Shiga-toxin *Escherichia coli* Contamination in Cattle Post Harvest

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Shiga toxin-producing *Escherichia coli*: The biology and virulence factors

Escherichia coli are gram-negative, rod-shaped, and facultative anaerobic bacteria. They belong to the family of *Enterobacteriaceae*. This group of bacteria is commonly present in the intestine of humans and warm-blooded animals. Most strains are not pathogenic to their host, but some strains are pathogenic and a significant threat to public health (Janda & Abbott, 2006)

There are three major groups of *E. coli*, characterized by their genetic makeup and their associated clinical illness: commensal, diarrheal, and extra-intestinal (Russo & Johnson, 2000). Commensal strains coexist within the intestinal tract and do not cause diseases in their hosts. They typically share mutual benefits with their hosts; however, they are capable of causing clinical infection in immunocompromised individuals. In contrast, diarrheal strains are not commonly encountered in the intestinal tract of humans and animals, and thus, colonization of these strains could lead to gastroenteritis or colitis in a naïve host. Six types of diarrheal strains that can affect human intestines are enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC). EPEC are an important cause of infant diarrhea in developing countries. EHEC cause bloody diarrhea (hemorrhagic colitis), non-bloody diarrhea and hemolytic uremic syndrome (HUS). ETEC cause watery diarrhea, a major cause of childhood diarrhea in developing countries and diarrhea in travelers to developing countries. EAEC cause persistent diarrhea in children and adults in both developing and developed countries. EIEC are genetically and pathogenically related to *Shigella*; they cause invasive inflammatory colitis,

dysentery, and watery diarrhea. DAEC cause diarrhea in children less than 1 year of age. Finally, extra-intestinal strains are pathogenic strains that colonize and infect other tissues, such as the kidney, bladder, and brain. These strains are also known as extra-intestinal-pathogenic *E. coli* (ExPEC) (Chattopadhyay & Sokurenko, 2013; Kaper, Nataro, & Mobley, 2004; Russo & Johnson, 2000).

Based on microscopic structure, pathogenic *E. coli* have three important antigenic characteristics. They are O (lipopolysaccharide -LPS- on the cell wall) antigen, H (flagella) antigen, and K (capsule) antigen. A specific combination of O, H, and—sometimes—K antigens defines the serotype of pathogenic *E. coli*; while serogroup classification is based on the possession of O antigen (Kaper et al., 2004). There are six serogroups (O26, O45, O103, O111, O121, O145) and one serotype (O157:H7) that will be discussed more specifically in this review because they are responsible for the majority of foodborne illness in the United States (Centers for Disease Control and Prevention, 2017).

The term Shiga-toxin producing *E. coli* (STEC) was coined to refer to *E. coli* serotypes able to produce one or more Shiga-toxins (Guth, Prado, & Rivas, 2010). There are over 100 *E. coli* strains that can express Shiga-toxins (Karmali, 1989). Shiga toxins (*stx*) are cytotoxins that have the ability to inhibit protein synthesis in eukaryotic cells. In the 19th century, Dr. Kiyoshi Shiga was the first scientist who identified Shiga-toxin from *Shigella dysenteriae.* In honor of his discovery, the toxin was named after him (Trofa, Ueno-Olsen, Oiwa, & Yoshikawa, 1999). This toxin was first identified from *E. coli*, as having cytotoxic activity in Vero cells. The term Verotoxigenic *E. coli* (VTEC) was coined to refer to the activity of this toxin (Konowalchuk, Speirs, & Stavric, 1977). Later findings revealed that this toxin biologically and structurally resembles Shiga-toxin produced by *Shigella dysenteriae* type 1 (O'Brien et al., 1984; O'Brien,

LaVeck, Thompson, & Formal, 1982). Therefore, the term STEC is more commonly used in the current reports rather than VTEC. STEC and VTEC, however, are equivalent terms as both refer to strains that produce one or more Shiga-toxins.

There are two immunologically distinct types of Shiga-toxins produced by STEC strains, and they are *Stx*$_1$ and *Stx*$_2$. *Stx*$_1$ is almost identical to Shiga-toxin produced by *S. dysenteriae* type 1, whereas *Stx*$_2$ is antigenically distinct. However, *Stx*$_2$ has the same mode of action with *Stx*$_1$; they bind to the same receptor to inhibit protein synthesis in eukaryotic cells (Scheutz et al., 2012). *Stx*$_2$ are linked to more severe disease in humans than *Stx*$_1$ (Luna-Gierke et al., 2014). Variants of the *Stx*$_1$ include *Stx*$_{1a}$, *Stx*$_{1c}$, and *Stx*$_{1d}$; whereas *Stx*$_{2a,}$ *Stx*$_{2b,}$ *Stx*$_{2c,}$ *Stx*$_{2d,}$ *Stx*$_{2e,}$ *Stx*$_{2f,}$ and *Stx*$_{2g}$ belong to the *Stx*$_2$ group (Scheutz et al., 2012). Certain variants are rarely present in humans. *Stx*$_{2e}$, for example, is commonly isolated from swine and causes a fatal neurological disease known as edema disease (Weinstein et al., 1988). STEC harboring *Stx*$_{2c}$ have a stronger association with human infections that progress to hemolytic uremic syndrome (HUS) while *Stx*$_{2d}$ and *Stx*$_{2e}$ are more likely to be associated with milder disease with minimal risk of HUS (Friedrich et al., 2002).

The surface of target cells for Shiga-toxin has a specific glycolipid receptor (Gb_3). The concentration of Gb_3 in a host determines its susceptibility to STEC infection. Gb_3 receptors are abundantly present in humans, especially on the renal tubular cells as well as microvascular endothelial cells of kidney, gut, and brain (Paton & Paton, 2006). During STEC infection, the bacteria release Shiga-toxins into the gut lumen. If sufficiently produced, translocation of the toxin into the underlying tissues leads to bloody diarrhea due to the toxic effect of *Stx* on the endothelial cells of the intestines. *Stx* translocation and absorption into the bloodstream results in the systemic distribution and binding to the renal endothelium and renal tubular cells. *Stx* damages renal endothelial cells and occludes microvasculature through a combination of direct toxicity and

production of local cytokines and chemokines results in renal inflammation. This condition may lead to Hemorrhagic uremic syndrome (HUS) (Kaper et al., 2004; Paton & Paton, 2006). Intimin contributes significantly to the very efficient adherence of bacteria to the intestinal mucosa, and thus, facilitate the introduction of *Stx* to the systemic circulation and to the development of HUS (Hauswaldt, Nitschke, Sayk, Solbach, & Knobloch, 2013).

