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Mini-Review

Boundaries That Prevent or May Lead Animals to be Reservoirs of *Escherichia coli* O104:H4

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ABSTRACT

Escherichia coli O104:H4, a hybrid serotype carrying virulence factors from enteroaggregative (EAEC) and Shiga toxin-producing (STEC) pathotypes, is the reported cause of a multicountry outbreak in 2011. Evaluation of potential routes of human contamination revealed that this strain is a foodborne pathogen. In contrast to STEC strains, whose main reservoir is cattle, serotype O104:H4 has not been commonly isolated from animals or related environments, suggesting an inability to naturally colonize the gut in hosts other than humans. However, contrary to this view, this strain has been shown to colonize the intestines of experimental animals in infectious studies. In this minireview, we provide a systematic summary of reports highlighting potential evolutionary changes that could facilitate the colonization of new reservoirs by these bacteria.

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Commensal *Escherichia coli* is a component of the gut microbiome in warm-blooded animals and humans. However, some strains express virulence genes that cause diarrhea or extraintestinal diseases (Ogura et al., 2009). Based on genotypic and phenotypic traits and clinical infections related to enteric and diarrheal bacteria, the following pathogenic types have been identified: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffuse-adhering *E. coli* (DAEC). Other pathogenic *E. coli* are responsible for extraintestinal infections, such as septicemia, neonatal meningitis, and urological disorders (Nataro and Kaper, 1998).

Diarrheagenic *E. coli* pathotypes have adhesion, invasion, and colonization capacities in the host based on multiple virulence factors including adhesins, host cell surface-modifying factors, invasins,

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toxins, and secretion systems, which determine the ecology of these bacteria, and individually the behavior of each pathotype that commonly affect human health. Some strains of ETEC are associated with animal disease (such as neonatal diarrhea) in farms. Cattle are a common reservoir of EPEC and EHEC, which have been isolated from both symptomatic and asymptomatic animals, while EAEC, DAEC, and EIEC are less likely to be found in animal hosts (Bekal et al., 2020; Kolenda et al., 2015).

EAEC strains are classified into typical and atypical groups based on the presence or absence of *aggR* (a transcriptional activator of aggregative adherence fimbria), respectively. Both typical and atypical strains have been isolated from humans while animal isolates usually correspond to the classified as atypical strains (Okhuysen and DuPont, 2010). The available information on the relationship between human and animal strains is inconclusive and does not confirm whether animals are reservoirs of EAEC. However, typical EAEC strains have been previously isolated from domestic pets in Brazil, such as dogs and cats, supporting the theory that pets serve as possible incidental reservoirs due to their close relationship with humans on a daily basis (Puño-Sarmiento et al., 2013).

EHEC are Shiga toxin-producing *E. coli* (STEC) causing hemorrhagic colitis (HC) and complications such as hemolytic uremic syndrome (HUS) via the production of Stx1 and/or Stx2. Cattle are natural reservoirs of these strains together with other ruminants, such as sheep and goats. Bacterial spread in the environment occurs from feces, leading to the occurrence of interspecies infection through water and/or food consumed by humans. EHEC is also frequently detected in domestic pets (dogs and cats) and wild nondomesticated ruminant species including llamas, alpacas, antelopes, and yaks (Etcheverria et al., 2016).

Evolution of *E. coli* through the genetic mobility of virulence factors carried by these bacteria has led to the emergence of new strains containing genes from different pathotypes. In 2011, a multicountry outbreak of *E. coli* causing high levels of HUS was reported, which originated from the highly virulent hybrid strain O104:H4 (Muniesa et al., 2012). This Shiga toxin-producing (STEC/EHEC) strain possesses an aggregative adherence plasmid (pAA) characteristic of the enteroaggregative *E. coli* (EAEC) pathotype as well as a plasmid encoding an extended-spectrum β-lactamase (ESBL). At time of the outbreak, humans were considered the only reservoir of this serotype, as a result of never being reported in animals and food (European Centre for Disease Prevention and Control and European Food Safety Authority, 2011).

In this review, we provide a summary of scientific literature related to *E. coli* O104:H4 with focus on its physiological characteristics, its ability to infect the intestine of animal models, its epidemiologic surveillance, and ecological preferences, that prevent or may lead animals to be reservoirs of *Escherichia coli* O104:H4.

Epidemiological and clinical implications caused by *E. coli* O104:H4

From May to June 2011, more than 800 patients were diagnosed with HUS. That was the result of an outbreak caused by *E. coli* O104:H4 that began in Germany and ultimately developed into an epidemic affecting 3842 individuals (Bielaszewska et al., 2011).

During the outbreak, sick patients were interviewed to establish their food consumption habits, travel history, and contact with other people or animals with diarrhea (Gault et al., 2011). Following comprehensive analysis of the possible routes of bacterial transmission, it was concluded that fenugreek sprouts were the main vehicle of infection (Buchholz et al., 2011). Contact with infected individuals (human-to-human transmission) was identified as a secondary contamination route.

Analysis of this outbreak revealed unique characteristics as compared to previous EHEC outbreaks; adults were mainly affected (88%; median age, 42 years), in particular, women (68%); humans were identified as the sole reservoir (Beutin and Martin, 2012; Frank et al., 2011) and HUS development was increased in patients (25%). Although only 90 of the affected cases were children, this event was recorded as the largest known pediatric HUS epidemic (Derad et al., 2016; Kemper, 2012).

The unusual epidemiological behavior is related to the combined characteristics of *E. coli* O104:H4 serotype, where its aggregative adhesion properties increased the internalization of Shiga toxin 2 by the intestinal epithelium. This results in high rates of complications, such as hemolytic uremic syndrome (HUS) with thrombocytopenia, fragmentation of red blood cells, and acute brain and kidney injury in infected patients (Frank et al., 2011; Navarro-Garcia, 2014).

The overall symptoms were highly variable. However, a median of 5 days was estimated from the onset of diarrhea to the development of kidney complications. It is known that up to 10% of patients with HUS go on to develop renal sequelae, among which 4% are diagnosed as long-term kidney disease and 12% progress to end-stage kidney disease or death (Borgatta et al., 2012). Also, coinfections with *Clostridium difficile* or norovirus and neurologic problems were frequently detected (Ullrich et al., 2013).

