Fall 12-1-2015

Detection Methods and Intestinal Adherence of non-O157 Shiga Toxin-Producing *Escherichia coli*

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DETECTION METHODS AND INTESTINAL ADHERENCE OF NON-O157 SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI*

by

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A DISSERTATION

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Major: Integrative Biomedical Sciences

Under the Supervision of Professor Rodney A. Moxley

Lincoln, Nebraska

December, 2015

DETECTION METHODS AND INTESTINAL ADHERENCE OF NON-O157 SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI*

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University of Nebraska, 2015

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Shiga toxin-producing *Escherichia coli* (STEC) are enteric pathogens of humans. Cattle serve as a reservoir and harbor STEC in their intestines. Intimin-positive STEC are referred to as enterohemorrhagic *E.coli* (EHEC). Seven serogroups (O26, O45, O103, O111, O121, O145, and O157) account for the majority of illness due to STEC and are hereafter referred to as STEC/EHEC-7. To improve detection, enrichment broths were compared for supporting growth of STEC-7 and STEC O104:H4 (STEC-8). In pure culture, STEC enriched in trypticase soy broth (TSB) had significantly greater growth compared to TSB containing antimicrobials. In fecal samples, *E. coli* broth enrichment yielded growth of STEC-8 that was significantly greater than in TSB. Optimized culture conditions allow for greater detection of EHEC-7 in cattle. To determine the prevalence of EHEC-7 in feedlot cattle, culture-based methods and molecular screening assays were used. In 576 feedlot cattle, EHEC-7 prevalence in hide samples as detected by NeoSEEK (NS) was 80.7% compared to 1.2% by culture. The prevalence of EHEC-7 on carcasses was 6.0% detected by NS. Additionally, EHEC-7 prevalence was determined in 100 culled dairy cows. The EHEC-7 prevalence in feces, hides, and carcasses, respectively,

was 6.5%, 15.6%, and 1.0% by culture, and 25.9%, 64.9%, and 7.0% by NS. These studies provide evidence that EHEC are ubiquitous on cattle hides and to a lesser extent feces and carcasses. Given the discordant results, continued improvement in EHEC-7 detection methods is needed. Comparison studies were performed using CHROMagar STEC, Possé differential agar (Possé), Possé with modified antimicrobials, STEC heart infusion washed blood agar with mitomycin C (SHIBAM), and SHIBAM with modified antimicrobials. CHROMagar STEC performed better than Possé or SHIBAM for detection of EHEC-7 in cattle feces, but modifications of the antimicrobials in the latter two media resulted in significant improvements. STEC attachment facilitates colonization of the intestine. All STEC-8 strains tested adhered to bovine colonic explants and Caco-2 cells. One strain invaded both bovine colonic epithelial cells and Caco-2 cells. STEC O104:H4 had significantly higher levels of adherence on Caco-2 cells compared to most STEC. Interventions which block adherence may be effective for pre-harvest control.

DEDICATION

This work is dedicated to Randy and Sue Stromberg.

ACKNOWLEDGEMENTS

I would like to thank Dr. Rodney Moxley for giving me the opportunity to further my education and for his support over my entire graduate career. I would also like to thank members of my graduate committee: Dr. Raul Barletta, Dr. Dennis Burson, Dr. Greg Somerville, and Dr. Harshavardhan Thippareddi, who provided excellent guidance. In addition, I would like to thank current and former members of Dr. Moxley's lab: Gentry Lewis, Matt Schaich, Prageeth Wijemanne, Fernanda Coura, Nikki Bestul, Rob Fenton, Hannah Hamilton, Alexander Mueting, Brandon Stewart, John Chatterton, Marcus Chlopek, Lauren Deuser, Quentin Jorgensen, Taylor Katt, John Keck, Anthony Kohtz, Ryan Larson, Trey Nygren, Spencer Randazzo, Anne Sorensen, and Sarah Wallis.

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CHAPTER 1

LITERATURE REVIEW

Shiga toxin-producing *Escherichia coli* (STEC)

Introduction

Escherichia coli (*E. coli)* belongs to the genus *Escherichia* and family Enterobacteriaceae. *E. coli* is a Gram-negative, facultative anaerobic, non-sporulating bacterium that is found in the environment, intestinal tract and extra-intestinal sites of mammals (Kaper *et al*., 2004). *E. coli* was first isolated in 1885, by Theodor Escherich from the feces of a healthy human (republished in English; Escherich, 1988). Several different strains of *E. coli* exist that inhabit different ecological niches (Kaper *et al*., 2004). Strains of *E. coli* are 2.0 µm long rods that possess peritrichous flagella and are motile. *E. coli* can grow rapidly at 37°C with a doubling time between 20 to 30 minutes in rich media, and is used as a model organism in genetics and biochemistry studies because it can be grown easily and it has a well described genetic system. Biochemically, *E. coli* are positive for indole, methyl red, and lactose, and negative for Voges-Proskauer reaction and citrate test.

The gastrointestinal tract of humans is colonized after birth with bacteria, and typically *E. coli* is found within the first week in the colonic flora (Nataro and Kaper, 1998). It is found in relatively low concentration compared to other commensal bacteria (Berg, 1996). Recently, delayed colonization of *E. coli* in newborns has been reported (Nowrouzian *et al*., 2003). Reduced exposure to bacteria and reduction in fecal bacteria

in hospitals has been suggested to account for why some infants are not colonized until six months after birth (Nowrouzian *et al*., 2003). Strains can persist for months to years in the intestinal microflora or can colonize transiently. Strains with genes encoding virulence factors were more likely to persist for at least three weeks in the microflora (Nowrouzian *et al*., 2003).

Commensal *E. coli* play vital roles in maintaining the gastrointestinal tract. *E. coli* produces vitamin K (Kindberg *et al*., 1987), helps breakdown food, aids in food absorption, and helps to prevent colonization by pathogenic bacteria (Tenaillon *et al*., 2010). The external layer of the gastrointestinal tract, the mucosa, is constantly exposed to bacteria. The mucosa and the associated bacteria help protect the gastrointestinal tract from the external environment (Tenaillon *et al*., 2010). Goblet cells play a role in protection by continuously secreting mucus to act as a lubricant along the gastrointestinal tract and to prevent bacterial adherence (Birchenough *et al*., 2015).

In the harsh environment of the gastrointestinal tract, *E. coli* must be equipped not only to survive, but also replicate and thrive. One way *E. coli* is equipped to survive is by expressing acid resistant genes that allow for protection and survival in the low pH environment of the intestine (Hong *et al*., 2012). *E. coli* must not only survive the extremely acidic environment, but also acquire nutrients for growth. Strains are provided with ample nutrients from the breakdown of food that allow for their growth in the gastrointestinal tract. In order to grow and thrive in the gut, *E.coli* uses simple sugars from the breakdown of complex polysaccharides by commensal anaerobes (Alteri and Mobley, 2012). Additionally, mucus can provide *E. coli* with sugars that allow growth (Tenaillon *et al*., 2010). In conditions where sugars are limited, *E. coli* can use

gluconeogenic substrates such as aspartate and tryptophan (Miranda *et al*., 2004), which are important for survival in the varying environment of the intestine. Due to the association with the gastrointestinal tract, *E. coli* has been used as an indicator for fecal contamination (Gruber *et al*., 2014).