Certain STEC strains may carry a large pathogenicity island named Locus Enterocyte Effacement (LEE). These LEE-positive strains carry the *eae* gene that encodes for intimin. Intimin is a virulence factor necessary for adherence of the bacteria to the enterocytes of the colon and leads to a characteristic histopathological lesion described as "Attaching and Effacing" (A/E) lesion. Destruction of enterocyte microvilli is the characteristic sign of the A/E lesion and the clinical manifestation is bloody diarrhea. This gene is also presents in EPEC strains and produces identical A/E lesion in the intestine of their hosts (Donnenberg et al., 1993; Jerse, Yu, Tall, & Kaper, 1990; McDaniel, Jarvis, Donnenberg, & Kaper, 1995). As noted earlier, the term STEC refers to *E. coli* that only produce *Stx*; whereas the term EHEC refers to a subset of STEC that also carry the *eae* gene (Kaper, Nataro, & Mobley, 2004). One of the most important characteristics of STEC O157 isolates is that almost all of them express intimin and are able to induce A/E lesions in any type of tissue. Thus, this strain belongs to the EHEC group (Law, 2000).

Although *Stx* could cause a systemic reaction, in most systemic cases, the infection does not depend solely on *Stx* production and distribution. The combined presence of virulence genes for *Stx* and intimin (*eae*) are predictive for severe cases in humans. Stx_2 and intimin (*eae*) are believed to act synergistically to develop HUS cases in humans (Boerlin et al., 1999; Ethelberg et al., 2004). Moreover, being a child ($\leq$ 7 years old) and having bloody diarrhea are also determinant factors of the development of HUS (Ethelberg et al., 2004).

The *ehxA* (enterohemolysin) has been proposed to contribute to the clinical pathogenicity of STEC. *EhxA* is a member of repeat toxin (RTX) family and this toxin has a capability to lyse red blood cells (H. Schmidt & Beutin, 1995). Although STEC strains commonly express the *ehxA* gene (Arthur et al., 2009), many non-pathogenic STEC strains in the environment also carry and express the *ehxA* gene (Boczek, Johnson, Rice, & Kinkle, 2006). The role of this toxin in disease pathogenesis is still uncertain. Moreover, there is no strong association between the presence of *ehxA* gene in STEC and clinical symptoms in humans. A study of 343 STEC isolates in clinically infected humans showed that 77% had the *exhA* gene, but it was not associated with the occurrence of HUS or bloody diarrhea (Ethelberg et al., 2004).

STEC/EHEC can be isolated from a wide variety of animals, such as sheep, goats, pigs, cattle, birds, dogs and cats (Wallace, et al, 1995; Beutin, et al., 1995). STEC and EHEC have also been detected from shellfish (Bennani et al., 2011). In adult ruminants, STEC are non-pathogenic; therefore, healthy ruminants are considered to be asymptomatic reservoirs. Yet, STEC can be pathogenic for nursery pigs (Fricke et al., 2015).

In the feedlot, *E. coli* have capability to survive in manure, water, soil, and other surface areas for extended periods. Furthermore, contaminated manure can easily contaminate soil and ground water through natural processes, such as infiltration of rainfall and storm water runoff. The use of contaminated-irrigation water or contaminated-animal manure as fertilizer in vegetable production has been reported to cause *E. coli* outbreaks from vegetable and fruit consumption (Erickson & Doyle, 2007; Chalmers, Aird & Bolton, 2000). An experimental study reported that *E. coli* O157 from manure-contaminated soil were able to enter lettuce plant root systems and migrate to the edible part of the vegetable (Solomon, Yaron, & Matthews, 2002).

Human illnesses: importance of STEC

Since the first STEC human outbreak reported in the United States in 1983 (Riley et al., 1983), these serogroups continue to cause outbreaks worldwide, and these outbreaks are associated with different vehicles and sources of transmission. A systematic review estimated that globally, STEC cause 2,801,000 acute illnesses annually, lead to 3,890 cases of HUS, 270 cases of end-stage renal disease, and 230 deaths. Compared to alveolar echinococcosis, another foodborne pathogen, STEC appear to cause more cases; nonetheless, STEC is associated with fewer cases than typhoid fever, foodborne trematodes, and nontyphoidal salmonellosis (Majowicz et al., 2014).

In the United States, STEC O157:H7 is estimated to cause 63,153 cases; 2,138 hospitalizations; and 20 deaths each year (Painter et al., 2013; Scallan et al., 2011). STEC non-O157 are the other STEC serogroups that contribute to foodborne illness in humans. According to the CDC, the six most frequent non-O157 STEC associated with human illness are O26, O45, O103, O111, O121, and O145 (Centers for Disease Control and Prevention, 2017). Non-O157 STEC are estimated to cause 112,752 cases and 217 hospitalizations annually in the U.S. (Painter et al., 2013; Scallan et al., 2011).

The epidemiological risk factors of STEC vary between countries. In Germany, for example, the four most common serotypes causing human outbreaks from 1997 to 2013 were O26, O91, O103, and O157. These serotypes are somewhat similar to the most frequent serotypes reported in the U.S (Fruth, Prager, Tietze, Rabsch, & Flieger, 2015).

The interval between ingestion of the bacteria and the clinical manifestation of the disease may range between 2-12 days (Talarico, Aloe, Monzani, Miniero, & Bona, 2016). Ingestion of a small number of STEC O157 (100 cells or less) may result in clinical symptoms in humans

(Caprioli, Morabito, Brugère, & Oswald, 2005; Strachan, Fenlon, & Ogden, 2001). However, some people may carry STEC as part of their intestinal microbia without being sick (Al-Gallas, Bahri, & Aissa, 2006; Silvestro et al., 2004). Clinical signs and symptoms of STEC infection may range from non-bloody diarrhea, hemorrhagic colitis, hemolytic uremic syndrome (HUS) (Kaper et al., 2004; Paton & Paton, 2006).

