, whereas the hook and the filament extend beyond the cell surface (94). Flagella provide motility and chemotaxis (41), increase adhesion (20, 41) and are involved in protein translocation (20). Motility is considered a virulence factor that promotes establishment of infection and transmission (36). Flagellin, the structural component of the flagellar filament, also plays a major role in the induction of the host immune responses. Flagellar expression is subject to phase variation and some pathogenic bacteria produce flagellin in order to promote colonization and mucosa invasion (41). In EPEC strains, flagellin and BFP are produced simultaneously during infection. Like BFP, the flagellum is also directly involved in local adherence, contributing to A/E lesion formation (20). Because EPEC flagella are involved in adherence and microcolony formation, it is required a coordinated transcription of the flagellar system and the virulence genes encoded by the LEE, which are the main responsible for the development of A/E lesion (48). In addition to the TTSS, bacteria can use the flagellar system as an additional mechanism for the export of virulence factors (41).

EHEC H7 flagella are composed of flagellin subunits of about 66,000 Da and they have also been demonstrated to be immunogenic (30). Flagellin of EPEC is an important adhesive structure, which is highly induced upon interaction between epithelial cells and, probably, a secreted signaling molecule of eukaryotic origin (20). The authors could not demonstrate that EHEC H7 was able to mediate attachment to epithelial HeLa cells. However, Bretschneider *et al.* (4) demonstrated that *E. coli* O157:H7 strains lacking flagellin expression did not effectively colonize the intestine of adult beef cattle. In addition, animals inoculated with a non-flagellated variant of *E. coli* O157: H7 were more susceptible to a second challenge with a motile *E. coli* O157: H7 than those originally inoculated with motile strains. These findings suggested that this appendage has an essential function in colonization. Later, Mahajan *et al.* (30) confirmed the presence of abundant H7 flagella in physical contact with the terminal rectum epithelial cells, demonstrating that H7 flagellum acts as an adhesin, which is crucial in the initial step of colonization of *E. coli* O157:H7.

Hayashi *et al.* (22) demonstrated that flagellin is the ligand for toll like receptor (TLR)-5. TLR-5 is able to detect both extracellular-luminal and invasive-flagellated pathogenic bacteria (41). Recognition of flagellin by TLR-5 induces the expression of the pro-inflammatory cytokine IL-8, the recruitment of neutrophils to the site of infection, and the activation of dendritic cells (41). Flagellin is a potent stimulator of humoral immune mechanisms, a hallmark of Th2 responses. Moreover, stimulation of an antibody response has been proposed to be related to the direct activation of dendritic cells within the mucosa by flagellin (41). Similar to EPEC, flagella from *E. coli* O157:H7

also induced IL-8 secretion in a colonic epithelial cell line (36). It was confirmed that H7 flagellin proteins, and not the production of Shiga-like toxins, are responsible for the up-regulation of pro-inflammatory chemokine production and sub-epithelial influx of neutrophils (7).

Microcolony formation and adhesion by EPEC depend on the coordinated transcription of flagellum and LEE genes (48). In addition, Girón *et al.* (20) demonstrated that EPEC strains that are mutated in the LEE-encoded genes are impaired in the expression of flagella. This was interpreted by the authors as indication of an association between flagellation, motility and the TTSS. Furthermore, Bretschneider *et al.* (2) found that *E. coli* O157:H7 *tir* deletion mutans were unable to express flagella and suggested a regulatory link between flagellum and Tir expression. Finally, Spears *et al.* (51) suggested that because of the importance of LEE-TTSS in the colonization process and the structural relationship between LEE and flagellar-TTSS, EHEC intestinal colonization is likely to be mediated by a regulatory cross-talk between both systems.

CONTROL OF EHEC INFECTION IN CATTLE

Vaccines should induce high titers of specific IgA at mucosal surfaces to prevent cattle infection and shedding of EHEC into the environment and/or colostral IgG responses for passive protection of neonates (53). During the course of infection, adult cattle are able to mount a serological response against *E. coli* O157:H7-type III-secreted proteins, intimin and LPS (5). Overall, these authors detected a positive association between the pattern of fecal shedding and the serum IgG titers to Tir, intimin or O157 LPS. However, for EspB, the IgG response was robust and persistent throughout infection, irrespective of the level of *E. coli* O157:H7 shedding. A positive relationship between hydrophilicity and immunogenicity was previously shown by Noya *et al.* (34). In this regard, bioinformatics analysis of *E. coli* O157:H7 LEE proteins, revealed the presence of two main hydrophilic domains in the central and carboxy-terminal regions of EspB (3). An occasional and very low mucosal IgG and IgA responses were also detected on mucosal scrapings of terminal colon and rectum of experimentally infected adult cattle. However, it is unclear the role of these antibodies in the protection to the infection (6).

Intimin would be a good candidate for vaccination. Nevertheless, these vaccines would require the incorporation of several intimin subtypes, mainly β, γ, and ε, to protect against the prevalent EHEC serotypes in cattle (53).

A glycoconjugate vaccine containing O157 LPS did not protect against intestinal colonization of mice with *E. coli* O157:H7 (27). Although specific O antibodies are detected in the serum of experimentally infected calves, correlation with bacterial clearance is not observed (24).

Potter *et al.* (39) demonstrated that a vaccine composed of *E. coli* O157:H7 culture supernatant enriched with type III-secreted proteins was effective in reducing intestinal colonization of cattle. Peterson *et al.* (38) determined that a three-dose regimen of this vaccine elicited a significant humoral response to Tir and LPS and reduced *E. coli* O157:H7 colonization of the terminal rectum under natural exposure. Recently, Smith *et al.* (50) concluded that a two-dose regime of the same vaccine was also effective in reducing the probability (92%) of *E. coli* O157:H7 colonization of the terminal rectum of commercially fed cattle at harvest. Type III-secreted proteins are relatively conserved among EHEC serotypes. Therefore, it was proposed that this formulation would have the advantage of providing cross-protective immunity (39). However, preliminary results of a recent study by the same research group suggest that protection, for the most part, is serotype-specific (3).

The use of H7 flagellin, as a component of a systemic vaccine, reduced the colonization rates and delayed peak of O157:H7 shedding in calves; however, it did not affect total bacterial shedding. This immunization effect was correlated with serum and mucosal antibody responses (31). Immunological strategies, targeting H7 flagella, are actually under study (30).

Passive protection by the use of recombinant antibodies directed to EHEC colonization factors in food-producing farm animals has also been proposed as a measure to reduce EHEC intestinal carriage before slaughter (53).

Furthermore, probiotic mixtures have also shown some effectiveness in reducing intestinal colonization of experimentally inoculated young calves and sheep. A two-year clinical trial in a research feedlot with a direct- fed microbial showed that cattle were 35% less likely to shed *E. coli* O157:H7 than untreated cattle (37). The use of lytic bacteriophages and bacteria capable of inhibiting EHEC growth in the gastrointestinal tract might also be useful for the control of EHEC in ruminants prior to slaughter, although the development of genetic resistance poses a problem to the long-term use of these control measurements (53). The USDA Food Safety and Inspection Service has recently approved the use of bacteriophage treatment for *E. coli* O157:H7 in live cattle. Bacteriophages are applied as a mist, spray or wash on live animals prior to slaughter.

Finally, it is important to consider that preharvest intervention strategies, for instance, vaccines against *E. coli* O157:H7 in cattle, must be produced at low cost to be adopted by farmers.

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