Routes of *E. coli* O104:H4 contamination

Although contaminated food is the primary vehicle for transmission of STEC strains, during the 2011 outbreak, *E. coli* O104:H4 was only isolated from clinical samples and not from animals or suspected foods (Gault et al., 2011). However, Buchholz et al. (Buchholz et al., 2011) reported that 25% of the HUS patients had eaten sprouts and cucumbers had been consumed by 88% of the patients (King et al., 2012) . Similarly, in another study on a group of individuals who had visited a specific restaurant, 20% developed symptoms of infection and all cases were correlated with the consumption of sprouts. Furthermore, the same food was similarly identified as the potential vehicle of infection in reported cases from France.

The sprouts were produced from fenugreek seeds imported from Egypt to Germany, and from there, it was distributed to France, Austria, Spain, and the United Kingdom. Although some lots of these fenugreek seeds were analyzed, *E. coli* O104:H4 could not be isolated from these samples (European Food Safety Authority., 2011). However, Xiao et al. (Xiao et al., 2014) demonstrated the ability of *E. coli* O157:H7 and O104:H4 strains to grow rapidly during sprouting, reaching levels of up to 8.1 log CFU/g in five days.

Notably, *E. coli* O104:H4 is typically an EAEC strain, for which humans are the only known reservoirs (Beutin and Martin , 2012). However, the strain also produces Shiga toxin, generated by strains of the EHEC pathotype, which can utilize both domestic and wild animals (in particular, ruminants) as hosts (Caprioli et al., 2005). Also, EHEC has been detected in cattle feces (Karmali et al., 2010), which are likely sources for contamination of food and water (Ferens and Hovde, 2011). These multiple properties warrant consideration of other possible transmission routes for *E. coli* O104:H4.

Animals colonized by *E. coli* O104:H4

Despite significant focus on the detection of this pathogen in the environment (Cabal et al., 2015; Paddock et al., 2013), very few studies have been conducted in pets and production animals to date. Intriguingly, although several *in vivo* studies on animal models (mice, rabbits, sheep, and calves) indicate the ability of *E. coli* O104:H4 to colonize their gastrointestinal tracts (GITs), this strain has not been detected in animals (Auvray et al., 2012; Wieler et al., 2011) or livestock settings (Fig. 1).

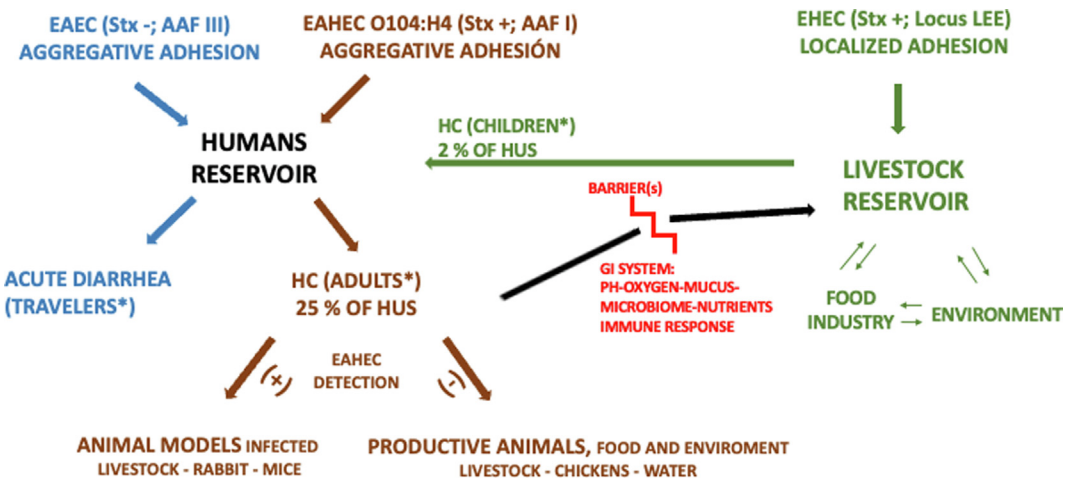

Figure 1. Diagram showing the epidemiological behavior of enteroaggregative *E. coli* (EAEC [blue]), enterohemorrhagic *E. coli* (EHEC [green]), and the hybrid strain *E. coli* O104:H4 (EAHEC [brown]). * = individuals most affected; HC = hemorrhagic colitis; HUS = hemolytic uremic syndrome; stx2a = shiga toxin 2a gene; AAF = adherence aggregative fimbria. GI = gastrointestinal.

The wide genomic plasticity of *E. coli* supports the extraordinary ecological possibility of adaptation to different environments (Etcheverria et al., 2016). However, it remains unclear why the O104:H4 strain, with a high rate of recombination and genetic exchange, cannot colonize new environmental niches or hosts naturally. One possible explanation is that this strain, as observed for other microorganisms, is highly sensitive to fluctuations in conditions such as pH, microbiome (Ribeiro et al., 2019), tissue oxygenation (Ventura et al., 2012), chemical compounds (Aurass et al., 2011), and immunological conditions of the hosts, which may alter the crucial interactions that maintain the functionality of biomolecules, thus modifying their cellular integrity (Stalb et al., 2018).

Quantitative polymerase chain reaction (qPCR) directly targeting virulence genes from fecal samples is the method generally used for the detection of *E. coli* O104:H4 (Guy et al., 2014). A culture-based approach followed by molecular testing has also been widely employed (Cabal et al., 2013). The implementation of similar protocols together with the analysis of potentially risky geographic sites as sources of contamination support the epidemiological research to date (Weiser et al., 2013). These studies have shown that cattle harbor *E. coli* O104 serotypes (other than H4) in the intestine, which are ultimately discharged in the feces (Rump et al., 2012). In 1994, the O104:H21 serotype (carrier of the *stx2* gene) was implicated in an outbreak in Montana (USA) associated with the consumption of raw milk (Feng et al., 2001), leading to the speculation that cattle were the source of contamination.