Karmali and colleagues in 1983-1985 were the first to establish the association between STEC infection and the development of HUS in humans (Karmali et al., 1985). HUS is a thrombotic microangiopathy characterized by thrombocytopenia, non-immune mediated microangiopathic hemolytic anemia, and acute renal failure. Although not every HUS case is associated with STEC infection, STEC/EHEC are the most common infectious agents causing HUS in humans. STEC can affect multiple organs such as kidney, gastrointestinal tract, and central nervous system. The strain of STEC/EHEC, virulence factors produced by the pathogens, and therapeutic approaches administered to the patients influences the outcome of the disease. Certain patients may require renal dialysis therapy or renal transplant. Patients with genetic disorders of complement regulation or underlying diseases are more likely to develop severe disease (Scheiring, Andreoli, & Zimmerhackl, 2008). The STEC-associated disease may occur at any age, but infants, young children less than 7 years, and older adults are considered to be at highest risk (Ethelberg et al., 2004; Rivas, Chinen, Miliwebsky, & Masana, 2015).

STEC are easily transmitted through multiple modes of transmission, such as contaminated water and food, direct contact with infected animals, person-to-person, and laboratory activity related to these strains. Transmission from person-to-person contact is possible because certain individuals are able to be long-term shedders and may carry the bacteria for long periods of time even after they are clinically recovered. A study in Germany reported that patients may shed STEC

O157 in their feces for 2 to 124 days. Patients that developed HUS were more likely to shed the pathogens for a longer period than patients with diarrhea or hemorrhagic colitis. The study suggested that all long-term shedders are super-shedder (bacteria concentration were greater than 10^6 CFU/g stool) (Karch, Rüssmann, Schmidt, Schwarzkopf, & Heesemann, 1995). Person-to-person transmissions in nursery school outbreaks have been reported in Germany, Japan, France, and the United Kingdom (Al-Jader et al., 1999; Boudailliez et al., 1997; Karch et al., 1995; Terajima, Iyoda, Ohnisi, & Watanabe, 2015). Due to the concern of long-term shedders, one proposal to reduce further dissemination of STEC in nursery outbreaks of STEC is stool culture examination of all children attending daycare and family members in the same household as the children (Karch et al., 1995).

Although rarely reported, engagement in risky behaviors related to contaminated environments may result in STEC infection. Trips to petting zoos or county fairs with direct contact with infected animals (Bender & Shulman, 2004; Keen, Wittum, Dunn, Bono, & Durso, 2006), camping in a contaminated environment (Howie, Mukerjee, Cowden, Leith, & Reid, 2003), swimming in or drinking contaminated water (Bentancor et al., 2012) as well as occupational exposure with the reservoir or raw meat have exposed human to STEC infections (McPherson et al., 2009). Personal hygiene related to hand-to-mouth activity and hand washing is the most important way to prevent these infections (Bender & Shulman, 2004).

In the United States, foods are the most frequently identified vehicle for STEC O157 outbreaks. Between 1982 and 2002, contaminated foods were associated with the majority (52%) of the outbreaks. Ground beef was the most frequent vehicle (41%) for the outbreaks, with fresh produce being the second most common vehicle (21%) (Rangel, Sparling, Crowe, Griffin, & Swerdlow, 2005). This report is not surprising since the main reservoir of STEC is cattle and the

concentration of *E. coli* is usually higher in ground beef compared to other beef retail cuts (Eisel, Linton, & Muriana, 1997).

Eating ground beef that has not been cooked sufficiently to kill STEC is thought to be the primary cause of infection. The first large outbreak of STEC O157 in the U.S. occurred in four western states, and this outbreak had a major impact on educating the public about good practices for food handling and led to the establishment of improved guidelines to control and prevent incidents regarding this pathogen (Bell et al., 1994; Tuttle et al., 1999). Outbreaks involving ground beef peak in summer months, mostly from May to August (Rangel et al., 2005).

Ecology and epidemiology in different cattle production systems

Although more than 500 STEC have been identified from cattle (Blanco et al., 2004), they are not known to be pathogenic (Buncic & Avery, 1997). Unlike humans, cattle lack Gb_3 receptors in the gastrointestinal tract; the receptors are only present in the brain and kidney of cattle. Stx_1 and Stx_2 were produced in STEC O157 colonized calves and cattle; but, since the toxins could not bind to the intestinal tract, they do not translocate and systemically distribute. The absence of Gb_3 receptors in the gastrointestinal tract provides a reason for the non-pathogenic nature of STEC colonization in cattle (Pruimboom-Brees et al., 2000).

Beef and dairy products are the major sources of human foodborne *E. coli* O157:H7 outbreaks in the United States; among them, ground beef is the most common vehicle (Rangel et al., 2005). Most meat products recalled due to the STEC contamination were beef products, such as veal products, boneless beef, beef primals, and ground beef. Ground beef comprised about 15% (23,215 pounds) of the total meat products recalled (144,547 pounds) in 2017 (U.S. Department of Agriculture, 2018).

Cull dairy cows comprised approximately 9.6% of the total U.S. cattle slaughtered (30.6 million head) in federal-inspected plants during 2016 (U.S. Department of Agriculture, 2017). Although the majority of the end product of cull dairy cows is marketed as ground beef (Faith et al., 1996), beef cattle and low-value cuts from finishing steers and heifers are also sources of ground beef. Nonetheless, control measures to reduce human exposure to STEC in cull dairy cattle production is important because approximately 17% of the total ground beef production comes from cull dairy cows (Troutt & Osburn, 1997).

A report from a longitudinal study in Finland provided valuable information about the dynamic presence of *E. coli* O157 in cull dairy cattle. The herd used in the study consisted of about 300 post-weaning bull calves from several dairy farms. All sampled calves (n=134) were negative on the arrival day and the animals were positive for *E. coli* O157 a day later. Based on pulsed-field gel electrophoresis (PFGE) analysis, all positive isolates were matched or related to the isolates obtained at the feedlot before the animals arrived. This result suggested that incoming dairy calves might acquire horizontal infection from the environment in the feedlot. The study also reported the lowest concentration of fecal shedding was in the first month after arrival and the highest was when the bulls were 6 to 9 months old, suggesting that the fecal concentration of *E. coli* O157 may increase over time. The author suggested implementation of an all–in / all-out system of cohort management combined with a long period before introduction of new animals to the same pens to reduce STEC infection in cull dairy cattle (Lahti, Ruoho, Rantala, Hänninen, & Honkanen-Buzalski, 2003). Moreover, they supported the results from other studies that reported that cattle shed more *E. coli* O157 during warmer months (Ekong, Sanderson, & Cernicchiaro, 2015).