To represent the diversity of *E. coli* strains, the *E. coli* collection of reference (ECOR) strains was initiated by Ochman and Selander (1984). The ECOR strains were used to classify *E. coli* into phylogenetic groups A, B1, B2, D, and E. Commensal *E. coli* are associated with groups A and B1, extraintestinal *E. coli* are associated with B2 and D, and it is unclear if the diarrheagenic *E. coli* (DEC) associate with a particular phylogenic group. In addition to genetic differences, DEC colonize the intestine compared to extraintestinal *E. coli* which colonize sites outside the intestine including surgical sites, the blood stream, and the urinary tract (Smith *et al*., 2007). Group E is a separate clade made up of Shiga toxin-producing *E. coli* (STEC) O157:H7 and enteropathogenic *E. coli* O55:H7 (Leimbach *et al*., 2013). Recently, Mosquito *et al.* (2015) found that DEC strains from patients with diarrhea were more frequently associated with group D compared to DEC strains from healthy patients. STEC strains commonly belong to groups A, B1 (Donnenberg and Whittam, 2001; Escobar-Páramo *et al*., 2004), and D (Bidet *et al*., 2005), but may fall into any phylogenetic group.

Pathogenic *E. coli*

In order to adapt to different environments, *E. coli* has altered its genome through acquisition of plasmids, pathogenicity islands, transposons, genes from phage, or by gene subtraction (black holes or pseudogenes; Maurelli *et al*., 1998; Leimbach *et al.*, 2013).

The genomic plasticity of *E. coli* allows it to survive in a variety of ecological niches and be a versatile species. *E. coli* can enhance pathogenicity through gene acquisitions or deletions. Acquisition of virulence factors may change a non-pathogenic strain into a pathogen. The core genome of *E. coli* is fairly conserved across phylogenetic groups, but diversity can arise through differences in the flexible or dispensable genome (Touchon *et al*., 2009). Even within strains, diversity can occur through point mutations and accessory elements (Leimbach *et al*., 2013). Since *E. coli* colonizes the gut, a diverse gene pool exists for horizontal gene transfer from other microbes. Thus, *E. coli* can draw from foreign genes in their environment that may increase survival and ability to thrive in the gastrointestinal tract. Addition of genes must not disturb the organization of essential function such as replication, transcription and translation in the bacterial chromosome. Therefore, there may be regions in the chromosome that are more permissive to gene acquisition such that these regions remain undisturbed (Leimbach *et al*., 2013). Deletion of genes can enhance pathogenicity by eliminating factors that are unfavorable to pathogenicity such as deletion of a gene that is involved in production of a toxin inhibitor (Maurelli *et al*., 1998). *E. coli* can change their genomes to fit the environment by interacting with viruses to acquire genes crucial for their survival (Goldenfeld and Woese, 2007).

Some strains of *E. coli* can cause disease in humans. Globally, it is estimated that pathogenic *E. coli* kills approximately two million people per year (Tenaillon *et al*., 2010). *E. coli* can infect the gastrointestinal tract, urinary tract, enter the blood stream and cause sepsis, or cause meningitis, particularly in neonates (Tenaillon *et al*., 2010). Bray (1945) first described the association between strains of *E. coli* and patients with diarrhea

in what was called “summer diarrhea”. Bray (1945) noted that there was a seasonal pattern to illness and that these strains were not seen in healthy infants. This led to the hypothesis that there were both pathogenic and non-pathogenic strains of *E. coli*. This was tested by giving adult volunteers cultures of *E. coli* isolated from infants with diarrhea. Healthy volunteers suffered similar illness to infants, including diarrhea, vomiting and abdominal discomfort when given *E. coli* from infants with diarrhea (Kirby *et al*., 1950). When *E. coli* from healthy infants was given to volunteers, no illness occurred (Kirby *et al*., 1950).

DEC have been subdivided into pathotypes. Nataro and Kaper (1998) classified DEC in six major pathotypes as follows: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) based on the virulence and molecular markers found in each pathotype. EPEC are similar to EHEC in that they carry intimin as a major adherence factor, but EPEC lack Shiga toxin (Stx) and have the bundle forming pili (*bfp*). EHEC strains evolved from EPEC through acquisition of genes that encode for Stx (Feng *et al*., 1998). A large EHEC outbreak was caused by a strain that had a combination of EHEC and EAEC characteristics by an *E. coli* O104:H4 strain (Brzuszkiewicz *et al*., 2011). This outbreak strain demonstrated how dangerous new pathogenic *E. coli* variants can be.

STEC

STEC are also referred to as verocytotoxin-producing *E. coli* (VTEC). The term VTEC originated from the observation that some strains of *E. coli* caused irreversible

cytopathic effect in Vero cells, but not in Y-1 or CHO cells (Konowalchuk *et al*. 1977). It is now known that Stx caused cytopathic effects in Vero cells (Karmali, 1989). Vero cells were susceptible to Stx along with HeLa, Daudi, and human liver cells, likely because they have the receptor for Stx and were given a sufficient dose of Stx, but Y-1, CHO, Henle 407, WI-38, BHK, and other cell lines are not susceptible because they lack the receptor for Stx (Karmali, 1989). Shiga toxin is the main virulence factor for STEC, but some strains may also have a major adherence factor, intimin. Intimin along with secreted proteins encoded on the locus of enterocyte effacement (LEE) cause attaching-effacing (A/E) lesion formation in the large intestine (Gyles, 2007).

Intimin-positive STEC are known as classical enterohemorrhagic *E. coli* (EHEC; Croxen *et al*., 2013). Intimin-negative STEC strains that have caused hemorrhagic colitis in humans are termed non-classical EHEC. EHEC O157 is the prototype EHEC strain. Comparative genomics have revealed key findings of EHEC. EHEC have much larger genomes (5.5-5.9 Mb) compared to K-12 *E. coli* strains (4.6 Mb; Perna *et al*., 2001). On average, an *E. coli* strain will contain 4,800 genes (Alteri and Mobley, 2012). EHEC are divided into two major lineages with EHEC O55 and O157 forming EHEC 1 and non-O157/O55 EHEC forming EHEC 2 (Sadiq *et al*., 2014). EHEC O157:H7 has been proposed to be derived from an EPEC O55:H7 ancestor (Feng *et al*., 1998). The EPEC O55:H7 ancestor strain gained the Stx2 phage, a mutation in the *rfb* gene changed the O-group from O55 to O157, and a single point mutation (SNP) occurred in *uidA* gene, which encodes for β-glucuronidase. Next, the Stx1 phage was gained and sorbitol fermentation was lost, and then loss of β-glucuronidase activity occurred. These changes made EHEC O157:H7 distinct from other EHEC.

Epidemiology of STEC in humans

STEC is transmitted by the fecal-oral route to humans and can cause hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS; Croxen *et al*., 2013). STEC was first recognized as a cause of HUS in 1982 when it was associated with consuming undercooked meat (Riley *et al*., 1983). The estimated infectious dose of STEC O157 is <50 bacterial cells (Tilden *et al*., 1996). STEC have a low infectious dose due to the potency of Stx. In contrast to STEC, the infectious dose for EPEC is 10^8 bacterial cells (Mellies *et al*., 2007) and for *Salmonella* Typhi at least 10^5 cells (Leggett *et al*., 2012).

STEC is the leading cause of kidney failure in children less than five years old (Mayer *et al*., 2012). In the overall population, the incidence of STEC is one to three per 100,000 and increases to six per 100,000 in children less than five years old (Cheung and Trachtman, 2014). Humans play a role in the transmission of STEC. Asymptomatic carriers can transmit STEC leading to outbreaks, with person-to-person transmission occurring in about 15 to 20% of outbreaks (Persad and LeJeune, 2014). STEC O157:H7 accounts for the majority of clinical infections due to STEC, but other serogroups such as STEC O26, O45, O103, O111, O121, and O145 (STEC-6) are frequently detected and associated with HC and HUS (Brooks *et al*., 2005). STEC other than STEC O157 are termed non-O157 STEC (Bettelheim, 2007). Recently, there has been an increase in detection of non-O157 STEC in clinical illness with STEC-6 causing over 70% of the illnesses due to non-O157 STEC (Brooks *et al*., 2005). Collectively, STEC-6 and STEC O157 are referred to as "Top7" (Delannoy *et al*., 2013) or STEC-7, and intimin-positive STEC-7 referred to as EHEC-7 in this dissertation.

