Chapter 5
Enterohemorrhagic (Shiga Toxin-Producing) *Escherichia coli*

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Summary Enterohemorrhagic (Shiga toxin-producing) *Escherichia coli* (EHEC/STEC) is a zoonotic food- and waterborne pathogen that can cause human infections ranging from asymptomatic carriage or mild diarrhea to hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). The isolates belong to a large number of O:H serotypes, and O157:H7 is the most prevalent serotype associated with large outbreaks and sporadic cases of HC and HUS in many countries. Advances on the knowledge of microbial pathogenesis, pathophysiology of the associated diseases, epidemiology, and risk factors have contributed to the development of several strategies trying to prevent food and environment contamination, and consequently transmission to humans. However, prevention of EHEC (STEC) infection has been difficult because of the broad spectrum of contaminated sources and the limited effectiveness of the different interventions used. The availability of effective vaccines to reduce carriage in livestock as well as for preventing human disease is a pending challenge. Specific targeted therapies against this pathogen group are another area of concern. A new risk scenario has emerged in the last decades due to the bacterial evolution that gave rise to the emergence of hypervirulent O157 clones with a worldwide distribution and other EHEC (STEC) strains with unusual combinations of pathogenic features, such as the O104:H4 strain. Because of the severity and the long-term sequelae of EHEC (STEC)-associated illnesses, they have a high social and economic cost for both the affected families and the health system. Therefore, all efforts should be directed to reduce the burden of these diseases.

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1 General Concepts About EHEC (STEC)

Enterohemorrhagic (Shiga toxin-producing) *Escherichia coli* (EHEC/STEC) comprise a group of zoonotic food- and waterborne pathogens whose hallmark is the ability to produce one or more cytotoxins of the Shiga toxin (Stx) family (Melton-Celsa 2014). The clinical manifestations related to EHEC (STEC) infections can range from symptom-free carriage or mild diarrhea to more severe clinical presentations like hemorrhagic colitis (HC) and a life-threatening syndrome known as Hemolytic Uremic Syndrome (HUS), affecting mainly infants and children (Tarr et al. 2005). Although the incidence of EHEC (STEC) infections varies over the world, the importance and impact of HC and HUS outbreaks on public health is enormous, being responsible as the main cause of acute renal failure in children in many countries (Tarr et al. 2005; Rivas et al. 2006a).

HUS was originally defined as a combination of microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure. Recently, the definition of HUS has come to include documented hemolysis rather than anemia, platelet consumption rather than thrombocytopenia, and signs of renal damage rather than renal failure (Ardissino et al. 2014). There is no specific treatment for HUS, and patients are generally given supportive care for water imbalance, anemia, hypertension, and renal failure. The frequency of patients dying during the acute phase of disease is still 1–2 % (Mele et al. 2014), and almost 30 % of patients develop long-term renal damage (Spinale et al. 2013).

The discovery and history of the emergence of this *E. coli* pathotype had recently been reviewed (Kaper and O'Brien 2014). It is fascinating that more than 30 years after its first association with a human disease, our knowledge on EHEC (STEC) epidemiology, virulence properties, pathogenesis, host interactions, and molecular evolution is continuously evolving.

Escherichia coli O157:H7, linked to the first outbreak of HC in the United States in 1982 (Riley et al. 1983) and responsible for outbreaks and sporadic cases of HUS in several countries (Rivas et al. 2006a; de Souza et al. 2011; Vally et al. 2012; Terajima et al. 2014), is the prototype of this group of pathogens originally named enterohemorrhagic *E. coli* (EHEC), and nowadays still represents one of the most important and prevalent serotypes responsible for the more severe cases of disease worldwide. However, knowledge that more than 400 *E. coli* serotypes can harbor *stx* genes has led to a more general classification of the group as Shiga toxin-producing *E. coli* (STEC), but epidemiological studies carried out all over the world have demonstrated that only a proportion of them have been implicated in human disease. In addition to O157:H7, some other serogroups as O26, O45, O103, O111, O121, and O145 have been recognized as responsible for the majority of the serious cases of infection (Gould et al. 2013; Terajima et al. 2014).

In EHEC (STEC) infections, the most significant virulence factors are the Shiga toxins (Stx). Stx family comprises several toxins, related to Stx from *Shigella dysenteriae*, sharing similar structure and biological activity. Another common charac-

teristic present among these toxins is that the *stx* operon is usually found within the sequence for an inducible, lysogenic, λ-like bacteriophage (Melton-Celsa 2014). Albeit these similarities, a high degree of diversity has been identified among these proteins, and therefore, in *E. coli* two major toxin subfamilies Stx1 and Stx2 are classified, and each is composed by several variants. Although the nomenclature of these variants was rather confusing as several systems had been proposed, a consensus has been reached in more recent years and a sequence-based nomenclature has been developed for detection and subtyping of *stx* genes (Scheutz et al. 2012). According to this scheme, members of the Stx1 subfamily include Stx1a, Stx1c, and Stx1d; whereas Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f, and Stx2g belong to the Stx2 group. More than sequence differences, it has been recognized that some of the variants are of clinical relevance as they have been associated with more severe cases such as HC and HUS, while others have been related to uncomplicated diarrhea or are probably not produced by strains causing human disease. In this respect, those producing Stx2a, Stx2c, or Stx2d have been reported to be more pathogenic than those strains producing Stx1 variants alone or both Stx1 and Stx2 (Scheutz 2014). On the other hand, the variants Stx2e, Stx2f, and Stx2g are rarely involved in human disease. In fact, Stx2e is responsible to cause edema disease of swine, a serious neurological disorder that is frequently fatal, whereas Stx2g and Stx2f have been mainly detected in animal reservoirs (Persad and LeJeune 2014).

A considerable amount of data exists to explain the stronger association of Stx2 with more severe diseases. Earlier studies had proposed differences in cytotoxicity among Stx1 and Stx2. It has been observed that while Stx1 is more toxic to mammalian cell lines, such as Vero cells, Stx2 is more potent in animal models. It had been shown that only Stx2-treated mice developed renal complications and death, and similar observations were seen in nonhuman primate models (Baboons) in which treatment with Stx2 caused HUS, while equal doses of Stx1 had no effect (Siegler et al. 2003). Moreover, comparison of the effects of the two toxins also showed different pro-inflammatory responses and different timings of organ injury. Stx1 induced a stronger and earlier pro-inflammatory response in baboons, while the Stx2 response was gradual and delayed by several days (Stearns-Kurosawa et al. 2010). Another study, also using a nonhuman primate model, showed that both Stx1 and Stx2 affected kidney function, and although Stx2 inflicted more severe damage to the kidney than Stx1, the damage caused on the kidney by Stx1 was significant (Stearns-Kurosawa et al. 2013). In parallel, a differential susceptibility of endothelial cells to Stx1 and Stx2 has been described (Bauwens et al. 2011), but the real basis of these differences is not well understood. It has been suggested that holotoxin stability, enzymatic activity, and receptor affinity may be related to the differential toxicity of Stx1 and Stx2.