The persistence and predominance of *E. coli* O157 strains in the environment and cattle have been established by many studies conducted in feedlots (LeJeune et al., 2004), dairy farms

(Faith et al., 1996), and cow-calf operations (Worley et al., 2017). LeJeune et al., (2004) assumed that the incoming animals might not contribute to the introduction of the persistent and predominant strains in the feedlot. However, other studies reported that the incoming animals might also carry and introduce new EHEC strains into the feedlot (Jones, Octavia, Lammers, Heller, & Lan, 2017; Joris, Verstraete, De Reu, & De Zutter, 2013). The newly introduced strains could become the predominant strains in the feedlot depending on the balance between the prevalence of the new strains and the fitness of these strains versus the resident strains to compete in the cattle farm and the bovine gastrointestinal tract (Sanderson et al., 2006).

Interestingly, STEC persist in extreme environments as well. *E. coli* O157:H7 survived in low-moisture (<10%) dairy cattle manure for extended periods of time (70, 56, and 49 days) and at various temperature (5, 22, and 37^{o}C). The lower the temperature (5^{o}C), the longer the STEC survived (70 days) (Wang, Zhao, & Doyle, 1996). Another study also found that *E. coli* O157:H7 survived in a bovine manure pile for 21 months, and the concentration of the bacteria was maintained at a level from $<10^2$ to 10^6 CFU/g (Kudva, Blanch, & Hovde, 1998).

At the individual cattle level, the concentration of *E. coli* O157 normally shed in feces could range from 10^2 to 10^6 colony-forming unit/gram (CFU/g) feces (Robinson, Wright, Hart, Bennett, & French, 2004). The majority of cattle, however, shed at a low level (less than 10^2 CFU/g) (Pearce et al., 2004). Individual and daily shedding is variable (Robinson et al., 2004). Certain animals have been called "super-shedders" based on one time fecal shedding of *E. coli* O157 at a concentration higher than 10^3 CFU/g (Low et al., 2005) or higher than 10^4 CFU/g (Omisakin, MacRae, Ogden, & Strachan, 2003). Introduction of a " super-shedder" to a naïve population is believed to influence the level of *E. coli* O157 excreted at the pen level, indicating the significant influence of "super-shedder", or at least high shedding animals, on STEC

contamination in the feedlot (Cobbold et al., 2007). Modeling data suggest that individual "super shedder" cattle are not necessary to generate the observed pen prevalence distributions (Chen, Sanderson, & Lanzas, 2013).

Experimentally, adult Holstein and Jersey cattle could be infected by a relatively high dose. A study reported that the infectious dose necessary for the cattle to establish shedding *E. coli* O157:H7 ranged from 10^4 to 10^7 log CFU (Cray & Moon, 1995). However, another study reported a smaller infectious dose, <250 CFU of *E. coli* O157:H7, was sufficient to infect juvenile cattle who were 2 to 24 months of age (Hancock, Besser, & Rice, 1998). This concentration is normally present in the cattle feedlot environment, thus, raising the possibility for cattle to acquire horizontal infection from their environment.

The fecal prevalence of STEC in cattle depends on many factors. A systematic review and meta-analysis reported fecal prevalence of *E. coli* O157 in North America differed by cattle types and seasonality. Fecal prevalence of fed beef, adult beef, and adult dairy were 10.68%, 4.65%, and 1.79%, respectively. Summer and winter month fecal prevalence for adult beef were 7.86% and 4.21%, respectively; whereas for adult dairy were 2.27% and 0.36, respectively. The term fed cattle, in this study, referred to young cattle being fed for slaughter; the term adult beef referred to breeding cows and cull cows from those herds; and the term adult dairy referred to lactating cows and cull cows from those herds (Ekong et al., 2015)

The presence of house flies appears to play a role in the dissemination EHEC O157:H7 among beef cattle and dairy cattle within a farm and to the surrounding environment including residential communities (Alam & Zurek, 2004; Burrus et al., 2016). House flies are also able to carry non-O157 STEC. Of the 463 house flies collected from feedlots and dairy farms from six states, 34.3% carried at least one of six serogroups of interest, O26, O45, O121, O145, O104,

O103. However, STEC was found in only 1.5% of house flies from feedlots. STEC isolated from the feedlots were *E. coli* O103, O104, and O45. All serotypes carried by house flies harbored *Stx1* and *ehxA* genes, and *E. coli* O45 also harbored *eae* gene (Puri-Giri, Ghosh, Thomson, Zurek, & Kaufman, 2017). Therefore, the management of house flies at farms is an important part of pre-harvest food safety.

Infection at dairy farms and feedlots may occur due to the contaminated drinking water or contaminated water troughs (Faith et al., 1996; Sargeant, Sanderson, Smith, & Griffin, 2003). *E. coli* O157 survived experimentally for longer than 6 months in contaminated water troughs. These surviving strains remained infectious to 10-week-old Holstein calves, and the calves excreted the pathogens up to 87 days after challenge (LeJeune, Besser, & Hancock, 2001). These pathogens may survive longer in colder temperatures. In an observational study of dairy farms, *E. coli* O157:H7 survived in cattle drinking water for 8 days at 5^{o}C, and for 4 days at 15^{o}C (Rice & Johnson, 2000). Thus, contaminated water and contaminated water troughs may serve as significant sources of infection, especially during the colder months when the pathogens are rarely detected in cattle feces.