Given the finding that *E. coli* O104:H4 has the ability to colonize the intestines of animals under controlled laboratory conditions, but not in those intended for production, we have reviewed the current knowledge on available animal models and environmental detection reports and put forward hypotheses to explain why these bacteria cannot cross the human/animal barrier.

EHEC and EAEC adhesion and colonization processes

Bacterial colonization initially depends on interactions with gastrointestinal glycan structures on the host glycocalyx at the site of animal- or human-specific mucin oligosaccharide formation, providing attachment sites for microorganisms via adhesins, which leads to lowering of the critical defense barriers of the host (Quintana-Hayashi et al., 2018).

EHEC strains located in the small intestine activate the expression of virulence genes and attach to epithelial cells, causing flattening of the microvilli (attaching and effacing phenomenon). This event is mediated by interactions of intimin, a bacterial outer membrane protein, and its receptor Tir (translocated intimin receptor). Tir and other effector proteins are delivered into host cells through the type III secretion system T3SS (Prager et al., 2014; Saeedi et al., 2017). Bacteria induce localized adherence through actin rearrangement of enterocytes and formation of pedestals (Franzin and Sircili, 2015).

On the contrary, EAEC strains carry aggregative adherence fimbriae (AAF, 5 different subtypes) encoded by the pAA plasmid, capable of mediating *in vitro* aggregative adherence in a "stacked-brick" pattern (Karch et al., 2012). The pathogenicity of this pathotype has been extensively explored in cell lines, animal models, and human volunteers (Huang et al., 2004; Nataro et al., 1995; Roche et al., 2010). Strains possessing these genetic traits are known to colonize the gastrointestinal tract and cause diarrhea, along with mild histopathological changes. *In vivo*, the bacterium penetrates the mucus barrier that overlays intestinal cells to reach the unstirred layer (a region approximately 50 μm above the mucosal surface) and the epithelium itself (Hicks et al., 1996; Strocchi and Levitt, 1991). Based on this typical colonization process, *E. coli* O104:H4 was characterized as EAEC expressing AAF/I responsible for the characteristic stacked-brick pattern of adherence. However, *E. coli* O104:H4 also produces Shiga toxin with a more complex adhesion process than that observed for EAEC strains, conferring stronger adherence to host cells due to the presence of AAF/I (Schiller et al., 2021).

In terms of virulence, the Stx phage-cured *E. coli* O104:H4 showed 3–6 times greater aggregative adherence to HCT-8 epithelial cells relative to EAEC strains (AAF/III). This superior aggregative adhesion by AAF/I results in a greater systemic absorption of Stx, thus in a high prevalence of HUS (Haarmann et al., 2018).

Animal models to study the intestinal colonization of *E. coli* O104:H4

Various animals have been used as models to mimic the asymptomatic intestinal carriage of *E. coli* O104:H4 in humans (Maura et al., 2012). The vast majority of models have utilized young or microorganism-free animals (Table 1). Studies in the literature on animal colonization by *E. coli* O104:H4 are described below.

Mice. BALB/c mouse intestine could be effectively colonized by *E. coli* O104:H4 strain C227-11. In that model, the animal excreted high levels of bacteria in feces (up to 10^9 CFU/g) for at least 3 weeks. In addition to localization in the intestinal lumen, the bacteria formed

Table 1
In vivo animal models colonization by *E. coli* O104:H4

Animal species	*E. coli* strains	Oral inoculation	Strategy for efficiency of colonization (oral consumption)	Response observed	Reference
7-week-old female mice BALB/c	55989Str (Stx-, AAF/III)	10^6 CFU/animal	Streptomycin sulfate (5 g/mL) in drinking water for 2 days BIno.	Recovery of bacteria from small (10^8 CFU/g) and large (10^9 CFU/g) intestine (respectively), forming aggregates on the surfaces of intestinal epithelial cells.	(Maura et al., 2012)
C57BL/6 and BABL/c mice	C227-11 (Stx+, AAF/I)	10^9 CFU/C57BL mice 10^{11} CFU/BABL/c mice	Ampicillin (5 g/liter) in drinking water for 2 days and 12 h BInf and 4 h PInf.	BABL/c mice treated with the antibiotic in water 2 days PInf, showed increased levels of bacteria in intestine.	(Zangari et al., 2013)
C57BL/6 mice	C227-11 and C227-11 ΔpAA (Stx+; without AAF/I)	10^9 CFU/animal	Ampicillin (5 g/liter) in drinking water 12 h BInf	Mice fed the ΔpAA strain maintained viability and weight compared with the wild-type strain.	(Boisen et al., 2019)
BALB/c mice	LB226692 (Stx+, AAF/I)	10^3 CFU/animal	Streptomycin sulfate (5 g/mL) in drinking water for 5 days BInf along with Mitomycin (0.75 mg/Kg) PInf	Progressive weight loss, clinical symptoms, and death.	(Secher et al., 2015)
Germ-free Swiss-Webster mice	TW16133 (Stx+, AAF/I)	8 x10^6 CFU/animal	-	High cecal colonization density, but no signs of renal acute tubular necrosis ATN.	(Safadi et al., 2012)
BALB/cYJ mice	55989Str	10^6 CFU in 200 mL/animal	Streptomycin sulfate (5 g/mL) in drinking water for 2 days BIno	Colonized mouse intestine excreted up to10^9 CFU/g in feces for at least 3 weeks.	(Maura et al., 2012)
New Zealand White infant rabbits	C227-11	10^9 CFU/animal	Ranitidine intraperitoneally (2 mg/g) BIno	Diarrhea and/or intestinal fluid accumulation by 3 days PIno.	(Munera et al., 2014)
Specific-pathogen-free 3-4 day-old New Zealand White rabbits	BL211 (Stx-; AAF/I)	10^9 CFU/animal	Ranitidine intraperitoneally (5 mg/Kg) and 2 h BInf	Colonization was significantly reduced in ileum and cecum	(Giles et al., 2018)
6 week-old weaned lambs	BL211	5 x10^9 CFU/animal	-	Low bacterial levels were recovered from intestine at 4 days PInf.	(Giles et al., 2018)
Cattle	LB226692	10^{10} CFU intrarumen/animal	-	The strain was recovered until 24 days PIno. At day 4 PIno, bacteria were isolated from intestinal contents and associated with intestinal mucosa.	(Hamm et al., 2016)