These toxins are characterized by an AB_5 structure with one enzymatically active A subunit non-covalently linked to a pentamer of B subunits responsible for the globotriaosylceramide (Gb3) binding. The Stx-Gb3 interaction leads to the internalization of the toxin which is transported via the retrograde pathway to the Golgi apparatus, endoplasmic reticulum, and nuclear membrane. An active A1 fragment

of the A subunit is retro-translocated into the cytoplasm where it binds to the large ribosomal subunit and inhibits protein synthesis by cleaving off a single adenine residue from the 28S rRNA. The action of Stx on target cells goes beyond the inhibition of protein synthesis. Further, it is well-known that the toxin also acts on cell signal transduction and immune modulation (Lee et al. 2013). The damage caused to the ribosome by Stx induces a ribotoxic stress response that is both pro-inflammatory and pro-apoptotic (Jandhyala et al. 2012). Indeed, the mechanisms of Stx1 and Stx2-induced apoptosis in various cell types have been extensively studied (Cherla et al. 2003). Recently, Basu and Tumer (2015) reviewed the participation of B subunits and the potential role for the A1 subunits in the differential toxicity of Stx1 and Stx2. Bentancor et al. (2013) studied the ability of the machinery of eukaryotic cells to recognize stx_2 sequences and to produce biologically active Stx2 in Vero cells transfected with a plasmid-encoding Stx2. Their results support the hypothesis that in the context of the inflammatory response of the colon during the EHEC (STEC) infection, phagocyte cells (macrophages/neutrophils) could incorporate stx_2 genes and produce active toxin as alternative source of Stx2.

The colon is the primary site of histological lesions caused by EHEC (STEC), and when the tissue is swollen, increases the Stx passage through the intestinal barrier, and the development of HUS is linked to the cytotoxic action exerted by Stx when passing from the intestine to the systemic circulation. Schüller (2011) reviewed the Stx interaction with the human intestinal epithelium and proposed that Stx uses different routes of translocation through the human intestinal epithelium in the early stages of infection: (1) Gb3-independent transcytosis, possibly enhanced by EHEC (STEC) infection; (2) paracellular transport during neutrophil (PMN) transmigration; (3) induction of Gb3 expression by EHEC (STEC) infection, retrograde transport, and Stx release after cell death; (4) Gb3-dependent translocation by Paneth cells; and (5) transcytosis by M cells. Sandvig et al. (2014) showed that the different steps of transport used by Stx have specific lipid requirements that confer specificity to Stx action.

Different studies have shown that kidney and brain are the most affected organs in HUS patients, due to the high sensitivity of the endothelial cells and renal microvasculature to Stx by the elevated expression of Gb3. Renal or neurological sequelae are consequences of irreversible tissue damage during the acute phase. Moreover, the tumor necrosis factor (TNF-α), mainly released by monocytes/macrophages, increases the expression of Gb3 in endothelial cells, and Stx is also able to increase and extend renal injury favoring endothelium interaction with leukocytes and platelets. Edema and detachment of endothelial cells of the basement membrane is seen in the histopathological examination of renal glomeruli and also formation of microthrombi of platelets rich in fibrin, causing the injury known as thrombotic microangiopathy (TMA). In addition to the direct effects of Stx on the renal epithelium and endothelium causing cell injury, the toxin induces an extensive inflammatory response and promotes the release of pro-inflammatory cytokines such as interleukin-1 (IL-1), TNF-α, and IL-6 in human renal epithelial cells and increases the expression of chemokines, cytokines, and molecules of adhesion in epithelia and

endothelium, which contribute to renal injury observed in HUS. Stx2 also induces the increase of IL-8 and monocyte chemoattractant protein-1 (MCP-1) and fractalkine/CX3CL1 in human endothelial cells, promoting adhesion and leukocyte chemotaxis (Zoja et al. 2010).

Other virulence factors, such as lipopolysaccharide (LPS), are necessary for the full development of HUS. It is known that the joint action of Stx and LPS increases the production of nitric oxide (NO) and reduces levels of catalase. Gómez et al. (2013) have demonstrated that Stx induces an oxidative imbalance, evidenced by renal glutathione depletion and increased lipid membrane peroxidation. The enlarged production of reactive oxygen species by neutrophils could be one of the major sources of oxidative stress during Stx intoxication. The authors concluded that Stx generates a pro-oxidative state that contributes to kidney failure, and exogenous anti-oxidants could be beneficial to counteract this pathogenic pathway.

On the other hand, one should consider that the differences in virulence observed among EHEC (STEC) isolates may also be associated to a diversity of Stx expression. As the production of Stx relates to the level of phage production, considerable information about the characteristic and behavior of *stx* phages, the factors involved in their induction and dissemination, has contributed to our understanding on how phage variability may affect pathogenesis and disease (reviewed in Krüger and Lucchesi 2015). Moreover, the effect of some antibiotics on *stx* phage induction and Stx production presents adverse clinical consequences and directly impacts therapeutic approaches (McGannon et al. 2010). As such, studies on novel strategies trying to detect conditions that repress phage induction will certainly contribute to diminish the risk of development of HUS (Keen 2012).

The importance of Stx in the development of disease is unquestionable, but a diverse array of additional virulence factors, including adhesins, other toxins, and proteases, are involved in the establishment and maintenance of an infection.

The ability to colonize the host intestinal epithelium is considered a key step in pathogenesis. The presence of locus of enterocyte effacement (LEE), a chromosomal pathogenicity island (PAI), encoding a type III secretion system (TTSS), an outer membrane protein called intimin, and effector proteins responsible for the characteristic attaching-and-effacing (A/E) lesion is pathognomonic of disease related to EHEC (STEC). The A/E lesion is a histopathologic alteration characterized by the effacement of microvilli and the formation of pedestal-like structures in a process driven by the actin polymerization. The loss of the absorptive capacity of microvilli probably contributes to the diarrhea induced by these bacteria. Both O157:H7 (Wick et al. 2005) and non-O157 EHEC (STEC) strains (Karmali et al. 2003; Wickham et al. 2006) contain a variable repertoire of virulence determinants, including a collection of non-LEE encoded effector (*nle*) genes that encode translocated substrates of TTSS. The TTSS facilitates the persistence, host-to-host spread, and virulence of EHEC (STEC). However, although LEE has been linked to EHEC (STEC) strains belonging to major serotypes causing more than 80 % of HC and HUS cases in Europe (ECDC 2015a) and the United States (CDC 2013), it has been noticed that possession of LEE is not a mandatory condition for the occurrence of

exacerbated infections, as some LEE-negative strains are also capable of causing outbreaks and sporadic cases of HUS (Paton et al. 1999; Karch et al. 2005; Bielaszewska et al. 2011). Thereafter, an increasing number of adhesins composed by several fimbrial proteins, as well as different members of autotranporter proteins (AT) family, have been identified in EHEC (STEC) strains, some of them exclusively among LEE-negative strains, which were demonstrated to be involved in adherence to human epithelial cells as well as in biofilm formation (McWilliams and Torres 2014).

Besides Shiga toxins, the contribution of other toxins as the cytolethal distending toxin V (CDT-V) and subtilase cytotoxin (SubAB), produced by some particular serotypes, in the development and pathogenesis of HUS has been addressed (Bielaszewska et al. 2005; Paton and Paton 2010). Amaral et al. (2013) compared the effects of SubAB with those caused by Stx2 on primary cultures of human glomerular endothelial cells (HGEC) isolated from fragments of human pediatric renal cortex. Both toxins decrease the cell viability, but Stx2 caused a necrosis significantly higher than that induced by SubAB. Stx2 increased apoptosis in a time-dependent manner, while SubAB increased apoptosis at 4 and 6 h but decreased at 24 h. Pre-incubation of HGEC with C-9, a competitive inhibitor of Gb3 synthesis, protected HGEC from Stx2 but not from SubAB cytotoxic effects. These data provide evidence of how SubAB could cooperate with the development of endothelial damage characteristic of HUS pathogenesis.

In addition, a plasmid-encoded enterohemolysin (EhxA), which is a pore-forming cytolysin, has been identified at high frequencies among several EHEC (STEC) strains, and frequently associated with diarrheal disease and HUS. Despite EhxA contributing to disease by damaging the membrane of erythrocytes and other cells, its role in the hemolytic anemia of HUS patients has not been ascertained. Nevertheless, the enterohemolytic phenotype has been used as a good marker in the identification of EHEC (STEC) (Beutin et al. 1989).