Frequent cleaning has been recommended to reduce survival of these pathogens in water troughs (Hancock et al., 1998). Unchlorinated drinking water appears to maintain the presence of *E. coli* O157 longer than chlorinated water experimentally (LeJeune, Besser, & Hancock, 2001). However, a longitudinal study reported that chlorinated drinking water was not significantly effective to reduce fecal *E. coli* O157 prevalence in feedlot cattle. Organic matter originated from drinking or standing activity of the cattle at the water troughs may provide the reason for chlorine neutralization (LeJeune et al., 2004). Moreover, the contamination level of STEC in the water trough was associated with the proximity of the water trough to the feed bunk and protection of

the trough from direct sunlight (LeJeune, Besser, Merrill, Rice, & Hancock, 2001). Redesigning feedlot water troughs could be an effective strategy to minimize drinking water contamination. Moreover, supplementation of drinking water with chlorate is also suggested because chlorate was experimentally capable to reduce the concentration of *E. coli* O157 and generic *E. coli* in the rumen and feces of non-lactating adult dairy cattle before harvest; adding another pre-harvest strategy to reduce STEC contamination in cull dairy cattle (Callaway et al., 2002).

Commercial feeds may serve as the source of infection for feedlot cattle (Hancock, Besser, Lejeune, Davis, & Rice, 2001). Moreover, a study in the U.K. reported STEC contamination in fresh grasses (6.3%) originated from fields where cattle had grazed within the previous month. These fresh grasses were free from fecal contamination; this indicates the persistence of the bacteria in the environment (Hutchison, Thomas, Walters, & Avery, 2006). A study done in the midwestern U.S. reported 14.9% of feed bunks sampled were contaminated with *E. coli* O157. However, the plausible source for feed contamination is still unknown since the study authors believed that cattle feces were not the source of feed contamination (Dodd et al., 2003).

Other possible environmental sources for STEC infection in the feedlot is fecal contamination from wildlife that live in the same geographic location. A study in two range cattle production facilities located in Kansas and Nebraska isolated *E. coli* O157 from wild opossum, whereas samples from deer, raccoons, and birds were negative. Small sample size in this study limits the ability of the study to provide a reliable estimation of *E. coli* O157 prevalence from those species (Renter, Sargeant, Oberst, & Samadpour, 2003). In Michigan, however, STEC O98 isolates were recovered from white-tailed deer and cattle in a shared agro-ecosystem. The isolates shared similar genomic classifications as confirmed by PCR profiling and multi-locus sequence typing (MLST) analysis, indicating a possibility for interspecies transmission (Singh et al., 2015).

Contamination at harvest

At harvest of cattle, the slaughter process is generally divided into seven steps: the arrival of cattle at the slaughter plant, hide removal, decontamination after hide removal (first decontamination), evisceration, second decontamination, chilling, and carcass fabrication. Carcass fabrication is the process of cutting carcasses into standard wholesale and retail cuts. In plants that slaughter beef cattle, the major products are primal and subprimal cuts of meat. As a result of deboning to obtain these cuts, trim from muscles and fat are separated and collected as a byproduct. Whereas in plants that slaughter cull dairy cattle, trim is the major product that results from deboning. To produce ground beef with a desired fat content, it is necessary to combine trim from fed beef cattle and cull dairy cattle because they have distinctly different levels of 'leanness' that is defined by the fat in the animals. In the U.S., 60% of trim comes from fed steers and heifers intended for meat production; the remainder comes from cull cows and bulls originally used for milk and breeding production (U.S. Department of Agriculture, 2002). During all these steps, there are many opportunities for STEC contamination of the carcass or trim to occur.

Upon arrival at the slaughter plants by truck from feedlots, cattle are placed in holding (lairage) pens until they are slaughtered. Comingling cattle during transportation and in the lairage pen at slaughter plants appears to increase STEC hide contamination (Moxley & Acuff, 2014). Lairage pens contaminated with feces and lairage pens positive for *E. coli* O157 are associated with increasing numbers of hides that test positive for *E. coli* O157 (Dewell et al., 2008). Moreover, transportation distance has been shown to affect the number of hide samples that test positive for *E. coli* O157; the longer distance of transportation the more positive samples were detected (Dewell et al., 2008). A study utilized pulse-field gel electrophoresis detection for *E. coli*

O157 showed that transfer of bacteria occurring in the lairage pens contributed to hide and carcass contamination (Arthur et al., 2008). Those study are supported by meta-analysis (Ekong et al., 2015); which estimated the number of enumerable hides (≥40 CFU/100 cm^2) were notably increased from 1.74% when sampled at feedlot to 23.81% when sampled at the plant.

However, other studies concluded that transportation and lairage time have no impact on the number of STEC detected at the individual animal level. A randomized complete block design study reported that there was no significant effect in the prevalence and the concentration of *E. coli* O157 shed in the feces between the transported and the non-transported cattle (Aperce, Alvarado, Miller, Van Bibber-Krueger, & Drouillard, 2014). Similarly, Stanford et al., (2011) found that transportation factors, such as duration of transportation, temperature-humidity index, and loading density did not affect the perineal swab prevalence between the feedlot and the slaughter plant. This study, however, has a drawback because they controlled the cleanliness of the trailer they used in this study to an extent that may not be consistent with the current standards. Studies conducted in beef cattle in Australia showed similar results. Transportation and lairage time at the abattoir did not increase the concentration of *E. coli* O157 shed in the feces of beef cattle (Fegan, Higgs, Duffy, & Barlow, 2009). These two studies (Fegan et al., 2009; Stanford et al., 2011) suggest that carcass contamination was more likely because of contamination at feedlot than at processing plant.

A risk assessment model suggested that reducing the preharvest fecal prevalence, especially in cohorts with higher level of fecal prevalence, may contribute in reducing the risk of carcass contamination at harvest (Dodd, Sanderson, Jacob, & Renter, 2011). As noted earlier, the presence of "super-shedders" or high shedding individuals in feedlot pens might influence the higher shedding of *E.coli* O157 at the pen level (Cobbold et al., 2007). At the processing plant,

other studies reported consistent findings; the presence of a “super-shedders” was more likely associated with carcass contamination (Fegan et al., 2009; Fox et al., 2008).

There is insufficient data to conclude an association between stress and fasting during transportation and prevalence of STEC on hides and fecal prevalence in slaughtered cattle (Schuehle Pfeiffer et al., 2009). The stunning box is believed to be a potential site for cross contamination during the slaughter process because brisket areas of all cattle hides touched the same spot on the stunning box floor when each animal is stunned, (Avery, Small, Reid, & Buncic, 2002). However, reports regarding stunning box contamination to draw conclusion about the association are lacking.