EAEC 55989Str = spontaneous streptomycin-resistant derivative from the 55989 strain; *E. coli* O104:H4 str. C227-11 = isolated from a German patient in Denmark during outbreak; C227-11 ΔpAA = plasmid pAA cured; *E. coli* O104:H4 str. LB226692 = isolated from a patient during the outbreak in Germany; *E. coli* O104:H4 str. TW16133 = isolated from a patient in Michigan, EUA; *E. coli* O104:H4 str. BL211 = Stx2 deletion mutant of strain C227-11; PIno = postinoculation; PInf = postinfection; BIno = before inoculation; Binf = before infection.

aggregates on the surfaces of intestinal epithelial cells. However, to obtain a good infection rate, administration of water with ampicillin postinoculation (PIno) was necessary (Zangari et al., 2013). Treatment with this antibiotic induced an increase of inoculated bacteria 2 days postinfection (PInf). On day 4, mice lost >5% weight, along with an increase in morbidity and mortality rates. In contrast, untreated mice showed intact commensal flora PInf, gained weight, remained healthy, and apparently remained unaffected by the strain.

Higher infection rates in mice were observed with antibiotic-resistant *E. coli* O104:H4. Secher et al. (Secher et al., 2015) used a streptomycin-resistant O104:H4 strain (LB226692) to infect pathogen-free BALB/c mice that were treated with streptomycin sulfate (5 g/mL) and mitomycin C (MMC, 0.75 mg/kg) 5 days before infection (BInf) and during the experiment. Progressive loss of body weight was observed ($P < 0.05$) along with high mortality rates, while the control (untreated with MMC) group showed few symptoms. Mitomycin C administered PInf or streptomycin sulfate (5 days BInf and PInf) additionally caused loss of weight, development of clinical symptoms, and death (Boisen et al., 2019; Maura et al., 2012). Similarly, a streptomycin-treated mouse model was applied in a separate study to demonstrate *in vivo* colonization of a bioluminescent strain of *E. coli* O104:H4. The results obtained indicate that the bacterium persistently colonizes the murine cecum over time (Torres et al., 2012).

To study the infectious process of *E. coli* O104:H4 *in vivo*, a plasmid-cured *E. coli* O104:H4 strain (C227-11, without pAA plasmid) was used to infect BALB/c mice. Plasmid pAA encodes the transcriptional activator AggR, which has a regulatory effect over around 20 genes incorporated by this plasmid, including the expression and function of aggregative adherence fimbriae, and it is supposed to regulate the chromosomal loci AAI type VI secretion cluster. Animals infected with the wild-type strain showed greater weight loss after 21 and 28 days, although similar mortality rates were observed for both wild-type and mutant strain-infected groups, suggesting that genes encoded on the pAA plasmid primarily play a role in morbidity. This effect may be exacerbated by the activity of Stx2a, which is released to a greater extent in wild-type strains according to results obtained from the evaluation of systemic effects. This finding suggests possible regulatory effects of pAA plasmid on Stx2 to enhance its delivery *in vivo* (Boisen et al., 2019).

Germ-free Swiss-Webster mice could also be colonized by *E. coli* O104:H4, consequently triggering kidney damage. In contrast to other protocols, mice remained bacterium-free until inoculation (10^6 CFU) with EHEC O157:H7 or O104:H4. Following euthanasia at 7 days postinoculation, higher cecal colonization density and bacterial aggregation were observed in the O104:H4-infected group, probably representing an early stage of a biofilm formation *in vivo* (PrüB et al., 2006), which was absent in O157:H7-infected mice. Interestingly, the latter group showed reduced renal function and acute renal tubular necrosis (ATN) while mice infected with *E. coli* O104:H4 with greater cecal colonization did not display signs of ATN (Safadi et al., 2012). Gene expression related to biofilm formation in cecum colonized by O104:H4 was consistent with reduced expression of *pgaA* (encoding PGA protein, the main component of the self-produced extracellular matrix in biofilm) and diguanylate cyclase (producing the biofilm-promoting second messenger c-di-GMP related to host inflammation). These findings indicate that low levels of exopolysaccharide with pro-inflammatory activity are insufficient to facilitate Stx systemic internalization and kidney damage (Richter et al., 2014; Wang et al., 2004).

In these earlier experiments, two additional groups of mice were infected with *E. coli* O157:H7 and O104:H4 for 15 days. Longer infection periods with *E. coli* O104:H4 resulted in 80% of cases developing ATN, increased severity of kidney injury related to *pgaA* and c-di-GMP overexpression producing an extensive biofilm, and potential inflammation in the cecum at day 15 PIno (Richter et al., 2014; Safadi et al., 2012), which induced paracellular pathways for Stx2 internalization. This finding could explain why despite overexpression of *stx2* in the first week of PIno, no signs of ATN in the kidney were observed (Ibarra et al., 2013).

Rabbits. *E. coli* O104:H4 can effectively colonize newborn New Zealand white rabbits, leading to diarrhea and/or intestinal fluid accumulation on day 3 PIno (Munera et al., 2014). In view of the controversy regarding the contribution of plasmid encoding beta-lactamases (pESLB; 88.5 Kb) to the pathophysiology of *E. coli* O104:H4, Giles et al. (Giles et al., 2018) examined its relevance in intestinal colonization in rabbits. The group identified a pESLB cured derivative of this strain, which displayed significantly increased adhesion in the ileum and cecum (160- and 215-fold, respectively) after 7 days of PInf, compared with its wild-type counterpart, suggesting that pESLB affects the fitness of *E. coli* O104:H4 during colonization and hinders long-term persistence of the bacterium, particularly in the upper regions of rabbit intestine.