The zoonotic character of EHEC (STEC) infections is well-established. Bacteria are largely distributed in the gastrointestinal tract of a wide diversity of animal species, normally as asymptomatic carriers. Several ruminant animals, especially cattle, are considered as the main natural reservoir, but other livestock species, domestic and wild mammals, birds, and fishes, have also been implicated in EHEC (STEC) carriage. The role of animals as reservoirs for infection or as spillover hosts has recently been reviewed (Persad and LeJeune 2014). As consequence, the transmission routes of EHEC (STEC) to human can occur either by the food chain, direct contact with animals or their environment, or by person-to-person spread. Infections have also been caused by drinking or swimming in contaminated water (Launders et al. 2013; Luna-Gierke et al. 2014). The panel of foods implicated as vehicles of EHEC (STEC) transmission is highly diverse. The main risk factors for EHEC (STEC)-associated human infections identified in earlier case-control and population-based studies were dietary behaviors and beef consumption. However, in recent years, other risky exposures have also emerged, like the consumption of fresh produce and sprouts, responsible for important outbreaks of HUS in several countries in the last 10 years (Beutin and Martin 2012; Rivas et al. 2014).

Therefore, several interventions targeting EHEC (STEC) related to animal handling, from farm to slaughter, as well as the implementation of food safety throughout production, processing, and distribution of fresh produce have been developed in past years looking for the improvement of the microbiological quality of foods.

The search for effective pre-harvest food safety practices for application to live cattle to reduce the contamination with *E. coli* O157 and other EHEC (STEC) strains of both foods of bovine origin, and related environmental contamination, has been reviewed by Besser et al. (2014). Different interventions, like feed ingredients, probiotics, and vaccines, have been identified with significant impact on *E. coli* O157 cattle shedding. But the authors concluded that the impact of these potential interventions remains insufficient due to their limited efficacy, practical difficulties with their implementation, and inconsistency in their results, leading to limited uptakes by producers. Also, the peri- and post-harvest interventions in the control of EHEC (STEC) in beef and in the agro-food chain were compiled by Moxley and Acuff (2014) and Duffy and McCabe (2014), respectively.

Since EHEC (STEC) does not cause disease in cattle, or triggers a local and protective immune response in the gastrointestinal tract, the goal of the EHEC (STEC) eradication from the bovine reservoir designing vaccines or other practices is not an easy task. Vaccines targeting a number of STEC O157-specific antigens have been tested in animal challenge studies. Some products have demonstrated efficacy to reduce the prevalence of cattle O157 shedding, but their efficacy to control the transmission in the environment during the natural exposure is doubtful (reviewed by Smith 2014).

Another strategy to decrease human-associated diseases involves the vaccination of the affected population, especially children. There are two possibilities: (1) a vaccine to control or prevent EHEC (STEC) infections; (2) a vaccine to prevent the systemic complications due to Stx action.

Because no licensed vaccine or effective therapy is presently available for human use, Mejias et al. (2014) recently developed a novel immunogen based on the B subunit of Stx2 and the enzyme lumazine synthase from *Brucella* spp. and they demonstrated the protection of mice against Stx2-associated damage by maternal immunization with the BLS-Stx2B chimera. Szu and Ahmed (2014) reviewed the human EHEC vaccines that have been studied clinically, in particular against *E. coli* O157. The LPS O157 conjugated to the recombinant exotoxin A of *Pseudomonas aeruginosa* (rEPA) has shown to be safe and induced high levels of anti-LPS, IgG antibodies, with bactericidal activity in adults and children (2–5 years old). On the other hand, a similar construct using the B subunit of Stx1 as the carrier protein elicited both bactericidal and toxin-neutralizing antibodies in mice.

There is a general agreement that patients with EHEC (STEC) infections should not be treated with antibiotics due to a higher risk of developing HUS as certain antibiotics induce expression of the Stxs. Recently, Agger et al. (2015) performed a systematic review in order to clarify the risk associated with the antibiotic treatment during acute EHEC (STEC) infections and in chronic carrier states. Among the ten clinical studies, four found an increased risk of HUS, four found no effect, and two found a reduced risk of HUS. In vitro and clinical studies suggested that DNA syn-

thesis inhibitors should be avoided, and certain protein and cell wall synthesis inhibitors reduced the toxin release from EHEC (STEC) isolates. The authors proposed that antibiotic treatment with protein and cell wall synthesis inhibitors could be considered when specific criteria (patient group, serotype, virulence profile, and duration of disease) are met. There are new therapeutic developments designed to limit Stx receptor generation or to prevent toxin binding, trafficking, processing, or activity within the cell (reviewed in Melton-Celsa and O'Brien 2014).

The identification of EHEC (STEC) strains of risk to public health is a challenge for diagnostic laboratories. A concept of molecular risk assessment (MRA) was developed by Karmali et al. (2003) and Coombes et al. (2008) as an aid to assess the role of genomic islands in contributing to the public health risk associated with different EHEC (STEC) strains, especially those found in foods, animals, or the environment. In this context, PCR-based methods could be used for identification of LEE-encoded effector and non-LEE-encoded effectors (*nle*) as an approach to define human virulent EHEC (STEC) types. Karmali et al. (2003) proposed a classification of STEC serotypes into five seropathotypes (A to E), taking into consideration the reported frequencies in human illness and associations with HC and HUS outbreaks. Seropathotype A (O157:H7 and O157:NM) is considered the most virulent and is related to the highest incidence in human disease and frequently involved in outbreaks. Seropathotype B (composed by O26:H11; O103:H2; O111:NM; O121:H19; and O145:NM) is associated at a lower frequency with severe human disease and uncommonly involved with outbreaks. Seropathotype C (O5:NM; O91:H21, O104:H21, O113:H21, and others) and D show a low incidence in human illness and are rarely associated with outbreaks, whereas seropathotype E is composed by many serotypes with no implication in human diseases so far demonstrated. Further, Coombes et al. (2008) identified 14 new *nle* genes in non-O157 STEC strains, grouped within three PAIs that correlated independently with outbreak and HUS potential for humans. Moreover, the authors showed an *nle* gene dosing effect in non-O157 STEC, where strains associated with severe human disease have an increased number of *nle* genes. Bugarel et al. (2010) have developed a low-density macroarray designed for simultaneous detection of genes encoding Stx_1 and Stx_2 (stx_1 and stx_2), intimin (*eae*), enterohemolysin (*ehxA*), and six different *nle* genes derived from genomic islands OI-71 (*nleF*, *nleH1-2*, and *nleA*) and OI-122 (*ent*, *nleB*, and *nleE*). The *nle* genes were found to be closely associated with certain serotypes and intimin genotypes in typical EHEC strains, including the new emerging EHEC (STEC) strains. The presence of *eae*, *ent*/*espL2*, *nleB*, *nleE*, and *nleH1-2* genes is a clear signature of EHEC (STEC) strains with high virulence for humans. Brandt et al. (2011) developed a PCR binary typing system (P-BIT) that could be used to aid in risk assessment and epidemiological studies of EHEC (STEC). They examined the distribution of 41 gene targets among O157 and non-O157 EHEC (STEC) isolates and found that P-BIT provided 100 % typeability for isolates, gave a diversity index of 97.33 % (compared with 99.28 % for *Xba*I pulsed-field gel electrophoresis [PFGE] typing), and produced 100 % discrimination for non-O157 STEC isolates. The authors identified 24 gene targets that conferred the same level

of discrimination and produced the same cluster dendrogram as the 41 gene targets initially examined. The P-BIT clustering identified O157 from non-O157 isolates and identified seropathotypes associated with outbreaks and severe disease. Using the MRA concept for screening EHEC (STEC) collections, an increasing number of emerging EHEC (STEC) types were detected (Bugarel et al. 2010). Internationally, the number of reported human diarrheal cases associated with non-O157 EHEC (STEC), including those leading to HUS, is rising rapidly, mainly due to increased surveillance for these pathogens.