During the dehiding process, the animal is moved from the stunning box to the main floor of the slaughter plant. In this process, the hide, horns, hocks (joints of leg to foot), and udder are removed. This process can lead to carcass contamination because during the process of removal, the carcass may easily get exposure to the STEC contamination directly from the animal’s own hide or feces, or indirectly from cross contamination from other animals via the machinery in the plant, equipment used by the abattoir’s workers, or STEC-contaminated aerosols in the plant environment (Elder et al., 2000; J. W. Schmidt, Arthur, Bosilevac, Kalchayanand, & Wheeler, 2012; U.S. Department of Agriculture, 2002). A study that utilized surrogate microorganisms approved for approximating *E. coli* O157:H7 ecology demonstrated this cross-contamination theory. In the study, the surrogate microorganisms transferred to the same animal, to the adjacent carcass, and to other non-inoculated carcasses. The surrogates were also isolated from equipment samples (knives, meat hooks, hide pullers, and splitting saws), from environmental samples (floor, air, and walls), and from personnel garment samples (gloves, boots, and aprons) (Villareal-Silva et al., 2016).

In initial processing of cattle, *E. coli* contamination seems to be confined only on the carcass surface, especially when intact fascia covered the lean tissue (Dickson, Cutter, & Siragusa, 1994); yet, ground beef production using trimming and grinding the carcasses likely increase STEC distribution (Koohmaraie, Bosilevac, Da La Zerda, Mothlagh, & Samadpour, 2015).

Summary

Shiga-toxin producing *E. coli* (STEC) are zoonotic bacteria that have a significant impact in causing human illnesses and deaths worldwide. Clinical signs and symptoms of STEC infection may range from non-bloody diarrhea, hemorrhagic colitis, hemolytic uremic syndrome (HUS), and death. Classification of STEC are determined based on the possession of O antigen and shiga-toxin gene. Certain STEC subsets may also carry the *eae* gene, and thus are classified as Enterohemorrhagic *E. coli* (EHEC) because expression of this gene is generally capable to induce hemorrhagic lesson in the mucosal of intestine. The majority of human infections are due to the consumption of contaminated red meat or other food products. Children, older adults, and immunosuppressed individuals are the most likely to develop severe infection, although some outbreaks also reported severe cases in healthy adults.

Since the first human outbreak in 1983, studies have been conducted to prevent and control human infection. In the United States, from 1982 to 2002 ground beef remains the most frequently identified vehicle for STEC O157 foodborne outbreaks. Many studies have focused on reducing STEC contamination in the cattle production system at commercial feedlots as well as at commercial processing plants. Preventing and controlling these bacteria have remained a big challenge because cattle that enter the food production system show no clinical signs.

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Chapter 2 - Prevalence and Concentration of STEC-7 in Feces of Cull Dairy Cattle Processed in Commercial Slaughter Plants

Abstract

Among animal products consumed by humans, ground beef has been reported as one of the most common vehicles for STEC outbreaks in humans. In the United States, cull dairy cattle contribute as one of the primary sources for ground beef. The objective of this study was to determine the prevalence and concentration of 7 Shiga toxin-producing *Escherichia coli* serogroups (STEC-7; O26, O103, O111, O121, O45, O145, and O157) and associated virulence genes (Shiga toxin 1 and 2 (*stx*1, *stx*2), intimin (*eae*), and enterohomolysin (*ehx*A)) in the feces of cull dairy cattle processed in commercial slaughter plants during summer months. Fecal swab samples (n=183) were collected from three processing plants, one in California and two in Pennsylvania. At each plant at least 60 to 65 cattle were selected, and the samples were obtained by swabbing the mucosal surface of the recto-anal junction using a sterile cotton-tipped applicator. To determine prevalence, all samples were subjected to culture-based detection methods that included enrichment, serogroup-specific immunomagnetic separation and plating on selective media, followed by polymerase chain reaction for serogroup confirmation and virulence gene detection. Pre-enriched fecal samples were subjected to spiral plating to determine the concentration of STEC-7. A sample was considered STEC positive if a recovered isolate harbored one of the 7 target O genes, *stx*1, and/or *stx*2. Of the 183 fecal swab samples collected, 23 (12.6%) harbored at least one O157, O26, O103, or O111 serogroup, with their associated virulence genes. However, none of the fecal samples from this cattle population carried STEC at high-levels (>10^4 CFU/g). This study has provided important information on STEC-7 prevalence from dairy cattle that enter the ground beef processing system. However, there is still a need to determine prevalence

and concentration of STEC in cull dairy cattle during winter months as well as in other sources of ground beef production (e.g., imported lean beef, cull beef).

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are bacteria that are commonly present in the gut of animals, especially ruminants, which are considered their main reservoir. However, STEC can cause severe disease in humans and it has been estimated that annually, there are 265,000 human STEC-associated infections in the United States. Although, there are hundreds of STEC serogroups, 36% of human infections are caused by STEC O157, with the rest being caused by non-O157 serogroups. The six most frequently reported non-O157 serogroups that cause human infections are O26, O103, O111, O121, O45, and O145 (CDC, 2015). Among animal products consumed by humans, ground beef has been reported one of the most common vehicles for food-borne outbreaks. Cross contamination commonly occurs when bacteria from the cattle' intestines are spread to raw meat (Doyle *et al*, 2002). Eating ground beef that has not been cooked sufficiently to kill STEC is thought to be the primary cause of human infection. Outbreaks involving ground beef peak in summer months, mostly from May to August (Rangel et al., 2005). The objective of this study was to determine the prevalence and concentration of STEC-7 in feces of cull dairy cattle from a single sampling during summer months.