Livestock. Lambs can be colonized by *E. coli* O104:H4 with no symptoms of disease or effects on intestinal tissues, similar to a reservoir host. However, unlike the rabbit model, upon infection with bacteria carrying ESBL-plasmid, greater magnitude and duration of fecal shedding (>10 CFU/g/40 days) were observed, compared to the derivative lacking the pESBL plasmid (>10 CFU/g/12 days and <10 CFU/g/13-35 days), suggesting that species-specific differences in hosts, such as gastrointestinal tract, carbon, and energy source, could mitigate the requirement for particular bacterial factors and relative need for specific genes (Giles et al., 2018).

E. coli O104:H4 can also colonize calves. The most important aspect for public health is that after intraruminal inoculation (10^{10} CFU), bacteria are shed in feces at levels equivalent to those of EHEC O157:H7 (used as the positive control) and decrease to below the detection limit by day 28 (Hamm et al., 2016).

Epizootiological screening for *E. coli* O104:H4 in an agronomic environment

During the *E. coli* O104:H4 outbreak in Germany, the potential colonization of ruminants by the bacterium was investigated (Table 2). *E. coli* colonies isolated from fecal samples obtained from farms in close proximity to the outbreak were analyzed for the presence of genes associated with O104:H4 (*stx2, terD, rfbO104, fliCH4*), STEC (*stx1, stx2, escV*), and EAEC (*pAA, aggR, astA*). Twenty-seven among 2000 colonies exhibiting some similarity (ESBL phenotype) to the Germany outbreak strain were analyzed for the aggregation pattern in HEp-2 cells. Genes belonging to STEC were detected in 28% of the animals. However, *E. coli* O104:H4 genes (*stx2, terD, rfbO104*, and *fliCH4*) and those belonging to the EAEC pathotype or associated with aggregation pattern were not detected in any of the samples (Wieler et al., 2011).

In another study, fecal samples from cattle from various slaughterhouses were analyzed for the simultaneous presence of four genes detected in *E. coli* O104:H4 (*stx2*, *wzxO104*, *fliCH4,* and *aggR*). None of the 1468 samples contained all four genes concurrently, clearly indicating an absence or limited quantities of bacteria in the animals, although the incidence of the genes *stx2* (66.8%), *stx2/wzxO104* (21.7%), and *stx2/wzxO104/fliCH4* (6.1%) suggests that this bacterium may be circulating in these environments (Auvray et al., 2012).

Similarly, Cabal et al. (2015) studied the distribution of virulence genes of *E. coli* O104:H4 in cattle from various areas of Germany and Spain three years after the German outbreak. The surveillance study failed to detect isolates carrying the combinations of genes representative of this serotype. Another study conducted in the Midwest of the United States, which analyzed the presence of eight typical genes (*stx1, stx2, terD, eae, wzxO104, fliCH4, ehxA*, and *aggA*) for detection of serotype O104:H4 with EHEC and/or EAEC traits from cattle feces showed that 15% samples harbored the *wzxO104/fliCH4/stx2* gene combination while none carried *aggA* encoding the major AAF/

Table 2
PCR detection of genetic markers of *E. coli* O104:H4 in fecal samples from different origins

Country	Sample origin	Fecal samples	Method	Genetic markers evaluated	Genes detected	Reference
Germany	34 cattle German farms (100 animals)	100	2,000 *E. coli* colonies screened	O104:H4 (*stx2, terD, rfbO104, fliC H4*) STEC (*stx1, stx2, escV*) EAEC (*pAA, aggR, astA*)	28% were STEC 0% had the combination of genes specific for O104:H4	(Wieler et al., 2011)
France	Cattle Abattoirs (1,468 animals)	1,468	Enrichment and bacterial DNA extraction	O104:H4 (*stx2, wzxO104, fliCH4, aggR*)	66.8% *stx2* 21.7% *stx2/wzxO104* 6.1% *stx2/wzxO104/fliCH4*	(Auvray et al., 2012)
Germany and Spain	Germany, 6 Cattle Abattoir (640 animals) Spain, 15 Cattle Abattoir (330 animals)	Pooled into 134 samples according to origin	Bacterial DNA extraction	O104:H4 (*stx2, wzxO104, fliCH4, aggR*)	*stx2/aggR/wzxO104/fliCH4* were simultaneously detected in 6 fecal pools from one German abattoir No isolates harboring the full combination were cultured	(Cabal et al., 2015)
USA (Midwest)	8 Feedlots located in the Midwest	248	Bacterial DNA extraction with or without enrichment	O104:H4 *(stx2, terD, wzxO104, fliCH4, aggA)* STEC (*stx1* and *eae)*	20.6% O104 Serogroup specific gene 15% *wzxO104/fliCH4/stx2* 0% *aggA*	(Paddock et al., 2013)
Vietnam (southern)	Chicken farms	204 (chicken feces) 204 (farmers feces)	205 *E. coli* colonies screened	O104:H4 (*stx2, aggR, wzxO104, fliCH4*)	Chicken fecal samples: 0.5% *aggR* Human fecal samples: 0.4% *stx2* 6.8% *aggR*	(Trung et al., 2016)
Nigeria (Zaria)	Abattoir	200	37 *E. coli* colonies screened	O104:H4 (*stx2, aggR)*	5.4% *stx2*	(Kabiru et al., 2015)

pAA = aggregative adherence plasmid.
[1]The simultaneous presence of gene markets of *E. coli* O104:H4 was not confirmed in the above investigations.
[2]Genes (encoded protein)*: stx1* (Shiga toxin 1); *stx2* (Shiga toxin 2); *terD* (tellurite resistance); *eae* (intimin); *wzxO104* (O104-specific O-antigen flippase); *fliCH4* (H4-specific flagellum); *aggR* (transcriptional activator); *aggA* (pilin subunit of aggregative adherence fimbria 1, AAF/1).

I protein in EAEC, leading to the conclusion that none of the *E. coli* isolates matched the O104:H4 serotype (Paddock et al., 2013).