However, the usefulness of the MRA concept changed dramatically in 2011. From May to July 2011, a large-scale outbreak was observed in several European countries, mainly affecting northern Germany, comprising 3842 cases of human infection, 855 (22.3 %) HUS cases and 53 fatalities, and involving an emerging enterohemorrhagic *E. coli* O104:H4 strain (Askar et al. 2011). This EHEC (STEC) strain presents an unusual virulence pattern that combines the production of Stx2a with enteroaggregative adherence, which is encoded by genes of the pAA plasmid and chromosomally carried genes of enteroaggregative *E. coli* (EAEC) strains (Bielaszewska et al. 2011). This new type of EHEC was designated enteroaggregative hemorrhagic *E. coli* (EAEHEC), since it shares virulence markers of both EAEC and EHEC strains. An important lesson to learn from the EAEHEC O104:H4 infection outbreak was that LEE and the *nle* genes, the current MRA approach to define human virulent EHEC (STEC) types, can be substituted by EAEC plasmid-encoded aggregative adherence mechanisms to enable Stx2-producing EAHEC to cause HC and HUS in humans (Beutin and Martin 2012).

After the 2011 outbreak and the fact that of all confirmed EHEC (STEC) infections in the European Union during 2007–2010, more than 85 % of the isolates were not fully serotyped, a Panel on Biological Hazards (BIOHAZ) was asked by the European Food Safety Authority (EFSA 2013) to review the seropathotype concept of Karmali et al. (2003) and the scientific criteria regarding pathogenicity assessment. The BIOHAZ Panel concluded that the seropathotype classification does not define pathogenic EHEC (STEC) nor does it provide an exhaustive list of pathogenic serotypes. The panel pointed out that there is no single or combination of marker(s) that defines a pathogenic STEC. Strains positive for stx_2 gene, and *eae* (intimin) or *aaiC* (secreted protein of EAEC) plus *aggR* (plasmid-encoded regulator) genes, are associated with a higher risk of more severe illness than other virulence gene combinations. Recently, de Boer et al. (2015) have described a rapid screening algorithm, including both molecular and conventional methods, to determine the pathogenic potential of STEC. The purpose was to discriminate infections with less-virulent EHEC (STEC) from those with clinical relevance and risk for public health.

During the investigation of the large outbreak reported in Germany in 2011, Whole Genome Sequencing (WGS) was applied for the first time in an epidemiology public health problem. The application involved the use of rapid, bench-top DNA sequencing technology, open-source data release, and prompt crowd-sourced analyses. In less than a week, these studies provided the draft genome of this new

EAEHEC hybrid and its unusual antibiotic resistance (Brzuszkiewicz et al. 2011). Afterwards, numerous advances in the use of WGS applied to research in different aspect of EHEC (STEC) were published (Eppinger et al. 2011; Jenkins et al. 2015).

A real-time evaluation of WGS for routine typing and surveillance of EHEC (STEC) was communicated by Joensen et al. (2014). The bioinformatics analysis using web-tools for species determination, multilocus sequence typing (MLST), determination of phylogenetic relationship, and a specific detection of *E. coli* virulence genes were described (Center for Genomic Epidemiology. http://www.genomicepidemiology.org). WGS demonstrated to be a robust method for assigning *stx* subtypes and for a real-time clustering of isolates in agreement with epidemiology, enabling discrimination between sporadic and outbreak isolates. Dallman et al. (2015) described the validation of the WGS approach as a molecular typing tool for surveillance of O157 in the United Kingdom and demonstrated that it can be used in real-time to provide the highest strain-level resolution for outbreak investigation.

There is an international agreement for the implementation of WGS for diagnosis and typing in Public Health due to the additional improvement in bioinformatics tools and the easy use in laboratories. However, WGS implies great changes. Regarding technology, new equipment would be needed for laboratories, and also for sequence analysis. Other major change is the way in which diagnostics and typing must be modified. It is recognized that the transition is a great challenge for the organizations and a concern to balance the benefits of applying new and powerful WGS approaches with the risk of implementing these new technologies too quickly (Joensen et al. 2014; ECDC 2015b).

2 Recent Advances in EHEC (STEC) Research

2.1 Emergence of New EHEC (STEC) Strains

The profile of the EHEC (STEC)-associated diseases has changed in recent decades, and different factors have contributed to the emergence of new strains causing sporadic cases and outbreaks with different epidemiological characteristics and widespread epidemics.

2.1.1 Emergence of a New EHEC (STEC) O26:H11 Clone in Europe

In Europe, EHEC (STEC) O26:H11 is the most common non-O157 serotype, being in some countries the most common cause of childhood-HUS. Previously, EHEC (STEC) O26:H11 strains isolated from humans harbored stx_{1a} and rarely associated with $stx_{2a.}$ However, a new highly virulent stx_{2a}-positive *E. coli* O26:H11 clone has been circulating in Europe since the mid-nineties (Bielaszewska et al. 2013). This clone was also observed in Latin America (Rivas et al. 2006b) and in the United

States (Brooks et al. 2005). By MLST analysis, the O26:H11 strains were divided into two phylogenetic groups: ST21 (stx_{1a} alone or associated with stx_{2a}), associated with less-severe disease, and ST29 (new stx_2 clone), with an increased virulence and disease severity. Recently, Delannoy et al. (2015) studied 23 *E. coli* O26:H11 strains, isolated from pediatric patients in France during the period 2010–2013. From the strains, 69.6 % belonged to the new clone, but 12 strains with negative results for both plasmid and chromosomal genetic markers exhibited a ST29 genotype and related CRISPR (clustered regularly interspaced short palindromic repeat) arrays, and seven strains harbored the stx_{2d} gene. By WGS, the evolutionary phylogenetic relationship of EHEC (STEC) O26:H11/H$^-$ has been investigated on European strains. Using the 48 phylogenetically informative single-nucleotide polymorphisms (SNPs), four distinct clonal complexes (CCs) were observed and the highly virulent German O26:H11 stx_{2a} clone was identified in a single CC, different from the former described strains (Bletz et al. 2013). Ison et al. (2016) differentiated *stx*-positive strains from *stx*-negative strains to infer the phylogenetic relationships of 178 *E. coli* O26:H11 bovine strains. The cattle *stx*-negative strains displayed synonymous SNP genotypes with the stx_2-positive, ST29 human strains, meanwhile the stx_1, ST21 human and cattle strains clustered separately, demonstrating the close phylogenetic relatedness of these *stx*-negative cattle strains and human clinical strains.

2.1.2 Emergence of EHEC/EAEC and Other Hybrid Strains Worldwide

Since the large outbreak associated with EAEHEC O104:H4 strain, attention has been focused on this new *E. coli* pathotype. Other O104:H4 strains with different PFGE macro-restriction profile, AAF type, antibiotic resistance, plasmid profile, virulence gene sets, or SNPs on the genomic level have been described, such as the HUSEC041 strain causing HUS in Germany in 2001, two French strains isolated in 2004 and 2009, and five additional strains causing HUS in France in 2011. As was previously proposed, it can be assumed that several O104:H4 EHEC lineages emerged from O104:H4 EAEC ancestors which differ in their genetic background (Mellmann et al. 2011). Like the enteroaggregative *E. coli* 55989 strain, isolated in Central Africa in the late 1990s, the German HUSEC041 isolate, the European outbreak strain, and the two French O104:H4 isolates belonged to ST678 and carried wzx_{O104}, $fliC_{H4}$, *aggR*, *lpfA*, *pic*, *sepA*, and *sigA*, and *stx-A*/*B2* genes (Monecke et al. 2011). The European outbreak strain harbored an extended-spectrum-lactamase gene, *bla*CTX-M-15, an additional lactamase gene (*bla*TEM-1), and other antibiotic resistance genes. Analysis of the genome sequences showed that HUSEC041 was positive for *bla*TEM-1, while the 2004 and 2009 French isolates lacked *bla*TEM-1 and other resistance genes. Ferdous et al. (2015) have described the isolation of *E. coli* O104:H4 strains in 2013, from a patient with HUS and a second individual showing only gastrointestinal complaints. They demonstrated that the EAEHEC O104:H4 Stx2a-positive strains were highly similar to the 2011 outbreak strain in

their core genome, showing that this clone is still circulating and a proper surveillance is necessary to prevent further outbreaks with these potentially pathogenic strains.