Many STEC serogroups are geographically distributed, but certain serogroups are more prevalent in certain regions. In the U.S., outbreaks due to STEC O157 are more common in northern states compared to southern states separated by 37° N latitude (Heiman *et al*., 2015). The reason for this perceived difference is unknown. Factors such as cattle density, temperature, and diet have been suggested to play a role (Heiman *et al*., 2015). STEC O113 is commonly found in Australia and STEC O91 is frequently found in Europe (Bettelheim, 2007). Differences in STEC serogroup prevalence could be explained by differences in animal populations. One of the largest outbreaks due to STEC was caused not by a STEC-7 but by STEC O104:H4 associated with contaminated fenugreek sprouts (Bucholz *et al*., 2011). This strain was an enteroaggregative *E. coli* that acquired Shiga toxin, lacked intimin, but had characteristics of enteroaggregative *E. coli*.

After ingestion of STEC, an incubation period occurs for three to seven days before the first loose stool (Tarr *et al*., 2005). Typically one to three days of non-bloody diarrhea precedes bloody diarrhea (Tarr *et al*., 2005). HUS will occur in about 15% of patients with bloody diarrhea and the other 85% will recover (Tarr *et al*., 2005). Patients infected with STEC develop neutralizing antibodies for Stx, which can be used to diagnose infection (Karmali, 1989). In addition to severe disease, STEC illness is a tremendous economic burden. The average cost as estimated in 2003 dollars per patient with STEC O157 infection is $5,515 and increases with severity of disease (Frenzen *et al*., 2005). The average cost of patients who develop HUS and die is estimated to be $6,175,500 in 2003 dollars (Frenzen *et al*., 2005). In 2003, the annual cost to the U.S. economy for illness due to STEC O157 alone was $405 million (Frenzen *et al*., 2005). Certainly the total burden due to all STEC is much higher. Thus, interventions that reduce

STEC, novel therapeutics for STEC infection, and better detection systems for STEC in food are a priority.

Based on clinical studies, the use of antibiotics to treat STEC infections is not recommended (Tarr *et al*., 2005), but Agger *et al*. (2015) suggested that antibiotics could be useful to treat some STEC infections. Hydration is the main course of therapy for patients infected with STEC (Croxen *et al*., 2013). *In vitro* studies have demonstrated that STEC strains increase toxin production when under conditions with antibiotics such as ciprofloxacin compared to those without (Bielaszewska *et al*., 2012). Conversely, other antibiotics, such as chloramphenicol, meropenem, azithromycin, rifaximin and tigecycline significantly decreased induction of Stx (Bielaszewska *et al*., 2012). This study tested antibiotics on only one STEC strain, and should be interpreted with caution. Further studies would need to be done to warrant the use of antibiotics to treat STEC infection. Agger *et al*. (2015) suggested treatment by using a combination of antibiotics such as azithromycin and fosfomycin to inhibit protein and cell wall synthesis. However, this antibiotic treatment scheme is still in question. Other treatments, such as proteasome inhibitors have shown promise to prolong the survival of mice when given Stx (Hattori *et al*., 2015), but have not been thoroughly investigated for human use. The microbiome and certain species in the microbiome may have a protective effect. *Bifidobacteria* is associated with protection of STEC infection in mice, and the use of dietary modifications to increase *Bifidobacteria* could be a strategy to reduce the risk of STEC infection (Fukuda *et al*., 2011).

Regulatory concerns of STEC

Meat sold to consumers is inspected by the U.S. Department of Agriculture (USDA). In 1993, a large outbreak due to the consumption of undercooked hamburgers contaminated with EHEC O157:H7 occurred in a fast food chain (Tuttle *et al*., 1999). A year later in 1994, the Food Safety and Inspection Service (FSIS), an agency of the USDA, declared EHEC O157:H7 as adulterants in raw, non-intact beef. Then in 2012, EHEC O26, O45, O103, O111, O121, and O145 (EHEC-6) were declared as additional adulterants in raw, non-intact beef by the USDA FSIS (USDA, 2012). The USDA FSIS has imposed a "zero tolerance" policy on EHEC-7.

Ground beef can become contaminated with EHEC-7 and poses a risk to public health. The internal portions of ground beef patties may contain viable organisms if heat does not penetrate the meat and inactivate the bacteria. Also, meat from one patty comes from multiple cows making it difficult to trace back to one animal. A small fraction of contaminated animals can lead to large levels of contaminated ground beef. This has led to an increased risk of STEC contamination in undercooked hamburgers. Proper handling instructions are listed on packages of meat for consumer benefit (Reicks *et al*., 2008).

The Food and Drug Administration (FDA) regulates food except for meat and poultry products. Similar to the USDA FSIS, the FDA had previously focused on EHEC O157:H7 as the only regulated STEC, but have expanded their regulations to include any pathogenic STEC (Feng *et al*., 2011). Any pathogenic STEC found is of concern and needs to be additionally tested to discriminate between STEC that have not been associated with illness from those that are pathogenic (Feng *et al*., 2011).

Food products

Numerous food products have been associated with STEC illness. Beef products have been one of the most common sources (Hussein and Bollinger, 2005). In addition to beef products, STEC has caused large outbreaks from contaminated potatoes, radish sprouts, mesclun lettuce, alfalfa and clover sprouts, packaged spinach, fenugreek sprouts, sausage, cheese, mushrooms, and shellfish (Erickson and Doyle, 2007; van Overbeek *et al*., 2014). Contaminated liquids, milk, punch, unpasteurized apple juice, and water have also been suspected in exposing humans to STEC (Brooks *et al*., 2005).

Foods are contaminated through environmental exposure that can occur in the field or during processing. Processing equipment used in preparing foods can become contaminated, and in turn, cross-contaminate multiple food products (Erickson and Doyle, 2007). Survival studies show that STEC can tolerate refrigerated temperatures (4°C), low pH (pH < 4.0), and reduced water activity (Erickson and Doyle, 2007). The extreme versatility of STEC allows it to survive in multiple food products.

STEC in ruminants

Ruminants, most notably cattle are the primary reservoir for STEC (Gyles, 2007). For STEC O157, the colon and recto-anal junction are main sites of colonization in cattle (Naylor *et al*., 2003; Persad and LeJeune, 2014). Carriage of STEC has been found in cattle, buffalo, sheep, goat, deer, and non-ruminant animals such as pig, horse, pigeon, and fox (Persad and LeJeune, 2014). Petting zoos with ruminant animals have been linked to outbreaks (Heuvelink *et al*., 2002; DebRoy and Roberts, 2006). Ruminants carry STEC asymptomatically and no ruminant species have been identified to have the vascular receptor for Stx (Persad and LeJeune, 2014). Carriage of STEC in cattle depends

on a number of factors such as age, immune status, diet, environment, microbiome, and sanitation (Persad and LeJeune, 2014).