The lack of *E. coli* O104:H4 in feces of cattle does not necessarily eliminate potential hazard risk. Using an FDA - *E. coli* Identification DNA microarray (ECID), Shridhar et al. (Shridhar et al., 2018) examined gene contents, with the aim of assessing the virulence potential of bovine O104:H7 strains compared with genes identified from human serotypes (O104:H4, O104:H7, and O104:H21) isolated from fecal samples. These experiments disclosed that bovine O104:H7 strains harbor genes from STEC pathotypes (*stx1c, ehxA, terD, iha, fimH, and lpfA)* but are negative for *eae* and type III secretion system-related genes. As expected, a number of *E. coli* O104:H7 isolates from cattle and humans were closely related to each other but distinct from *E. coli* O104:H4 (Germany outbreak strain) and did not carry the *stx2* gene.

While detection of this strain in cattle has been a research priority, other animals have additionally been evaluated as possible reservoirs. In southern Vietnam, analysis of *E. coli* O104:H4 in poultry farms (chicken feces, farmers, and individuals not exposed to poultry farming) led to the detection of 6.8% isolates containing the *aggR* gene. However, none of the isolates simultaneously contained *stx2/wzxO104/fliCH4 (80)*. In addition, among water, vegetable, and slaughtered animal samples from Nigeria were confirmed 152 *E. coli* isolates, from which only two isolates from cattle harbored *Stx2* and no additional genes from *E. coli* O104:H4 were detected (Kabiru et al., 2015).

Could *E. coli* O104: H4 transmit from animals to humans?

Anatomical, physiological, and biochemical barriers of the host. Clarification of the anatomical, physiological, and biochemical differences in the gastrointestinal tracts of different animal species may provide insights into the factors underlying the limitations in bacterial colonization of species other than humans (Kararli, 1995; Quintana-Hayashi et al., 2018). For successful colonization of the gastrointestinal system of animals by *E. coli* O104:H4, several biological, biochemical, and physical barriers must be overcome. However, little information is currently available on the limitations of this serotype that impede colonization. In this case, the O157:H7 serotype has been shown to grow in the presence of crude bovine intestinal mucus and utilize mannose and galactose (mucin carbohydrates) as the carbon source, providing a growth advantage for EHEC due to higher carbohydrate catabolism and faster utilization of glycans than commensal bacteria. In contrast, crude bovine intestinal mucus has little or unreported benefits for the growth of *E. coli* O104:H4 to date (Bertin et al., 2013).

Intestinal environments are different among animal species, acting as a reservoir regulated by chemical and physical factors that affect adherence to the mucosa, and serve as a complex barrier to bacterial colonization (Fetissov, 2017). The intestinal microbiome is a diverse community of more than 100 trillion microorganisms that influence mucosal and systemic functions through the production of metabolites and virulence factors as well as interactions with other members of the microbiota (Mondot et al., 2013). For instance, an earlier study showed that interactions of *E. coli* O104:H4 with the intestinal microbiome of infected mice induced an increase in the population of *Firmicutes* after 5 days postinfection. In contrast, groups infected with EAEC did not display significant changes in microbiome populations, suggesting that the O104:H4 strain causes alterations in the microbiotal balance, even expressing Stx2a during the process (Ribeiro et al., 2019).

Other factors, such as pH, bile, pancreatic juice, and mucus, additionally interfere with the colonization process. The increase in tissue oxygenation (via hyperbaric oxygen) causes the greater diffusion of oxygen from the intestinal tissue, altering the microbiome of mice (Albenberg et al., 2014). However, humans contain a higher proportion of oxygen-tolerant organisms than mice (Ventura et al., 2012).

The intestinal binding site for enteric pathogens varies, depending on the following: 1) receptor expression by cells of target tissues, 2) quantitative and structural differences in carbohydrate side chains of

glycoproteins in intestinal epithelial cells, and 3) degree of cellular differentiation and maturation, which can cause changes in receptor concentrations (Deplancke and Gaskins, 2001), among other factors. These factors are crucial at the binding site (globotriaosylceramide GB3 is a membrane glycolipid receptor of the B subunit of Stx) of STEC, in particular for serotype O104:H4, which also expresses the aggregative adherence fimbriae that influence bacterial adhesion (Kang et al., 2001). The anti-aggregation protein dispersin (that binds noncovalently to the lipolysaccharide of the outer membrane), AggR protein, Pic protein (a mucinase with lectin-like activity that induces the production of mucus in the host intestine), and the presence of autotransporters (SepA, SigA) are other factors that promote colonization of *E. coli* O104:H4 in the gastrointestinal tract of hosts (Navarro-Garcia, 2014; Rasko et al., 2011).

Interestingly, Pic, and AggR (which is encoded by the aggregative adherence plasmid) proteins characteristics of EAEC interact with the immunologic system of the host, playing an important role in bacterial pathological processes. Pic was initially shown to be related to mucinolytic activity *in vivo*, but subsequent studies suggest that the protein stimulates rapid mucus secretion related to different host intrinsic stimuli, such as interleukins (IL), prostaglandin E2, acetylcholine, histamine, and neurotensin (Navarro-Garcia et al., 2010). Similarly, AggR facilitates coexpression of human IL-8 as a host immune response, as evident from the increased IL-8 release in feces of patients with diarrhea infected with aggR-positive strains of EAEC (Jiang et al., 2002). Interestingly, an earlier study by Sugawara et al. (1995) developed in a 48-well modified Boyden chamber (quimiotaxis test for neutrophils extracted from blood in response to recombinant IL-8) evaluated the main function of IL-8 (a neutrophil chemoattractant) from humans, monkeys, dogs, rabbits, rats, hamsters, and mice, and revealed weak chemoattraction of neutrophils by IL-8 in all animal species, except humans and monkeys. This finding suggests that AggR, which induces human IL-8 and promotes hypersecretion of mucus, improves intestinal inflammation and consequently, EAEC colonization (Harrington et al., 2005).