The emergence of EHEC/EAEC O104:H4 suggests that either certain EAEC serotypes might be more susceptible to acquire EHEC determinants or that there are certain EHEC/EAEC ancestors which successfully adapted to survival-specific selection conditions. In this context, other EHEC/EAEC strains of different serotypes were previously described associated with human disease. During an HUS outbreak in France in 1996, an O111:H2 strain was characterized as stx_2- and AAF-positive, and *eaeA*- and *ehxA*-negative, being the first EHEC/EAEC hybrid described (Morabito et al. 1998). Few years later, an O86:NM [H2] strain with stx_2 and AAF marker genes, but no *eae,* was isolated in 1999 from a pediatric patient with HUS and bloody diarrhea in Japan (Iyoda et al. 2000). In 2011, an O111:H21 strain associated with a household outbreak in Northern Ireland was detected. The strain harbored the stx_{2c} gene and the type V aggregative AAF fimbriae, but was *eae*-negative with a low level of resistance to ampicillin. It belonged to ST40, a sequence type comprising other *E. coli* pathotypes (STEC, EAEC, enteropathogenic *E. coli* [EPEC], and non-pathogenic *E. coli*). Genome sequencing revealed that the clonal complex, pAA plasmids, and phage encoded-*stx* genes were different in comparison with the O104:H4 outbreak strain (Dallman et al. 2012). Prager et al. (2014) screened about 2400 strains of the EHEC collection from the German National Reference Centre for *Salmonella* and other Bacterial Enteric Pathogens (NRC), corresponding to the period 2008–2012, for the presence of stx_1, stx_2, *eae,* and *ehxA* genes. Among 268 *eae*-and *ehxA*-negative strains, two strains exhibited both EHEC and EAEC marker genes and were stx_2- and *aatA*-positive. One strain, isolated from a bloody diarrhea patient in 2010 and serotyped as O59:H-[$fliC_{H19}$], harbored stx_{2a}, belonged to ST1136, and exhibited genes for type IV aggregative AAF fimbriae, and with resistance towards sulfonamides, streptomycin, and trimethoprim/sulfonamide. The *iha*, $lpfA_{O26}$, $lpfA_{O113}$, and *irp2* genes, frequently associated with EHEC, and *aggR*, *aap*, *set1a*, *set1b*, *pic*, *sigA,* and *iucA* genes in general related to EAEC were detected. The *astA* gene was not detected. The second strain isolated from a patient with diarrhea in 2012, harbored stx_{2b}, was typed as Orough:H2 and belonged to ST26. In Argentina, Carbonari et al. (2015) screened a total of 36 stx_2-positive, *eae*- and *ehxA*-negative non-O157 STEC strains, isolated from HUS and diarrhea cases, for the AraC-like regulator AggR. Nine (25 %) EHEC/EAEC O59:H-[$fliC_{H19}$] strains were identified, isolated from 8 HUS and one bloody diarrhea cases. The first O59 isolate corresponded to a HUS case from 2005. The stx_{2a}, *iha*, lpf_{O26}, lpf_{O113}, *aatA*, *aap*, *sigA* genes were detected. The presence of type IV aggregative AAF fimbriae was established by the amplification of the *agg4A* fimbrial subunit gene. The strains showed low toxicity on Vero cells and were resistant to streptomycin and trimethoprim/sulfonamides. By *Xba*I-PFGE, nine patterns were established, with 86.7 % similarity. A high clonal relationship (>85 %) with the EHEC/EAEC O59:H-[$fliC_{H19}$] German strain was established. Tozzoli et al. (2015) reported an outbreak caused by an EHEC/EAEC O127:H4 strain in Northern Italy in 2013. The analysis of 76 fecal samples from children and school staff was performed. Five Stx2-producing EAEC

O127 strains were isolated. By WGS, the strain was characterized as O127:H4 and ST678. It possessed the stx_{2a} gene and other EAEC virulence genes such as *aggR*, *aap*, *aat*, *aaiC*, *sigA*, *pic,* and *astA*. The stx_2-phage was inserted in *wrbA* site and was highly similar to that of the O104:H4 outbreak strain. Nyholm et al. (2015) have sequenced the whole genome of three (2 humans and 1 bovine) EHEC (STEC)/ETEC strains harboring both *stx* and *est* genes. They concluded that virulence genes of different *E. coli* pathogroups can coexist in strains of different phylogenetic lineages and the finding of novel hybrids is a challenge for the traditional diagnostic of *E. coli* infections.

2.1.3 Emergence of Hypervirulent EHEC (STEC) O157 Strains of Clade 8

Manning et al. (2008) showed that both outbreaks, in the United States in 1993 and Japan in 1996, had low rates of hospitalization and HUS in comparison with the 2006 North American spinach outbreak. Phylogenetic analysis identified 39 SNP genotypes in a broad collection of STEC O157 and allowed to separate the isolates into nine distinct clades. Patients with HUS were significantly more likely to be infected with strains of clade 8, which have increased in frequency over the past 5 years. It has been suggested that enhanced severity related to clade 8 strains may be explained by the overexpression of some genes, particularly stx_2 (Neupane et al. 2011). Moreover, Kulasekara et al. (2009) examined the genome of TW14359, the strain associated with the spinach outbreak in the United States, and they compared it to the genome of two other sequenced prototype strains (EDL933 and Sakai). They found seven coding sequences postulated as putative virulence factors that could be responsible for the high virulence of this strain. Lineage-specific polymorphic assay (LSPA-6), derived from octamer-based genome scanning by selecting six loci that are biased in their allelic distribution among *E. coli* O157 strains (Yang et al. 2004), allows the description of lineages I, II, and I/II that have a different distribution among different hosts and different geographical regions (Yang et al. 2004; Manning et al. 2008; Hirai et al. 2014; Mellor et al. 2015). Also, several studies have shown a marked difference on lineages distribution between human isolates and strains from the cattle reservoir (Whitworth et al. 2010; Franz et al. 2012). LI/II predominates in both clinical and bovine isolates in Argentina and Australia (Mellor et al. 2012). Pianciola et al. (2014) have described the almost exclusive circulation of *E. coli* O157 strains belonging to the hypervirulent clade 8 (>90 %) and also the presence of putative virulence factors in higher frequencies than those reported in Neuquén Province, Argentina, a region with one of the highest HUS incidence worldwide. Recently, the same group has demonstrated a high prevalence of EHEC (STEC) O157 clade 8 in human strains with the exclusive presence of LSPA-6 lineage I/II in strains from other regions of Argentina. This particular scenario may be originated in a similar situation in the bovine reservoir, with only slight differences. This homogeneity in EHEC (STEC) O157 genotypes detected in human and bovine strains contrasts with results reported in other countries (Pianciola et al. 2016).