The prevalence of STEC in cattle is variable and unpredictable. Most studies have focused on the prevalence of STEC O157, because it causes the majority of disease due to STEC infections in humans in the U.S. (Gould *et al*., 2013). Increased shedding of STEC O157 occurs during summer months and is decreased during winter months with the same pattern observed for frequency of human infections (Barkocy-Gallagher *et al*., 2003). Based on testing of fecal samples of feedlot cattle, this same seasonal pattern could not be established for EHEC-6 (Dewsbury *et al*., 2015). However, a low number of fecal samples were positive for EHEC-6 in the summer, and no EHEC-6 positive samples were found in winter months. Season is associated with STEC shedding in both beef and dairy cattle (Hancock *et al*., 1994; Hancock *et al*., 1997; Smith *et al*., 2005).

Colonization occurs throughout the gastrointestinal tract, but mainly in the large intestine (Keen and Elder, 2002; Gyles, 2007). Some STEC strains may proliferate in the rumen before reaching the large intestine (Free *et al*., 2012). For STEC O157, in both experimental and naturally infected cattle the terminal rectum was determined to be an additional site of colonization (Naylor *et al*., 2003). It was previously thought that STEC may not cause disease in adult cattle (Sandhu and Gyles, 2002). This has been disputed by cases of STEC infection causing attaching-effacing lesions and diarrheal disease in cattle over a year old (Wada *et al*., 1994; Pearson *et al*., 1999; Moxley *et al*., 2015).

Environmental factors influence the prevalence of STEC in cattle. In the environment, STEC can survive on vegetable plants (Xicohtencatl-Cortes *et al*., 2009a) and fruits (Fremaux *et al*., 2008). STEC are commonly found in soil where cattle have

been or are being housed, and can contaminate surrounding areas during periods of rain (Fremaux *et al*., 2008). Factors such as protozoa (Burow *et al*., 2005) and fly populations (Ghosh and Zurek, 2015) may also influence STEC prevalence. Protozoa in the rumen or in the environment can ingest STEC (Steinberg and Levin, 2007), which may account for decreased transmission. Conversely, flies are more prevalent during the summer and may serve as a vector for transmitting STEC (Ghosh and Zurek, 2015).

Cattle shed STEC O157 at levels between <1 CFU/g of feces to 1 x 10^7 CFU/g of feces (Chase-Topping *et al*., 2008). Animals shedding STEC O157 greater than 10^4 CFU/g of feces are a small fraction of STEC O157 positive animals, but are responsible for the majority of the environmental contamination (Arthur *et al*., 2009). These cattle have been termed "super-shedders" (Chase-Topping *et al*., 2008). Thus, use of cattle feces in fertilizer applications should be handled properly to reduce environmental contamination.

Calves shed STEC at higher concentrations compared to adult cattle. Although a few cases of disease have been reported in adult cattle, most cases of diarrhea associated with STEC occur in calves between one to three weeks (Moxley and Smith, 2010). Calves experimentally inoculated with STEC O157 developed watery diarrhea (Dean-Nystrom *et al*., 1999). Development of the rumen and the immune system in adult cattle may act to inhibit STEC colonization. Diet is an important factor in *E. coli* colonization. STEC O157 prevalence has been associated with the percent of distiller's grains in feed (Smith, 2014a). Feeding cattle a barley based diet, was associated with an increase in STEC O157 prevalence compared to corn-based rations (Berg *et al*., 2004). There are conflicting reports on whether forage or grain-based diets reduce STEC O157

colonization and shedding (Smith, 2014a). Diet does seem to affect STEC colonization, but its role in the process is not fully understood.

Cattle genetics may play a role in colonization. In experimental studies, some cattle were never colonized when orally inoculated with STEC O157 (Shere *et al*., 2002). This suggests that some genetic factors may lead cattle to be more resistant to STEC colonization. Shere *et al*. (2002) found that bulls were significantly ($P = 0.022$) more likely to shed STEC O157 at higher levels ($>10^5$ CFU) compared to steers, but there was no difference in the prevalence of STEC O157 between the two groups. However the number of cattle found shedding high levels was very small (Shere *et al*., 2002). They also examined six different breed groups to test the role of genetic factors. Brahman calves shed lower concentrations of STEC O157 compared to Angus, and resistance only existed in 100% Brahman calves (Shere *et al*., 2002). In this study, STEC O157 strains isolated from different cattle were tested for similarity. Strains of STEC O157 isolated from cattle shedding low, medium, and high levels had matching profiles based on pulsed-field gel electrophoresis (PFGE). This supports the hypothesis that genetic factors of cattle influence the shedding of STEC O157, and not strain specific factors.

Cattle type may also play a role in STEC colonization. Islam *et al*. (2014) evaluated the prevalence of STEC O157 in different cattle types using a meta-analysis. The prevalence of STEC O157 in intestinal samples (fecal, rectal swab, and intestinal content) from dairy cattle was 1.75%, 6.84% for beef cattle, and 19.58% for feedlot cattle (Islam *et al*., 2014). Dairy cattle are normally older animals that have been in the production setting longer and turnover is lower. The dairy operation reduces the number of new cattle introduced that could be colonized with STEC, and may reduce the

prevalence of STEC. STEC O157 shedding in dairy cattle can lead to both environmental and milk contamination. STEC can contaminate surfaces of dairy cow udders and equipment, which can lead to milk contamination (Mohammed *et al*., 2015). Contaminated raw milk, which does not employ a step to inactivate bacterial pathogens, poses as a source of pathogen exposure to humans.

STEC are geographically well distributed and found in cattle across most countries. The global prevalence of STEC O157 was estimated as 5.68% in intestinal samples (Islam *et al*., 2014). In North America, the prevalence of STEC O157 was slightly higher (7.35%) compared to the global estimate (Islam *et al*., 2014). Cattle sampled in Asia had a prevalence of 4.69%, 5.15% in Europe, 1.65% in Latin America and the Caribbean, and 31.20% in Africa (Islam *et al*., 2014). There were a limited number of studies used to determine prevalence in Africa, which may have led to overestimation in this region. Higher prevalence in certain regions may relate to increased risk of STEC infection from interaction with animals harboring STEC. It is unclear if cattle in North America have a higher prevalence of non-O157 STEC as well, because there is limited available data.

Cattle feces

After colonization and proliferation of STEC in the large intestines of cattle, these organisms are shed in feces (Gyles, 2007). STEC O157 can survive close to two years in undisturbed manure samples from the environment and at low temperatures (-20°C) for 100 days in cattle feces (Kudva *et al*., 1998). Generally, fecal samples are collected off of pen floors, from intestinal contents, or by rectal swab. There are conflicting reports on the

sensitivity for detection of STEC O157 between collection by recto-anal mucosal swabs and collection of rectal contents (Naylor *et al*., 2003; Khaitsa *et al*., 2005). The influence of sample collection method on sensitivity of non-O157 STEC detection has not been determined. Sample collection method is likely to be based on convenience and experimental design. Unless cattle are isolated, matching the pen floor sample to a specific cow can be difficult, but these samples are relatively easy to collect compared to rectal grabs and swabs.

A number of studies have estimated the prevalence of STEC in cattle feces over geographically different regions and times (Hancock *et al*., 1994; Elder *et al*., 2000; Cobbold *et al*., 2004; Renter *et al*., 2005; Thomas *et al*., 2012 Bosilevac *et al*., 2013). For beef cattle, STEC O157 prevalence has ranged from 0.2% to 27.8% at slaughter and for non-O157 STEC it was 2.1% to 70.1% at slaughter (Hussein and Bollinger, 2005).

Cattle are transient carriers of STEC and only rarely reported to consistently shed STEC over months to years. In one study, cattle were sampled 12 times over a year at approximately one month intervals for STEC O157 (Besser *et al*., 1997). Most cattle were positive for only a single sampling, but some cattle were repeatedly positive with two positive samples in sequential months (Besser *et al*., 1997). Similar heterogeneous shedding was observed in a longitudinal study sampling cattle 18 times at weekly intervals for STEC O157 (Williams *et al*., 2014).