In a separate study, Stalb et al. (2018) analyzed the adhesion of *E. coli* O104:H4 to jejunal and colonic intestinal epithelial cells (IECs) of human and bovine subjects. Their experiments demonstrated the affinity of *E. coli* for human IEC owing to strong interactions through flagella and AAF/I. In comparison, adhesion to bovine FKD-R 971 cells was sporadic but the strain exhibited the aggregative adherence phenotype that is also characteristic of human-associated EAEC strains. Although this finding supports the possibility of human host adaptation, induction of IL-6 in bovine IEC, potentially indicative of pro-inflammatory activity in the intestine, may lead to a lower likelihood of bacterial colonization *in vivo*. However, EHEC strains capable of colonization of cattle showed limited pro-inflammatory activity. Although both IL-8 and IL-6 are pro-inflammatory cytokines, IL-8 is also a powerful neutrophil chemotactic factor that regulates the production of adhesion molecules in host cells and bioactive lipids and amplifies local inflammation (Jacobi et al., 1998) functions that may facilitate EAEC colonization in humans.

Bacterial advantages and limitations. The acidity of gastric juices provides a line of defense against foodborne pathogens in many animal species. However, diarrheagenic *E. coli* strains have developed an adaptative acid tolerance response through activation of the VisP protein. At pH 3.0 and 7.2, O104:H4 did not display significant differences in *visP* expression. However, EAEC 042-Stx^{-} showed overexpression of this gene at low pH (3.0), supporting the involvement of VisP in the acid response in EAEC (Ribeiro et al., 2019).

EHEC O157:H7 can also survive in the gastrointestinal tract of ruminants through a different and potentially more efficient pH homeostasis system (Stevens et al., 2002). These adaptive capacities are supported by the following: 1) the buffering capacity of the bacterial cytoplasm, 2) low permeability of protons, and 3) extrusion of protons from the cytoplasm by means of a membrane-bound proton pump (Benjamin and Datta, 1995). Recently, Hu et al. (Hu et al., 2017) identified Asr protein as a regulator of acid resistance in EHEC. Furthermore, cadAB and AhpC (mediators of the lysine decarboxylation and oxidation-reduction processes, respectively) were reported to consume additional protons while maintaining a neutral pH value in the cytoplasm. However, limited information is available on the expression of Asr in *E. coli* O104:H4.

E. coli O104:H4 is a relatively sensitive serotype that cannot withstand environments poor in nutrients or toxic concentrations of chemical compounds. Under these circumstances, a viable but nonculturable stage (VBNC) develops, which complicates its detection from suspected sources when using culture-based methods (Bloch et al., 2012; Muniesa et al., 2012). In addition, the presence of copper ions (0.9% saline + Cu^{2}) or chlorine dioxide (from treated water) has been shown to cause loss of cultivability in just 3 days. Bacterial resuscitation is a more complicated process. To restore VBNC *E. coli* O104:H4 induced by the stress of copper ions to the viable stage, repeated washing with cold EDTA is necessary, suggesting that the existence and resuscitation of the VBNC stage in O104:H4 is a complex process and could negatively affect pathogen detection in animal, environmental, and food samples (Aurass et al., 2011).

Levels of protein in the host diet can affect colonization by *E. coli* O104:H4 (Roche et al., 2010; Zangari et al., 2013). C57BL/6 mice were inoculated with *E. coli* O104:H4 strain C227-11 and treated with a low protein diet (LPD) or water plus ampicillin (5 g/L) at 2 days PInf. Weight loss and mortality were more prevalent in animals treated with water plus ampicillin (3/5) compared to those treated with LPD (1/5). Reduction in the pathogenicity of *E. coli* O104:H4 on day 4 PInf was correlated with the bacterial count in feces. Lower counts (10^{4} CFU/g) were observed when animals were treated with LPD, in comparison with those treated with water plus ampicillin (10^{9} CFU/g, *89)*. In another study, when mice were treated with LPD 10 days prior to the bacterial inoculation, an increased intensity of EAEC infection in mice (1–4 logs increase for fecal shedding of organisms) was observed, when compared with control (normal diet, *67)*.

The pAA plasmid, a relatively unstable genetic element of *E. coli* O104:H4, is lost during disease in humans and mice and upon asymptomatic colonization in calves, resulting in a change in bacterial aggregation pattern from well-defined "stacked bricks" to individual bacilli or small groups consisting of 3–10 bacteria. The instability of pAA has clinical, diagnostic, epidemiologic, and evolutionary implications (Zangari et al., 2013; Zhang et al., 2013). Furthermore, acquisition by *E. coli* O104:H4 of the *astA* gene encoding heat-stable enteroaggregative *Escherichia coli* enterotoxin 1 (EAST1) in calves has recently been reported (Hamm et al., 2016).

Bacterial survival and colonization among EHEC, EAEC, and/or EAHEC O104:H4. According to the virulence factors of EAHEC O104:H4, it has been characterized as EAEC which acquired phage Stx2 from EHEC (Rasko et al., 2011). A Mauve progressive synteny, analyzing the chromosome of the strains EAEC 55989 and EHEC TW14359, showed that the EAHEC O104:H4 genome presents orthologous blocks with EHEC and EAEC, which align on the complementary strand (Darling et al., 2010). Two clusters related to the metabolism of mercury and copper which encoded CusF (CuI efflux system periplasmic) and PcoC (copper-resistant) proteins were also detected (Table 3), suggesting a possible link with Cu^{+}-induced VBNC reported in EAHEC (Aurass et al., 2011; Islam et al., 2016). This can make the bacterium difficult to detect in the environment.

Adhesion to eukaryotic cells is a highly differentiated virulence factor among the pathotypes analyzed. Although EAEC and EAHEC present aggregative adhesion due to the presence of pAA (which encoded AggR, AAF/I, and dispersin), Muniesa et al. (Muniesa et al., 2012) observed that this plasmid is not necessary for intestinal colonization. *E. coli* O104:H4 ΔpAA was able to infect infant rabbits showing a robust colonization due to the presence of chromosome-encoded autotransporters such as Pic and SigA. In contrast, EHEC uses intimin,

Table 3
Extracellular proteins encoded from unique and shared regions of EHEC and EAHEC chromosome identified by tandem mass spectrometry (Islam et al., 2016).