2.2 Advances in the Knowledge of LEE-Negative EHEC (STEC) Strains Associated with Human Disease

EHEC (STEC) O113:H21 is a LEE-negative strain prevalent in the environment, which has been isolated from foods and animals and also from patients with severe disease. Feng et al. (2014) have described a PCR microarray and *stx* subtyping PCR to characterize 65 strains isolated from various sources (environment, food, and clinical infections) and geographical locations (Argentina, Brazil, Canada, and the United States, among others). All the strains carried only Stx subtypes associated with human infections, suggesting that the environmental strains have the potential to cause disease. Most of the O113:H21 strains were closely related and belonged to the same ST223 clonal group, but CRISPR analysis showed a great degree of genetic diversity among the O113:H21 strains. In recent years, another LEE-negative EHEC (STEC) strain serogrouped as O178 have been isolated from cattle and food of bovine origin in South America and Europe. Miko et al. (2014) characterized 74 German and Argentinean *E. coli* O178 strains from animals, food, and humans and studied their serotypes, *stx*-genotypes, and 43 virulence-associated markers by a real-time PCR-microarray. Most ($n=66$) of the strains belonged to serotype O178:H19 and were mainly isolated from cattle and food of bovine origin, but one strain was isolated from an Argentinean patient with HUS. Genotyping the STEC O178:H19 strains by PFGE revealed two major clusters: Cluster A-strains ($n=35$), including the HUS-strain, carried genes associated with severe disease in humans (stx_{2a}, stx_{2d}, *ehxA*, *saa*, sub_{AB1}, $lpfA_{O113}$, *terE* combined with stx_{1a}, *espP*, *iha*), and cluster B-strains ($n=26$) showed a limited repertoire of virulence genes (stx_{2c}, *pagC*, $lpfA_{O113}$, *espP*, *iha*). Based on these results, the authors recommended that EHEC (STEC) O178:H19 strains should be considered with respect to their potential to cause diseases in humans.

2.3 EHEC (STEC) in the Environment

Lascowski et al. (2013) reported the frequency and characteristics of EHEC (STEC) in 1850 treated and untreated drinking water samples, collected in 41 municipalities in the north of Paraná State, Brazil, in the period February 2005–January 2006. A total of 12 isolates, 11 from untreated water and one from treated water, were positive for stx_1/stx_2 (5), stx_1 (2), and stx_2 (5). All stx_2-positive isolates presented the $stx_{2dactivatable}$ subtype, were *eae*-negative, but carried other virulence genes such as *ehxA* (100 %), *saa* (100 %), $lpfA_{O113}$ (75 %), *iha* (42 %), sub_{AB} (25 %), and *cdtV* (8 %). Multidrug resistance was identified in 25 % of the isolates. The strains belonged to seven distinct serotypes and PFGE revealed the presence of two clusters and two clones in the region. The authors concluded that the analysis of the drinking water supplies for pathogenic *E. coli*, as EHEC (STEC), may be useful to prevent waterborne outbreaks. Tanaro et al. (2014) reported the isolation of *E. coli* O157:H7 from

311 surface water samples exposed and not exposed to runoff from corrals, near 48 cattle feedlots, distributed in the Province of Entre Ríos, Argentina, in the period April 2009–July 2011. By multiplex PCR, 70.5 % of the exposed surface water (ESW) samples were rfb_{O157}-positive, and 62 *E. coli* O157 and 32 EHEC (STEC) O157:H7 strains were isolated. In the non-exposed surface water (NESW) samples, 60.0 % were rfb_{O157}-positive, 9 *E. coli* O157, and 6 EHEC (STEC) O157:H7 strains. Although no significant differences were found, these results showed that the ESW tended to be more contaminated with EHEC (STEC) O157:H7 than NESW.

These findings highlight the relevance of the persistence of EHEC (STEC) in the environment as a result of the extensive livestock farming, and the risk that pathogens contained in feedlot runoff may reach recreational waters and also contaminate produce through irrigation, increasing the potential dissemination of O157 strain and the subsequent risk for humans. Matheus-Guimarães et al. (2014) have determined the ability of 14 O157 and 8 non-O157 strains, isolated from bovine hide and carcass, to interact with biotic and abiotic surfaces. Biofilm formation assays showed that four O157 and two non-O157 strains were able to adhere to glass, and only one O157 strain to polystyrene. The data suggested that STEC strains can have different factors involved in the biofilm production on diverse surfaces. The ability of non-O157 LEE-negative strains to form biofilm highlights an industrial and health problem that cannot be ignored. Moreover, the detection of an O157 EHEC (STEC) strain that is able to form biofilm on different surfaces and adhere to and invade human cells indicates an important ability to persist in the environment and to interact with the host.

3 EHEC (STEC) in Latin America

3.1 Surveillance of EHEC (STEC) Infections Tends to Integrate Food Chain Surveillance Systems

In general, there are different types of food-borne surveillance systems, including event-based surveillance, indicator-based surveillance, and integrated food chain surveillance. Each country must determine the most appropriate structure for their surveillance system based on their available resources. In America, there are different implemented surveillance systems, depending on the country. The industrialized countries show integrated food chain surveillance systems established according to national regulations and including different networks that work together with standardized protocols. Integrated food chain surveillance is viewed as the optimal practice for conducting continuous risk analysis for food-borne diseases, but also requires significant ongoing resources and greater multidisciplinary collaboration compared to the other systems (Ford et al. 2015).

Different authors have described the surveillance implemented in the United States and Canada, demonstrating how the surveillance system has the capability to

help assess the magnitude of the food safety problem, define priorities for action, establish transmission pathways and food sources, provide different control options, define targets along the food chain, and measure the success of food safety interventions (Havelaar et al. 2007; Gaulin et al. 2014; Whitney et al. 2015).

In the United States, *E. coli* O157:H7 infections became nationally notifiable in 1995. Since 2000, all EHEC (STEC) infections that cause human illness are notifiable to the Nationally Notifiable Diseases Surveillance System (NNDSS). The Foodborne Diseases Active Surveillance Network (FoodNet) monitors the incidence of laboratory-confirmed infections caused by nine pathogens commonly transmitted through food, including O157 and non-O157. In 2014, 690 cases of non-O157 EHEC (STEC) and 445 cases of O157 were notified, with incidence rates of 1.43 and 0.92/100,000 population, respectively. Among 546 (79%) serogrouped non-O157 isolates, the top O-groups were O26 (31%), O103 (24%), and O111 (19%). Compared with the 2011–2013 period, the incidence of EHEC (STEC) O157 infections was lower, while the incidence of non-O157 infections increased. In 2013, a total of 87 cases of post-diarrheal HUS were reported among children aged <18 years (0.79 cases per 100,000). Of these, 46 (53%) occurred in children aged <5 years (1.55 cases per 100,000) (Crim et al. 2015).

In Canada, EHEC (STEC) infection has been classified as a notifiable disease since 1990. FoodNet Canada's (formerly known as C-Enter Net) is the comprehensive and integrated surveillance system that focuses on active surveillance of human cases of illness, coupled with monitoring possible sources of illness in food, animals, and water. In 2013, 470 *E. coli* O157 cases occurred (1.34 cases/100,000 populations), with 245 hospitalizations and 8 fatalities. For each *E. coli* O157 case reported to Canada's National Surveillance System, it is estimated that there are approximately 20 cases in the community (Government of Canada 2015).

3.2 Surveillance and Epidemiology of EHEC (STEC) Infectious Diseases in Latin America

In Latin America, the EHEC (STEC) surveillance systems are different in each country, and they were implemented according to priorities in public health and resources. In recent years, the countries have enhanced their strategies, working in agreement with different partners (other countries, PAHO, WHO, CDC, PulseNet, Sanger Institute). The need to respond to different epidemiological situations at national, regional, and international levels motivated countries to get an improvement in diagnosis and subtyping. Furthermore, since national surveillance systems for EHEC (STEC) have improved, an increased report in number of clinical cases and etiologic agent detection was observed.