Materials and Methods

Processing plant sampling

Samples were collected, during August and September 2017, from three commercial plants that processed cull dairy cattle and were willing to participate in this study. One commercial plant

was in California and the two others were in Pennsylvania. At each plant, a convenience sample of 60 to 65 matched- hide-on and fecal samples were selected. Hide samples were used for another study. Fecal samples were obtained by swabbing the mucosal surface of the recto-anal junction using a sterile cotton-tipped applicator. Mucosal surfaces were swabbed immediately before evisceration, when the rectums were secured to prevent fecal contamination (bunging). After sampling, each cotton-tipped applicator was placed into a pre-labeled tube containing 3 ml of *Escherichia coli* broth media. The samples were then placed in the cooler immediately after sampling. Ice packs were distributed in the cooler to keep samples refrigerated as the samples were shipped to the laboratory. Samples were processed within 48 h of collection at the Pre-harvest Food Safety Laboratory at Kansas State University, College of Veterinary Medicine.

Laboratory procedures for STEC detection

A 100 µL aliquot of the pre-enriched fecal suspension was added to a micro-centrifuge tube containing 900 µL of *Escherichia coli* broth. The fecal suspension was enriched by incubating at 40^{o}C for 6 h. Enriched fecal samples were then subjected to an immunomagnetic separation (IMS) procedure in a Kingfisher™ Flex Magnetic Particle Processor (Thermo Scientific, Waltham, MA) using individual IMS beads (O157; Abraxis® [Warminster, PA]), and pooled combinations of O26, O45, and O111 IMS beads (Pool 1; Abraxis®), and O103, O121, and O145 IMS beads (Pool 2; Abraxis®). For individual IMS beads (O157), 980 µL of enriched sample were mixed with 20 µL of individual IMS beads and subjected to the IMS procedure. For pooled IMS bead treatments (non-O157 pools), 940 µL of enriched sample were mixed with 20 µL of each IMS beads in the pool and subjected to the IMS procedure. Twenty-five µL of each pooled non-O157 and individual O157 bead suspension were spread-plated onto Modified Possé medium and CT-SMAC media, respectively. Plates were then incubated for 20 to 24 h at 37°C.

After incubation, isolates were subjected to colony confirmation. Up to ten chromogenic colonies (mauve, green, blue or purple) from the modified Possé medium and six sorbitol-negative colonies from CT-SMAC were picked, plated onto blood agar plates, and incubated at 37°C for 24 h. For non-O157, ten colonies from each sample were pooled in distilled water, boiled for 10 minutes, centrifuged at 9,300 Xg for 5 m, and the lysate containing the DNA was tested by an 11-plex PCR assay (Bai *et al*., 2012) targeting 7 serogroups and 4 virulence genes (*stx*1, *stx*2, *eae*, and *ehx*A). If the pooled colonies were positive for any of the six non-O157 serogroups, then the ten isolates were individually tested by the 11-plex PCR. For O157, non-sorbitol-fermenting colonies were tested for the O157 antigen by latex agglutination (Oxoid, Basingstoke, United Kingdom); if positive, a spot-indole test was performed. Colonies positive for agglutination and indole production were tested by a 6-plex PCR assay (Bai, Shi, & Nagaraja, 2010a) that targets the *rfb*E, *fliC*$_{H7}$, *eae*, *stx*1, *stx*2, and *ehx*A genes. Isolates confirmed to be positive for one of the seven serogroups were stored on cryogenic beads (CryoCare™, Key Scientific Products, Round Rock, TX).

Laboratory procedures for STEC quantification:

A 100 µL aliquot of the pre-enriched fecal suspension was subjected to a spiral plate procedure for quantification of STEC-7. Using an Eddy Jet Spiral Plater (IUL Instruments; Barcelona, Spain), 100 µL of the pre-enriched fecal suspension were spiral-plated onto 2 different media:

a. Sorbitol MacConkey agar containing cefixime (0.05 mg/L) and potassium tellurite (2.5 mg/L; CT-SMAC) for STEC O157.
b. Modified Possé agar medium that includes novobiocin at 5 mg/L and potassium tellurite at 0.5 mg/L (MP) for non-O157 STEC.

Plates were incubated at 37ºC for 20-24 h. Plates were illuminated from below and colonies enumerated by placing a specialized counting grid overlay on top of the plate. Six non-sorbitol fermenting colonies from CT-SMAC medium and ten chromogenic colonies (mauve, green, blue or purple) from MP were picked, inoculated onto blood agar (Remel, Lenexa, KS) plates, and incubated at 37°C for 24 h.

The non-sorbitol-fermenting colonies from the CT-SMAC plates were tested for the O157 antigen by latex agglutination (Oxoid, Basingstoke, United Kingdom); if positive, a spot-indole test was performed. Colonies positive for agglutination and indole production were tested by a 6-plex PCR (Bai, Shi, & Nagaraja, 2010b) that targets the *rfb*E, *fli*CH7, *eae*, *stx*1, *stx*2, and *ehx*A genes. For a sample to be considered positive by PCR, it must have at least *rfb*E and either the *stx*1 or *stx*2 genes. Chromogenic colonies from the MP plates were pooled in 50 µL ddH2O and tested by an 11-plex PCR targeting 6 O-serogroups and 3 virulence genes (*stx*1, *stx*2, and *eae*).

Results

Characteristics of the study population

A total of 183 fecal swab samples were collected from cull dairy cattle from commercial processing plants located in California (1x), and in Pennsylvania (2x) in August and September 2017. The capacity of the processing plants ranged from 450 to 1,500 animals per day. Table 1 displays the date of collection and the number of samples collected in each of the processing plants.

Table 1. Date of sampling collection, number of samples collected, and plant capacity by processing plant

Plant ID	Date of collection	Number of samples collected	Plant capacity (cattle/day)
A	August 14, 2017	62	1,000
B	August 21, 2017	59	1,500
C	September 18, 2017	62	450-470
	Total # of samples	183	

Prevalence of STEC-7

Post-enriched samples were used to determine the proportion of positives for O157 and non-O157 serogroups and their virulence genes in this population of cattle. Samples were classified as O157 or one of the non-O157 serogroups (O26, O103, O111, O121, O45, O145) based on the presence of the O somatic antigen. Within-plant prevalence of EHEC O157 ranged from 1.7% to 3.2%; overall mean prevalence was 2.7%. Within-plant prevalence of EHEC non-O157 ranged from 0% to 25.4%; overall mean prevalence was 10.5% (Table 2). Table 3 depicts each of positive sample detected based on possession of O somatic antigen (O157 and non-O157) and its virulence genes (*stx1, stx2, fliCH7, ehxA* and *eae*). Samples were considered as Shiga-toxin producing *Escherichia coli* (STEC) if they possessed either one of the Shiga-toxin virulence genes (*stx*1 and/or *stx*2). If the STEC samples were also positive for intimin gene (*eae*), they were considered as enterohemorrhagic *Escherichia coli* (EHEC). All O157 positive samples had a *stx* and *eae* gene.