Numerous studies have estimated the prevalence of STEC O157, but few have estimated the prevalence of EHEC-7 in cattle feces or other cattle matrices. Some studies have investigated EHEC serogroups within EHEC-7, with EHEC O26 as one of the most studied non-O157 EHEC. EHEC O26 causes the majority of clinical cases due to non-

O157 EHEC in the U.S. (Brooks *et al*., 2005), and thus is of particular concern. In beef cattle, 10 out of 2436 fecal samples tested positive for EHEC O26 (Sasaki *et al*., 2011). In 160 fecal samples, Bonardi *et al*. (2015) found three EHEC O26, one EHEC O111, and five EHEC O157 positive fecal samples. Dewsbury *et al*. (2015) found the prevalence of EHEC O26 was 1.0%, EHEC O103 was 1.6%, EHEC O145 was 0.8%, and EHEC O157 was 41.4% in 576 fecal samples collected during summer months. In contrast, during winter months no EHEC-7 were recovered (Dewsbury *et al*., 2015). *E. coli* serogroups O26, O45, O91, O111, O121, O145, and O157 were isolated from fecal samples of Australian beef cattle, but only *E. coli* O157 contained the genes for Shiga toxin (*stx*) and intimin (*eae*). Hence, the serogroup is not enough to determine if a strain is likely to be an EHEC.

The pen effect can influence the prevalence and transmission of STEC between cattle. Animals in the same pen are more likely to shed STEC if they are in the same pen as an animal that is already shedding STEC (Tabe *et al*., 2008). Accurate analysis of prevalence must take into account if samples were collected from animals in the same or a different pen. The condition of the pen can influence the transmission and prevalence of STEC (Smith *et al*., 2005). A higher prevalence of STEC is found in pens that are dry and dusty or muddy compared to a condition in between these (Smith *et al*., 2005).

Wang *et al*. (2015) found that cattle carry bacteriophages in their gastrointestinal tract that are able to lyse non-O157 STEC. The specificity of these phages was restricted to one serogroup or exhibited activity against a broad number of serogroups (Wang *et al*., 2015). These bacteriophage could drastically change the prevalence of STEC in cattle feces. Their use for control of non-O157 STEC in cattle has yet to be demonstrated.

Cattle hides

Cattle hides are contaminated by fecal material containing STEC through direct interaction with feces, contact with other animals, dirt and dust particles carrying STEC, or other surfaces (Keen and Elder, 2002). Similar to fecal samples, seasonal variation of STEC O157 is observed with hide contamination more likely to occur in summer months compared to winter months (Barkocy-Gallagher *et al*., 2003). Antimicrobial washes such as lactic acid, hydrogen peroxide, or cetylpyridinium chloride have been used to reduce bacteria on cattle hides (Baird *et al*., 2006). Criteria have been developed to judge cleanliness of hides based on a 5-point scale from 1 (very clean) to 5 (very dirty) during processing (McEvoy *et al*., 2000). There was a relationship between hide cleanliness and reduction of total viable bacterial counts recovered on beef carcasses (McEvoy *et al*., 2000). In another study, no relationship was observed between hide cleanliness and bacterial counts from carcasses (Van Donkersgoed *et al*., 1997).

Arthur *et al*. (2011) demonstrated that STEC O157 can survive on cattle hides for nine days. The prevalence of STEC O157 has been shown to fluctuate on cattle hides from 84% to 0% in only two weeks (Arthur *et al*., 2009). Thomas *et al*. (2012) recovered EHEC O26 and O157 from hides during beef processing. Similarly, Bonardi *et al*. (2015) recovered EHEC O26 from two of 160 and EHEC O157 from four of 160 hide samples from cattle at slaughter.

Sampling multiple locations on cattle hides gives a clearer representation of prevalence of STEC. The back, hock, perineum and belly were all optimal recovery sites for STEC O157 in different studies (Keen and Elder 2002, Stephens *et al*., 2007;

Kalchayanand *et al*., 2009). Keen and Elder (2002) found high isolation rates of STEC O157 from five different sites on cattle hides. In this study, isolation rates from the hides exceeded those from the feces, and visible fecal contamination did not correlate with high relative STEC O157 isolation (Keen and Elder, 2002). It is possible that wind and dust can distribute STEC over the entire hide. Hide surfaces can serve as a means for animal-to-animal transmission of STEC.

Cattle carcasses

Cattle carcasses primarily become contaminated with STEC during the removal of STEC contaminated hides during processing (Elder *et al.*, 2000; Arthur *et al.*, 2002; Keen and Elder, 2002; Barkocy-Gallagher *et al.*, 2003; Arthur *et al.*, 2004; Koohmaraie *et al.*, 2005; Schmidt *et al.*, 2012). Dehairing of hides, hide washes, and bacteriophage to lyse STEC O157 are examples of interventions to reduce hide to carcass transfer of microbial contamination during processing (Barkocy-Gallagher *et al*., 2003; Nou *et al*., 2003; Arthur *et al*., 2004). Similar transfer of STEC occurs during processing of sheep (Thomas *et al*., 2013). Pathogens can also be transferred onto the carcass surface by direct contact with contaminated equipment (knives) and hands of operators (Thomas *et al*., 2012). By sampling air next to hide removal stations during processing, Schmidt *et al*. (2012) found that hide removal introduced pathogens into the air that could then contaminate the carcass surface. Most studies suggest that carcasses can be contaminated with STEC, but the prevalence is typically <3% (Duffy *et al*., 2014). Carcass surfaces are treated with antimicrobial washes to reduce microbial contamination.

As with other sample types, most of the studies have focused on the prevalence of STEC or EHEC O157 on carcasses. Thomas *et al*. (2012) found EHEC O157 on two of 301 carcass samples. A study that sampled Danish beef carcasses found a prevalence of 3.2% for STEC O157 (Breum and Boel, 2010). After carcass dressing and being held at cold storage for a week, STEC O157 was recovered from only one of 16 found positive (Breum and Boel, 2010). This suggests that proper dressing and chilling of carcasses may result in death of those organisms contaminating the carcass surface. Detection of STEC O157 after interventions poses a serious risk to food safety.

Carcasses have been sampled before and after interventions to determine if they were effective in reducing STEC. Final post-intervention carcass samples had a significantly lower prevalence of STEC O157 compared to pre-intervention carcass samples (Rivera-Betancourt *et al*., 2004). If STEC are found on carcasses after interventions, this would indicate ineffective controls and introduces a risk for the consumption of contaminated products from that carcass.

Ground beef

Since STEC are frequently found in cattle, beef processors are required to develop a hazard analysis and critical control point specific to their operation to help control for biological, chemical, and physical hazards (USDA, 2014a). Contamination of cattle carcasses from removal of STEC contaminated hides is the primary route that leads to beef contamination but otherwise can occur at different points during processing. A multi-hurdle approach is required to eliminate contamination in the final product.

Inactivation of STEC in food products is an important step in reducing foodborne illness. Properly cooking food products such as raw beef to a temperature of 160°F reduces the risk of STEC infection (Luchansky *et al*., 2013). Many consumers are unaware of the proper temperature required to inactivate bacterial pathogens, and do not use a thermometer to ensure that proper temperatures have been reached (Røssvoll *et al*., 2014). Consumers incorrectly evaluated hamburger doneness based on the color as reported by Røssvoll *et al*. (2014). Thus, it is important for the beef industry to ensure elimination of adulterants before the product reaches consumers.