The report of EHEC (STEC) infectious diseases and HUS cases is different in each country. In general, the report relies primarily on syndromic surveillance through the food-borne diseases surveillance System (Argentina, Bolivia, and

Paraguay) and/or through the acute diarrheal surveillance system (Argentina, Brazil, Chile, Costa Rica, Paraguay, and Peru). The official notification of HUS is mandatory in Argentina, Bolivia, Brazil, Chile, and Paraguay. Countries like Uruguay and Costa Rica do not have a formal surveillance system for HUS and EHEC (STEC) infections. In general, the syndromic surveillance is reinforced with laboratory-based surveillance through their National Networks. Depending on the laboratory capacity, resources, and infrastructure, molecular methods for EHEC (STEC) detection were implemented at different levels, in each country. Mostly, the preliminary results in local laboratories are still generated by isolation and phenotypic methods (serotyping, enterohemolysin detection, among others) and then strains are submitted to the National Reference Laboratory (NRL) for further confirmation (by PCR, MLVA, PFGE). The NRLs participate in the Network's External Quality Assurance System (EQAS; National Food Institute, Denmark / Global Foodborne Infections Network) for quality assurance evaluation for food-borne pathogens diagnosis and/or more specifically in the Quality Assurance for EHEC (STEC) diagnosis and subtyping (Staten Serum Institute, Denmark). Additionally, countries are evaluated for EHEC (STEC) subtyping by PFGE through the PulseNet Latin America and the Caribbean (PNALC) QAQC program (certification and proficiency testing) established according to the requirements of PulseNet International (PNInt).

In Argentina, post-diarrheal HUS is endemic and the prevalence is the highest worldwide. Data on human EHEC (STEC) infections are gathered through different strategies: (1) the National Health Surveillance System collects data of HUS cases, and since 2000, the report is mandatory and must be immediate and individualized; (2) the Sentinel Surveillance System through 25 HUS Sentinel Units; (3) the Laboratory-based Surveillance System through the National Diarrheal and Foodborne Pathogens Network; and (4) the Molecular Surveillance through the PNALC. Over the last 10 years, around 400 HUS cases were reported annually. In the period of 2010–2015, the median of incidence was 8.4 cases per 100,000 children <5 year of age and the lethality was between 2 and 5 %. Most (36 %) of the cases were children <5 years old and 56 % were female (Ministerio de Salud 2016. http://www.msal.gov.ar/index.php/home/boletin-integrado-de-vigilancia). In 2015, 337 HUS cases were notified and 190/257 (73.9 %) EHEC (STEC) infections were confirmed at LNR. O157:H7 (56.3 %) was the predominant serotype, with the stx_{2a}/stx_{2c}/*eae*/*ehxA* genotype, followed by O145:H-[$fliC_{H28}$] (13.4 %), stx_{2a}/*eae*/*ehxA*. Recently, an indirect diagnostic of antibodies against EHEC (STEC) O157, O145, and O121 by ELISA, using glycoconjugates (glyco-iELISAs), was implemented, representing an improvement in the diagnostic, especially in those cases where the isolation was not possible (Melli et al. 2015).

In general, HUS cases are sporadic; however, some outbreaks are reported through the surveillance system of HUS and associated diseases. In the period 2013–2015, 27 outbreaks of bloody diarrhea and HUS, associated with O157 and non-O157 STEC strains (24 in families, 2 in kindergartens, and 1 in the community), were identified. The first detection of hybrid strains (Carbonari et al. 2015) was a laboratory finding, and the NRL was forced to modify the screening workflow for a broader molecular characterization to enhance the sensitivity in the diagnosis.

In Chile, the clinical surveillance of HUS and laboratory-based surveillance for EHEC (STEC) infections are mandatory at national level and establish to send the isolates to the Instituto de Salud Pública as NRL for further confirmation. In the period 2007–2013, 599/2425 (24.7 %) of the strains received were confirmed as EHEC (STEC). The predominant serotypes were O157:H7 (52.7 %), O26:H11 (24.9 %), and O26:H− (7.3 %). The majority of cases were from the Región Metropolitana (74.0 %), 52.2 % were males and the most affected group corresponded to children aged 1–4 years old (Ministerio de Salud 2014. http://www.ispch.cl/sites/default/files/STEC.pdf).

In Uruguay, the report of HUS cases is included in food-borne diseases outbreaks or public health events of national significance and become immediate notification. The incidence of HUS is approximately 5/100,000 children <5 years old, with 12–15 new cases annually. Among 43 children with clinical diagnosis of post-diarrheal HUS studied (2002–2013), in seven cases EHEC (STEC) strains of different serotypes and genotypic profiles (O157:H7, *stx*$_2$/*eae*$_{\gamma1}$/*ehxA*, O26:H11, *stx*$_1$/*eae*$_{\beta1}$/*ehxA*, O26:H−, *stx*$_1$/*stx*$_2$/*eae*$_{\beta1}$/*ehxA*, O111:H−, *stx*$_1$/*stx*$_2$/*eae*$_{\gamma2}$/*ehxA*, O145:HNT, *stx*$_2$/*eae*$_{\beta2}$/*ehxA*, and ONT, *stx*$_2$/*eae*/*ehx*A) were recovered. In one case, a co-infection (O26:H11/O145:HNT) was detected (Varela and Schelotto 2015). In 2012, extra-intestinal O157:H7 infections in two elderly women were reported for the first time in Uruguay. The strains were characterized as *stx*$_1$/*eae*$_{\gamma1}$/*ehxA*/*fliC*$_{H7}$/*fimA* of phage type (PT) 39 and *stx*$_1$/*stx*$_2$/*eae*$_{\gamma1}$/*fliC*$_{H7}$/*fimA* of PT40 (Gadea et al. 2012). In 2010–2011, a descriptive study was conducted to determine etiology and clinical manifestations of acute diarrhea in children up to 5 years of age from high socioeconomic level households. Out of 59 diarrheal cases, two O26 and one O153 EHEC (STEC) strains were detected. A child infected with EHEC (STEC) O26 *stx*$_1$/*stx*$_2$/*eae*$_{\beta1}$/*ehxA* strain, who had bloody diarrhea, developed a complete HUS after 20 days, requiring dialysis in the acute stage (Varela and Schelotto 2015).

In Brazil, EHEC (STEC) infections are important public health issues in some regions, but in general the incidence is relatively low (Guth et al. 2010). The HUS surveillance is mandatory at national level and the EHEC (STEC) surveillance is performed through monitoring diarrheal diseases, targeting mainly the detection of diarrhea outbreaks. Furthermore, each state could have the own regulations with specific programs to reinforce the national surveillance. The HUS Laboratory Network consists of five Sentinel Laboratories and the Instituto Adolfo Lutz is the NRL, to which all strains are submitted for further characterization and subtyping. Human infections are linked mostly to sporadic cases of non-bloody diarrhea associated mainly with non-O157 strains. However, HUS cases associated with O157 as well as non-O157 infections have been described in São Paulo State. Almost half (46 %) of patients were <2 years old and female (61.5 %). EHEC (STEC) strains were isolated from 3/7 patients, and serotypes O26:H11 (*stx*$_1$/*eae*/*ehxA*), O157:H7 (*stx*$_{2a}$/*stx*$_{2c}$/*eae*/*ehxA*), and O165:HNM (*stx*$_{2a}$/*stx*$_{2c}$/*eae*/*ehxA*) were identified (de Souza et al. 2011). The results of the indirect diagnosis by LPS antibodies-ELISA showed that seven sera yielded positive signal for O157 LPS-antibodies and 2 for O111 LPS-antibodies (de Souza et al. 2011). In the Rio de Janeiro State, from a total

of 1154 strains received in the period 2013–2015 by NRL for Enteric Diseases at the Instituto Oswaldo Cruz (FIOCRUZ), 42 (3.6 %) were confirmed as non-O157 EHEC (STEC). The origin of the strains was human (24), foods (2), environment (1), and animal (15) (Rodriguez, personal communication).