Table 2. Cumulative prevalence of EHEC O157 and non-O157 serogroups by processing plant

Plant ID	Sample size (N)	Prevalence % (n/N)	
		O157	non-O157*
A	62	3.2 (2/62)	0.0 (0/60)**
B	59	1.7 (1/59)	25.4 (15/59)
C	62	3.2 (2/62)	6.5 (4/62)
TOTAL	183	2.7 (5/183)	10.5 (19/181)

*Each of positive sample corresponds to have at least one non-O157 somatic antigen.
**Two samples could not be processed by the IMS assay.

Table 3. Distribution of test positive samples for EHEC O157 and non-O157 by processing plant

Plant ID	Sample ID	O157	Non-O157
A	2	O157, *stx1, stx2, eae, ehxA, fliCH7*	-
	14	O157, *stx1, stx2, eae, ehxA, fliCH7*	-
B	79	-	O103, *stx1, eae*
	80	-	O103, *stx1, eae*
	83	-	O111, *stx1, eae*
	84	-	O111, *stx1, eae*
	92	-	O103, *stx1, eae*
	94	-	O103, *stx1, eae*
	105	-	O103, *stx1, eae*
	114	-	O103, *stx1, eae*
	116	-	O103, *stx1, eae*
	117	-	O103, *stx1, eae*
	120	-	O103, *stx1, eae*
	121	-	O103, *stx1, eae*
	123	O157, *stx2, eae, ehxA, fliCH7*	-
	125	-	O111, *stx1, eae*
	128	-	O103, *stx1, eae*
	130	-	O103, *stx1, eae*
C	152	-	O26, *stx1, eae*
	158	O157, *stx2, eae, ehxA, fliCH7*	-
	191	-	O103, *stx1, eae*
	195	-	O103, *stx1, eae*
	203	O157, *stx1, eae, ehxA, fliCH7*	-

Table 4 depicts cumulative prevalence results at the sample-level. Cumulative prevalence of EHEC O26, O103, and O111 serogroups were 0.6%, 7.7%, and 1.6%, respectively. Approximately 13% (23/183; data not shown) of samples contained one of the O serogroups and at least one *stx gene*

and the *eae* gene. No samples tested positive for O121, O45, and O145 serogroups, and no samples tested positive for *fliC*$_{H7}$, *stx*2, and *ehx*A genes.

Table 4. Percentage of test positives fecal samples for O157 (n=183) and non-O157 (n=181) serogroups and distribution of virulence genes

O serogroup	Positive O gene % (n)	O gene + *stx*1 % (n)	O gene + *stx*1 + *eae* % (n)
O157	2.73 (5)	2.73 (5)	2.73 (5)
O26	0.55 (1)	0.55 (1)	0.6 (1)
O103	7.65 (14)	7.65 (14)	7.7 (14)
O111	1.64 (3)	1.64 (3)	1.64 (3)
O121	0 (0)	0 (0)	0 (0)
O45	0 (0)	0 (0)	0 (0)
O145	0 (0)	0 (0)	0 (0)

None of the samples tested positive for *stx*2

Concentration of STEC-7

There were no enumerable samples for O157. The number of quantifiable *Escherichia coli* non-O157 in pre-enriched fecal swab samples is depicted in Table 5. Unfortunately, fecal swab samples from the first two plants sampled on the first two weeks were unable to be processed for quantification purposes. Among samples from plant C (sampled on week 3) there were 3 (1.6%) samples that were quantifiable for non-O157 serogroups, 2 had < 500 CFU/g and 1 had between 500 and $<10^4$ CFU/g.

Table 5. Number of quantifiable samples and concentration in CFU/g feces of non-O157 serogroups in pre-enriched samples

Plant ID	Sample size	Number of quantifiable fecal samples		
		< 500 CFU/g	500 < n < 10^4 CFU/g	≥ 10^4 CFU/g
A	62	N/A	N/A	N/A
B	59	N/A	N/A	N/A
C	62	2	1	0

N/A: Samples were unable to be processed

Discussion

Shiga-toxin producing *E. coli* is a major public health issue with ground beef as one common source of infection in humans. Within-plant prevalence of EHEC O157 ranged from 1.7% to 3.2%; overall mean prevalence was 2.7%. Within-plant prevalence of EHEC non-O157 ranged from 0% to 25.4%; overall mean prevalence was 10.5%. Small sample size in this study makes it difficult to generate conclusion about prevalence level of cull cattle during summer months. However, this report can be used to inform an estimate of prevalence in cull dairy processing plants.

Animals shedding > 10^4 CFU of *E. coli* O157 per gram of feces, may cause higher transmission among pen-mates and more carcass contamination in the processing plant (Doyle *et al*, 2002). Of the 62 samples in this study, there were no enumerable samples detected for O157. A small percentage of the samples in this study (1.6%) have enumerable non-O157 that were less than 10^4 CFU/g feces, indicating there is still a risk of food contamination from cull dairy cattle when entering processing plants. However, most of the samples were not eligible for quantification due to issues during shipping of samples to our laboratory.

In the current study, STEC serogroups O157, O103, O111, and O26 were the most prevalent in cull dairy cattle. Previous studies had reported similar results (Bonardi et al. 2015; Dewsbury et al. 2015). An epidemiologic study of STEC infection in the United States during 1983-2002 reported that O26, O111, and O103 were the most frequent non-O157 serogroups identified in outbreaks of human infection (Brooks *et al*. 2005).

This study provides evidence that feces of cull dairy cattle pose a potential risk to cross-contaminate raw meat products during processing, and therefore, represent a potential risk as the source of human infection. Further, this study provides important information on STEC-7

prevalence from cattle that enter the ground beef processing system. However, there is still a need to determine prevalence and concentration of STEC in cull dairy cattle during winter months as well as in other sources of ground beef production (e.g., imported lean beef, cull beef).

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