There is a tremendous economic loss to the beef industry associated with a recall due to EHEC-7 contamination. In 2014, 1,840,533 lbs of ground beef products were recalled due to EHEC-7 contamination (USDA, 2015a). High event periods are those events during processing where there are a higher than normal number of EHEC-7 positive trim samples (Wang *et al*., 2014a). High event periods may indicate a failure to implement a microbial control step. Sampling techniques for STEC detection use sponges to swab the carcass surface or hide surface of cattle. Sampling sites of cattle have been chosen based on locations that are primarily used for ground beef.

The prevalence of STEC in commercial ground beef has been evaluated by multiple studies. Bosilevac and Koohmaraie (2011) surveyed 4,133 ground beef samples from across the U.S. and found 1,006 samples positive for *stx*. However, only 10 STEC isolates that were considered to be pathogenic were isolated from 10 different samples. In a different study, a convenience sample of 1,129 commercial ground beef products were tested for STEC, but no isolates were recovered from this study (Liao *et al*., 2014). In

another study, 18 of 51 ground beef samples were positive for at least one of the regulated serogroups, but no STEC isolates were detected (Magwedere *et al*., 2013).

The USDA FSIS performs testing of raw ground beef and component samples for EHEC-7. In 2012, 0.53% of raw ground beef tested positive for EHEC O157 and 0.91% tested positive for one of the EHEC-6 organisms (USDA, 2015b). Of the samples testing positive for non-O157 EHEC, five were EHEC O26, seven were EHEC O103, and two were EHEC O111 (USDA, 2015b).

Veal

Of the 319 beef calf hides sampled, 54% tested positive for STEC O157 (Arthur *et al*., 2009). Hide contamination may lead to contamination of veal products. Different processing steps may be required for veal compared to beef, and the interventions used in veal processing have not been extensively evaluated (Wang *et al*., 2014b). Due to the high prevalence of EHEC in veal products, USDA FSIS reports EHEC prevalence in veal separately from raw beef samples. In 2012, the USDA found 0.60% of veal samples were positive for EHEC O157 and 7.89% were positive for one of the EHEC-6 (USDA, 2015b). A limited number of veal samples have been tested, with 166 and 38 tested for EHEC O157 and EHEC-6, respectively. Therefore, it is possible that the percentage of EHEC-6 positive veal samples is lower than expected due to the small sample size.

Flies and wildlife

Colonization by STEC is not restricted to ruminants. A number of other animals have been found to carry STEC. Flies are found around cattle production environments,

among other places, and have been found to carry STEC (Ghosh and Zurek, 2015). Flies may serve as vectors for STEC, carrying strains to multiple cows and to different farms. Janisiewicz *et al*. (1999) found that flies are internally and externally contaminated with STEC O157. Flies can also aggregate on cattle feed. Ghosh and Zurek (2015) found that flies carrying fecal coliforms can contaminate steam-flaked corn used in cattle feed which could then lead to contaminated cattle during consumption of feed.

Wildlife can interact with feedlots or environments contaminated with STEC. Many wildlife animals are consumed as meat products and therefore could be important transmitters of STEC to humans. STEC were isolated from deer, wild boar and hare meat and some strains were EHEC O26 and O103 (Miko *et al*., 2009). Deer have been implicated in the contamination of strawberries that led to an outbreak of STEC O157 (Laidler *et al*., 2013). Foxes have also been noted as carriers of STEC (Mora *et al*., 2012). Wild birds that carry STEC are of particular concern to produce farmers. STEC have been found in wild birds, and it has been suggested that they could transport STEC from feedlots and watersheds to produce fields (Cooley *et al*., 2013).

STEC characterization and risk assessment

Classical typing methods for *E. coli* have focused on the O, H, and K antigens and more recently mainly the O and H antigen. The O antigen makes up the outer most portion of the lipopolysaccharide and is attached to the core oligosaccharide. There are over 160 different O antigen structures and 53 H-types produced by *E. coli* (Bettelheim, 2007). There are approximately 400 known O:H combinations (serotypes), but only 100 of these have been associated with disease in humans (Bettelheim, 2007). Certain

serogroups such as EHEC-7 are more commonly associated with human illness (Brooks *et al*., 2005). Some of the biological roles that the O antigen is involved in include colonization of the intestine, resistance to antimicrobial peptides (Miyashita *et al*., 2012), and antiphagocytic ability of some strains of *E. coli* (Medearis *et al*., 1968). Sequencing of the gene that encodes for 6-phosphogluconate dehydrogenase (*gnd*) has also been used to assess the serogroup (Gilmour *et al*., 2007).

There are commercial reagents for testing of the O antigen for the most common serogroups in clinical disease. Serotyping is performed at reference laboratories commonly using antisera to test for the O antigen and restriction fragment length polymorphism for testing of the H-antigen (Joensen *et al*., 2015). H antigen determination has relied on the *fliC* gene that encodes for the flagellar filament structure protein. This has allowed H antigens to be typed even if the strain is non-motile. Serotyping can also be performed by whole-genome sequencing. Joensen *et al*. (2015) described the SerotypeFinder as a whole-genome sequencing tool for rapid identification of all known *E. coli* serotypes. Biologically, flagella allow movement of the bacteria and in some cases is an initial adherence factor to epithelial cells (Mahajan *et al*., 2009). The K antigen is a made up of polysaccharides or proteinaceous organelles and is a capsular antigen. A few of the functions of K antigens include resistance to serum-induced killing, interfering with complement (Johnson, 1991), and antiphagocytic abilities (Van Dijk *et al*., 1979).

Tracking and discrimination of strains is important for outbreak investigations. Multi-locus-sequence typing (MLST) is a technique that uses the sequence <10 highly conserved genes to compare the relatedness of strains (Sadiq *et al*., 2014). MLST can

define *E. coli* into sequence types, although higher discriminatory methods have been developed to discriminate strains within a sequence type. PFGE has been used to discriminate strains based on the migration of large DNA fragments under an electrical field (Sadiq *et al*., 2014). This has been the gold standard for typing STEC during outbreak investigations. Currently, surveillance of certain STEC outbreaks have used whole-genome sequencing to identify disease caused by a common strain from geographically dispersed outbreaks and outbreaks involving only a few patients (Dallman *et al*., 2015). The DiversiLab repetitive PCR can also be used for strain typing by amplifying regions of DNA between repetitive elements (Herbold *et al*., 2015).

Although classical EHEC of certain serogroups have received the most attention, non-classical EHEC O91, O104, O113, O128, and O146 have been isolated from clinical cases (Beutin and Fach, 2014). This has made predicting whether a STEC strain could be pathogenic to humans difficult. Molecular markers in combination with certain *stx* subtypes (stx_{1a} and stx_{2a}) and intimin have been associated with HC and HUS in patients (Scheutz *et al*., 2012). Based on molecular markers in O island (OI) 122, Karmali *et al*. (2003) developed the first molecular risk assessment (MRA) to assess the pathogenicity of STEC of different serogroups. Karmali *et al*. (2003) classified STEC into different seropathotypes. O157 was seropathotype A with a high incidence and common involvement in outbreaks. STEC O26, O103, O111, O121 and O145 were seropathotype B with moderate incidence and uncommon involvement in outbreaks. STEC O91, O104, O113 and others were seropathotype C with low incidence and rare involvement in outbreaks and multiple serotypes made up seropathotypes D and E with low and nonhuman incidence and rare and not applicable to involvement in outbreaks,

respectively. This classification scheme relies on the frequency of a serogroup to cause serious disease or known outbreaks, but does not fully describe the pathogenic potential of a strain. Coombes *et al*. (2008) expanded the MRA of STEC strains based on 16 genes from OI 36, OI 57, OI 71, in addition to OI 122. STEC strains associated with outbreaks strains had an average of 11.7 of 16 genes and non-outbreak strains had an average of 3.8 of 16 genes (Coombes *et al*., 2008). Strains that were from patients with HUS had an average of 8.4 of 16 genes and non-HUS strains had an average of 3.3 of 16 genes (Coombes *et al*., 2008).