Strains	Group	Protein function
EHEC O157:H7-specific proteins	Membrane proteins	IpgB2 (BfpT-regulated chaperone); OmpT (omptin); LpxR (lipid A 3-O-deacylase); Tir (translocated intimin receptor); Map (LEE-encoded effector); CesT (Tir chaperone); EspJ (non–LEE-encoded effector protein); LolA (lipoprotein carrier); EaeA (intimin)
	T3SS effector proteins	Esp [EspB, EspF, EspJ]; Nle [NleA, NleB, NleC]; Tir (Translocated proteins) and Intimin (adhesin)
EAHEC O104:H4-specific proteins	Membrane proteins	HchA (molecular chaperone), FyuA (pesticin/yersiniabactin receptor); NmpC (outer membrane porin protein LC); FimC (fimbrial chaperone protein); Fimbrial subunit AAF/1
	Resistance	β-Lactamase class A CTX-M; β-Lactamase class A TEM; TerD (tellurium resistance protein); EDTA (resistant nuclease)
	Mercury and copper Metabolism cluster	CusF (Cu[I]/Ag[I] efflux system periplasmic protein); PcoC (copper-resistant protein)
EHEC and EAHEC shared proteins	Membrane proteins[1]	FepA (receptor), TolC (protein), BamC (assembly factor); Ycel (polyisoprenoid-binding periplasmic protein); SlyB (lipoprotein); OmpX (protein)
	Iron-binding proteins[1]	CirA (catecholate siderophore receptor); FhuA (ferrichrome transporter); EfeO (iron uptake system component)
	Transport system substrate-binding protein[1]	ZnuA (zinc); LivK (branched-chain amino acid); PppA (oligopeptide); ModA (molybdate); GltI (glutamate/aspartate);
	Osmotic proteins[2]	OsmC (osmotically inducible protein); OpuC (osmoprotectant transport system substrate-binding protein)

EHEC = O157:H7 str. EDL933; EAHEC = O104:H4 str. TW16133.
[1] *E. coli* O157:H7 proteins with increased abundance in M9 medium compared to O104:H4.
[2] *E. coli* O104:H4 proteins with increased abundance compared to O157:H7.

Table 4
Genes identified in EHEC, EAHEC, and EAEC chromosome that encoded proteins associated to VBNC, acid tolerance, and adhesion / colonization.

Gene	Protein encoded and/or function	*E. coli* pathotypes			Reference
		EHEC	EAHEC	EAEC	
VBNC					
ompW	Outer membrane protein W	+	+	+	(Asakura et al., 2007)
rpoS	RNA polymerase sigma factor RpoS	+	+	+	
tufAB	Elongation factor, TU1	+	tuf*	tuf*	
mobA	Mo^+ cofactor guanylyltransferase	+	+	+	
Acid tolerance (associated with the Fe-S system)					
ykgJ	Putative metal-chelating domain	+	HProt	+	(Hu et al., 2017)
fes	Ferric enterobactin esterase	+	+	+	
ybfa	DUF2517 domain	+	HProt	+	
grxA	Reduced glutaredoxin 1	+	+	+	
Acid tolerance (transportation and membrane)					
ycaD	Putative transporter YcaD	+	+	+	(Dahan et al., 2005; Hu et al., 2017)
yjcB	Uncharacterized protein YjcB	+	MP	+	
yohJ	3-hydroxypropanoate export YohJ	+	HProt	CidA/Lrg*	
mokP	CKP encoded within cryptic prophage CP-933P	+	-	-	
Stress resistance					
asr	acid shock protein	+	HProt	+	(Hu et al., 2017; Ribeiro et al., 2019)
cadA	Lysine decarboxylase 1	+	+	+	
cadB	Lysine cadaverine antiporter	+	+	+	
visP	Sec-translocase	+	UHP	+	
Adhesion					
pAA	Aggregative adherence plasmid	-	+	+	(Munera et al., 2014; Valat et al., 2012)
pic	Pic protease	-	+	+	
esc	Type III secretion system (T3SS)	+	-	-	
eae	Intimin	+	A/E protein*	+	
espBDJ	T3SS translocator EspB; EspD; EspJ	+	-	-	
tir	Translocated intimin receptor protein	+	-	-	

* = protein with identical sequence but different annotation; CKP = cell-killing protein; HProt = hypothetical protein; UHProt = uncharacterized hypothetical protein; MP = membrane protein.

T3SS effectors Esp [EspB, EspF, EspJ]; Nle [NleA, NleB, NleC], and Tir to develop the adherence and colonization process (Valat et al., 2012).

No differences for most of the genes associated with the Fe-S system, transportation, membrane, and stress resistance were reported between EAEC 55989, EHEC TW14359, and EAHEC O104:H4 *(37, 65,* Table 4). Exceptionally MokP, a cell-killing protein encoded within cryptic prophage CP-933P, is only present in the EHEC genome. Dahan et al. (Dahan et al., 2005) identified EspJ (*E. coli*-secreted protein J), carried on the 5′ end of this prophage. In an *in vivo* mice study, the *ΔespJ* mutant of this strain exhibited higher levels of colonization in the gastrointestinal tract than the wild-type strain demonstrating that this protein plays an important role in host colonization and pathogen transmission. These authors suggest that the *espJ* gene exhibits "antivirulence" properties in EHEC, however, the presence of this and other genes in EAHEC and EAEC and its role in survival and pathogen transmission remain to be elucidated.

Conclusions and perspectives

Comprehensive surveillance studies have been carried out to detect the presence of EAEC, in particular, serotype O104:H4 in humans, animals, and the environment. The data support a considerable trend

toward humans as the only reservoir. However, it is important to note that research to date has not included all species and categories of wild and domestic animals that interact with humans. In this review, we have described the physiological characteristics, its ability to infect the intestine of animal models, its epidemiologic surveillance, and ecological preferences, that prevent animals to be reservoirs of *Escherichia coli* O104:H4. However, these unfavorable characteristics and conditions for the infection of animals could be modified or adapted, further increasing the reservoirs or targets of this bacterium. In view of the significant negative health impacts of this multiresistant serotype, further studies are necessary to monitor wild animals, bacterial mutations, changes in animal nutrition, and other factors that could potentially contribute to interspecies colonization.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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