In Bolivia, the surveillance has been improved through a South-South Cooperation Project with Argentina. In 2/3 HUS cases notified in 2014–2015, O157/stx_2 and O26/stx_1 strains were isolated from children <2 years old. Also, 30 EHEC (STEC) O157 and non-O157 strains were detected in ground beef during sampling procedures at retail stores, conducted in 2010–2013 by the Red de Laboratorios Oficiales de Análisis de Alimentos (RELOAA), in La Paz, Cochabamba, Tarija, Sucre and Santa Cruz Departments. The highest detection rate (56.6 %) was in La Paz and El Alto. All O157:H7 strains ($n = 9$) were stx_{1a}/stx_{2a}/*eae*/*ehxA*, except one that was stx_{2a}. The non-O157 isolates were O8:H19 stx_{2b}/*saa*/*ehxA*, O91:HNT stx_{2b}/*saa*/*ehxA*, O126:H27 stx_1/*saa,* and ONT:H28 stx_1/*saa*/*ehxA* (1, each one), while 18 strains were not serotyped. (Damiani and Montiveros, personal communication).

In Paraguay, the HUS notification is mandatory, immediate, and individualized. The EHEC (STEC) infections are gathered by the laboratorial surveillance of diarrheal and food-borne diseases (Ministerio de Salud y Bienestar Social, 2015. http://vigisalud.gov.py/wp-content/uploads/2015/12/GNVNPY.pdf). In 2013–2015, ten HUS cases without EHEC (STEC) isolation were notified. However, strains were detected in eight sporadic diarrhea cases and characterized as O26 stx_1/stx_2/*eae*, ONT:HNT stx_1/*eae*, ONT:HNT stx_2, and ONT:HNT stx_1/stx_2, ONT:HNT stx_1 (Weiler, personal communication).

In Costa Rica, EHEC (STEC) infections are not notifiable in the surveillance system. However, the Centro Nacional de Referencia de Bacteriología (CNRB) of Inciensa has implemented a differential diagnostic protocol for pathogens associated to diarrheal cases, mostly involved in outbreak or dead. Through this strategy in 2013–2015, 11 EHEC (STEC) strains O157:H7/stx_2/*eae* (2), O145/stx_2/*eae* (3), non-O157 stx_1/*eae* (4), and non-O157 *stx*1 (2) were isolated from sporadic childhood diarrheal cases. The CNRB also receive for confirmation purposes strains isolated from water and food from the Laboratory Network and from animals submitted by the Laboratorio Nacional de Servicios Veterinarios (LANASEVE) depending on the Servicio Nacional de Sanidad Animal (SENASA, Ministerio de Agricultura). Throughout sampling procedures in four exporting food plants, performed by the Dirección de Inocuidad de Productos de Origen Animal (DIPOA), using the USDA/FSIS guidelines, 18 non-toxigenic O157:H7, three O157 stx_1/stx_2, one O157 stx_2, and one non-O157 stx_1 strains were detected (Bolaños and Duarte, personal communication).

In Peru, the surveillance of EHEC (STEC) infections is performed through the monitoring of acute diarrheal diseases. The Instituto Nacional de Salud as NRL has implemented molecular protocols for laboratorial-based surveillance. Four O157:H7 strains genotyped as stx_2 (2), stx_1, and stx_1/stx_2 were isolated from diarrheal cases attended in Lima in 2014. One HUS case was reported in Lambayeque without EHEC (STEC) isolation (Zamudio, personal communication).

3.3 Technologic Improvement of Latin America Laboratories for the Integration of the Region in the Worldwide EHEC (STEC) Surveillance

Laboratory-based surveillance for detection of EHEC (STEC) is a key in the surveillance of associated diseases, globally. Actually, the international trends indicate that just the improvement of the national surveillance is not enough and it is essential to get a worldwide coverage. Collaboration and data sharing between organizations and countries is required due to the international dimension of food-borne pathogens and food trade in particular.

Reference Laboratories of Central and South America participate regionally in the Global Foodborne Infections Network (GFN) that give support in diagnosis capability and improve the response capacity of food-borne diseases. As part of PNLAC, NRLs contribute to the molecular surveillance of food-borne pathogens, in the framework of PNInt.

In the PNALC Regional Database, there are 498 PFGE patterns, corresponding to 985 STEC O157 strains isolated in 1988–2015 in five countries (Argentina, Chile, Cuba, Paraguay, and Uruguay). A high genetic diversity among the strains of different countries (63.1 %) is observed, and certain PFGE patterns highly related are detected in specific countries. Interestingly, there are strains with identical patterns circulating in Argentina and Chile. PNALC offers to the countries the possibility to join efforts, working with standardized protocols under a quality control system that let to participate actively in the worldwide surveillance. The network is continually working trying to get harmonization between countries in the implementation and performance of novel technologies. At present, the vision of PNInt is the worldwide use of WGS in all public health laboratories to identify, characterize, and subtype foodborne bacterial pathogens, replacing existing phenotypic and molecular methods as support of food-borne disease surveillance and thus reach the reduction of the burden of these diseases. This proposal includes mainly the use of wg-MLST as strategy for sequence analysis. Because of the public health risk, the first approach on wg-MLST was a pilot project on *Listeria* and now continues with EHEC (STEC). For this strategy, it is needed to build an allele database stored in a unique engine server. Standardized protocols, validation, and nomenclature designation are in progress in order to work on the harmonization of the strategies among the countries globally.

All members of PNInt, including PNLAC, agree to transition to WGS. The situation in countries of Latin America could be briefly described in three items, (1) the wide range of capability. The first steps in training were done by courses/workshops in the framework of PNALC in collaboration with the Wellcome Trust Sanger Institute. Moreover, some countries are in the stage of equipment acquisition or installation (Paraguay and Venezuela), and others have already implemented WGS, but the current use is just for research, no for routine surveillance (Argentina, Chile, Colombia, México, and Peru); (2) the availability of resources to WGS is variable among countries and maybe it is the major weakness to overcome; (3) some barriers, like bioinformatics capacity for analysis and storage, and connectivity issues should be improved.

At present, Argentina is participating as external laboratory, in a Pilot Project of WHO/FDA for WGS implementation to support public health surveillance, in the framework of the Genome TRAKR Project. Because of the endemic situation of HUS and EHEC (STEC) infectious diseases in the country, a specific project was included. In order to determine the concordance between routine tests and WGS results regarding detection and characterization issues for diagnosis and discrimination and relationships among strains for outbreak and clade detection, the LNR has run 16 EHEC (STEC) O157:H7 strains of different clades, sources, and different date of isolation. The obtained sequences had comparable coverage and genome size and passed QC assessment, and they were accessible at NCBI (BioProject PRJNA282762). An agreement with previous results, mainly regarding identification, characterization, outbreak, and clade detection, was observed. By WGS, additional information like other virulence factors, ST and phylogenetic tree, could be analyzed (Chinen et al. 2015).

4 Conclusions

Advances on the knowledge of pathogenesis, virulence determinants, and risk factors have contributed to the development of several strategies trying to prevent food and environment contamination, and consequently transmission to humans. The uses of new techniques, like WGS typing, has been useful in surveillance, diagnosis, and epidemiological studies, as well as the discovery of emerging genotypes and identifying the genetic differences between human pathogenic and nonpathogenic EHEC (STEC) strains.

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