Toxins

Shiga toxin is the main virulence factor of STEC. However, not all STEC are thought to be able to cause disease in humans. A myriad of other virulence factors in STEC have been associated with HUS and HC (Coombes *et al*., 2008). Factors to reduce the host immune response, inhibit cell death, and lyse cells to acquire nutrients all contribute to bacterial survival, persistence, and virulence.

Shiga toxin

Shiga toxin was first found in *Shigella dysenteriae*, which shares similarity to Stx found in *E. coli*. There are two major types of Stx termed Stx1 and Stx2. STEC strains can possess one or both types of the toxin. Shiga toxin genes are located on DNA from bacteriophage. After infecting *E. coli*, phage can grow lytically or undergo lysogenic growth by inserting into the bacterial chromosome. Toxin production can occur during initial lytic growth or after a signal induces lytic growth. Serra-Moreno *et al*. (2007)

described five main insertion sites plus additional sites used by *stx* when they integrate into the *E. coli* chromosome. Under conditions of stress, s*tx* can be lost from the chromosome (Croxen *et al.*, 2013). The toxin has two major subunits, subunit A and subunit B which make up an AB_5 structure (Kaper *et al*., 2004). The B subunit binds to globotriaosylceramide (Gb3) and the A unit is the catalytically active subunit. Once the A subunit is cleaved from the B subunit, it can bind to the ribosome and disrupt protein synthesis by cleaving a specific adenine (Kaper *et al*., 2004).

After a person becomes infected with STEC, Stx can cross the intestinal epithelium and enter the bloodstream (Croxen *et al*., 2013). The route that Stx takes to travel from the intestinal tract to the blood stream is unclear. It was shown that Stx binds to neutrophils, which could facilitate toxin transport to the kidneys where Stx will bind to Gb3 (Brigotti *et al*., 2008). Gb3 is found in abundance in the kidneys and to a lesser extent in the lung and brain (Obrig, 2010). Then, Stx is internalized and trafficked through the Golgi apparatus and the endoplasmic reticulum to the cytoplasm (Croxen *et al*., 2013). Once in the cytoplasm, the A subunit removes an adenine from 28S rRNA which inhibits protein synthesis (Croxen *et al*., 2013).

Stx2 is more toxic than Stx1in gnotobiotic piglets (Francis *et al*., 1989), in mice (Tesh *et al*., 1993), and is more often found in cases of human disease with severe illness (Law, 2000). Scheutz *et al*. (2012) developed a standardized nomenclature for Stx with three subtypes for stx_1 (stx_{1a}, stx_{1c}, and stx_{1d}) and seven subtypes of stx_2 (stx_{2a}, stx_{2b}, stx_{2c}, stx_{2d}, stx_{2e}, stx_{2f}, and stx_{2g}). Multiple variants of *stx* can be found in a single strain. Variability in subtypes is mainly in the B subunit effecting binding strength to Gb3 (Russo *et al*., 2014). The subtypes stx_{2a}, stx_{2c} and stx_{2d} are closely related (Scheutz *et al*.,

2012) and are found in isolates from patients with HUS (Feng and Reddy, 2013). At least three distinct sequence types of *stx*$_{2a}$ phages have been found (Yin *et al*., 2015). Diversity within subtypes arises from SNPs and insertion sequence element location (Yin *et al*., 2015). The sequence differences within subtypes may play a role in the amount of Stx produced (Yin *et al*., 2015). Subtype *stx*$_{2d}$ is also called mucus-activatable and is very toxic (Feng and Reddy, 2013). In pigs, *stx*$_{2e}$ has been isolated from animals with edema disease, and *stx*$_{2e}$ binds to globotetraosylceramide (Gb4) preferentially over Gb3 (Persad and LeJeune, 2014). Gb4 is found in porcine tissue but rarely in human tissues. Therefore, this subtype is rarely seen in human disease. In pigeon feces, *stx*$_{2f}$ is frequently isolated, but is rarely thought to be pathogenic to humans (Schmidt *et al*., 2000).

Stx can be found in bacteria other than *E. coli* and *Shigella*. Stx has been infrequently found in extraintestinal *E. coli* (Martínez-Castillo and Muniesa, 2014), and *Enterococcus* (Casas *et al*., 2011). Free Stx phage can be found in water contaminated with fecal material, human, cattle, and poultry wastewater and river water (Martínez-Castillo and Muniesa, 2014). Free Stx2 phage is more prevalent than Stx1 phage in extraintestinal environments (Grau-Leal *et al*., 2015). Stx phage can persist in the environment or in meat processing environment even with bacterial inactivation or disinfection treatments (Martínez-Castillo and Muniesa, 2014). Food processing steps to reduce bacterial contamination such as thermal treatment, salt, UV exposure and antimicrobials do not reduce the presence of Stx phage and could enhance Stx release from STEC (Allué-Guardia *et al*., 2014).

Cytolethal distending toxin (CDT)

CDT is produced by some Gram-negative bacterial pathogens, including *E. coli*. CDT has three subunits, CdtA, CdtB and CdtC. CDT is a cyclomodulin, which is term used to describe bacterial toxins and effectors that interfere with the eukaryotic cell cycle. CDT blocks the cell cyle transition between G_2 and M phases (Nougayréde *et al*., 2005). CdtB has DNase activity, while the other two subunits transport CdtB into the host target cell (Croxen *et al*., 2013). CdtB causes DNA damage leading to cell death, which has been demonstrated in multiple cell lines (Jinadasa *et al*., 2011). All three subunits of CDT in *E. coli* are thought to be required for cell death (Taieb *et al*., 2015). Five types of CDT are known, termed CDT-I through CDT-V. CDT-V was associated with strains causing diarrhea in humans (Croxen *et al*., 2013).

Cytotoxic necrotizing factor 1(CNF1)

CNF1 and its isoform CNF2 are AB-type cytotoxins. CNF is a cyclomodulin that promotes eukaryotic cell proliferation by activating DNA replication and the transition from G_1 to S phase of the cell cycle (Nougayréde *et al*., 2005). Although CNF1 is mainly found in uropathogenic *E. coli* and *E. coli* causing neonatal meningitis, it has also been found in STEC (Kaper *et al*., 2004). The C-terminal portion of CNF1 is involved in the deamination of a glutamine residue of Rho GTPases which causes reorganization of the cytoskeleton (Kaper *et al*., 2004). Reorganization of the cytoskeleton by CNF1 causes impairment of epithelial barrier function (Hopkins *et al*., 2003). CNF1 may also play a role in modifying the morphology of mitochondria to increase ATP-producing capacity for greater cell proliferation (Travaglione *et al*., 2014).

Enteroaggregative heat-stabile toxin (EAST1)

EAST1 is a heat-stable enterotoxin encoded by the *astA* gene found in ETEC, EPEC, and STEC. Both STEC O157 and non-O157 STEC tested positive for *astA* (Law, 2000). The role of EAST1 in pathogenesis remains unclear. It has been suggested that EAST1 contributes to the initial diarrhea seen in patients, because fluid secretion is stimulated in the rabbit model by EAST1 (Law, 2000). EAST1 and K88 fimbria were cloned and a constructed *E. coli* strain that lacked other toxins was used to assess the effect of EAST1 in K88 receptor-positive gnotobiotic piglets (Ruan *et al*., 2012). Gnotobiotic piglets did not develop diarrhea and showed no signs of dehydration after being challenged with 10^9 CFU of this strain (Ruan *et al*., 2012). This suggests that EAST1 alone does not play a role in diarrhea.

Subtilase

Subtilase (SubAB) is an AB_5 cytotoxin that causes apoptosis in human cortical renal tubular epithelial cells (Márquez *et al*., 2014). SubAB is located on a 165-kb plasmid first found in STEC O113 (Paton *et al*., 2004), but may also be found on the chromosome of some strains. There are variants of subtilase and a single strain can have multiple variants. The possession of at least two subtilase variants occurred in about half of the STEC strains isolated from sheep and wild ruminants (Nüesch-Inderbinen *et al*., 2015). The A subunit cleaves a chaperone of the endoplasmic reticulum. This leads to accumulation of unfolded proteins in the endoplasmic reticulum, which leads to stress-induced cell death (Croxen *et al*., 2013).

Plasmids

Plasmids are covalently-closed, circular DNA molecules that replicate independently from the chromosome and commonly carry virulence and antibiotic resistance genes. Plasmids can also integrate into the chromosome. Most STEC carry a plasmid known as pO157 that is 75-100 kb, and additionally have other plasmids. Plasmid pO157 is made up of prophage, parts of other plasmids, and other mobile genetic elements (Caprioli *et al*., 2005). Due to the number of mobile elements, genes are unevenly distributed among STEC strains (Ishii *et al*., 2007). It is thought that pO157 was acquired from *Yersinia pestis* due to the similarity found between pO157 in EHEC strains and pCD1 found in *Y. pestis* along with similarities to pO157 and sequences in *Y. pestis* chromosome (Johnson and Nolan, 2009). The large plasmid carries genes for EHEC-hemolysin (*ehxA*), catalase peroxidase (*katP*), serine protease autotransporter (*espP*), a type II secretion system (*etpC-O*), a zinc metalloprotease (*stcE*), and an adherence factor (*toxB*; Yan *et al*., 2012). The *toxB* gene shows considerable similarity to another adherence factor (*efa1*), which is found on the chromosome (Caprioli *et al*., 2005). Based on the nucleotide sequence, pO157 encodes for 35 proteins in total, including a type II secretion system, but not all of the functions are known (Burland *et al*., 1998).

The biological role of pO157 is not fully understood. In a *Caenorhabditis elegans* model of infection, pO157 was needed for full pathogenicity and killing of *C. elegans* (Youn *et al*., 2013). Its role in colonization of cattle is not well understood, and there are conflicting reports using cell lines (Law, 2000). Interestingly, in an inoculation

experiment involving cattle, strains without pO157 were not able to persist as well as wild type strains (Sheng *et al*., 2006).

A 121-kb plasmid (pSFO157) is found in sorbitol fermenting EHEC O157. Plasmid pSFO157 shares high similarity to pO157 and is predicted based on complete DNA sequence analysis to share a hypothetical ancestral plasmid (Rump *et al*., 2012). The major differences observed between pSFO157 and pO157 include the absence of *espP*, *katP*, and *toxB* in pSFO157. Also, pSFO157 has a *sfp* fimbria gene cluster that is absent from pO157 and accounts for most of the additional size (Brunder *et al*., 2006). Induction of this fimbria increased under conditions mimicking the colonic environment and facilitated adhesion to Caco-2 and HCT-8 cells (McWilliams and Torres, 2014).

A smaller 3.3-kb plasmid, termed pSP70 by Sharma and Stanton (2008) is also present in some EHEC strains. In EHEC O157, a 3.3-kb plasmid was found in 6.5% (Meng *et al*., 1995) and 8% (Haarmann *et al*., 1998) of strains tested. Haarmann *et al*. (1998) found sequence similarity between pSP70 and the antibiotic resistance plasmids NTP1 which confers ampicillin resistance and NTP16 which confers kanamycin resistance found in *Salmonella typhimurium* strains. It is unclear if pSP70 has any role in virulence.

In some LEE-negative STEC strains, a plasmid, pO113, named after STEC O113:H21 is found (Croxen *et al*., 2013). Plasmid pO113 has a size of 165-kb plasmid and contains many of the same genes found in pO157. By sequencing, Newton *et al*. (2009) found that pO113 encodes for known virulence factors *ehxA*, *espP*, *saa*, and *subAB*. A large transfer region accounted for most of the increase in size compared to

pO157 (Newton *et al*., 2009). Thus, plasmids in STEC contain genes for toxins, adherence factors, secretions systems, and other proteins with unknown functions.

Adherence factors

The LEE is a 35-kb pathogenicity island in EHEC and EPEC that encodes for intimin, Tir, and the type III secretion system (Jores *et al*., 2004). The LEE is arranged into five polycistronic operons, LEE1 through LEE5, and is required for A/E lesions (Kaper *et al*., 2004). Elliott *et al*. (1999) cloned the LEE from a STEC O157 strain into an *E. coli* K-12 strain, but the K-12 strain was not able to produce A/E lesions or secrete *E. coli* secreted proteins (Esp). The Tir-cytoskeleton factor protein (TccP) was found outside the LEE and required for A/E lesion formation (Garmendia *et al*., 2004). An A/E lesion is described by intimate attachment of bacteria to the host cell, effacement of microvilli, and polymerization of actin filaments underneath attached bacteria resulting in "pedestal" formation (Kaper *et al*., 2004). In addition to *E. coli*, the LEE is found in members of *C. freundii* and *H. alvei* (McDaniel *et al*., 1995). The LEE is speculated to be acquired by horizontal gene transfer because it has a lower GC content compared to that of the *E. coli* chromosome (Elliott *et al*., 1998). The LEE is optimally expressed at 37°C, while in the exponential growth phase (Rosenshine *et al*., 1996), and induced in cell culture media (Roe *et al*., 2003). The LEE encoded regulator is a master regulator of LEE transcription and activates transcription of each LEE operon.

Intimin

Intimin is a 95-kDa protein and serves as the major adherence factor for EHEC (Kaper *et al*., 2014). The 280 amino acids on the C-terminal end of the protein serve as the binding region to Tir (Frankel *et al*., 1995). A study involving human volunteers established the importance of intimin for the development of diarrhea (Donnenberg *et al*., 1993). Volunteers ingested 2 x 10^{10} CFU of an EPEC strain or an isogenic *eae* deletion mutant. All 11 volunteers who received the intimin-positive wild-type strain developed diarrhea, while only four of 11 who received the intimin-negative mutant strain developed diarrhea (Donnenberg *et al*., 1993). Different growth media has an influence on expression of intimin. Knutton *et al*. (1997) reported greater expression of intimin in cell culture media (DMEM) compared to Luria-Bertani broth.

Intimin types β, ε, γ1 and γ2 were found to be preferentially associated with certain serotypes of STEC. Adu-Bobie *et al*. (1998) found that *E. coli* O26:H11 was associated with *eae*-β, and *E. coli* O157:H7 with *eae*-γ. In total, 27 intimin types are known (Ito *et al*., 2007). In part, the intimin type determines the tissue specificity of a strain. Strains expressing *eae*-α and *eae*-β have similar tissue specificity in human *in vitro* organ cultures (Mundy *et al*., 2007). In gnotobiotic pigs, strains expressing *eae*-α produced A/E lesions in the small and large intestine, while those expressing *eae*-γ produced lesions only in the large intestine (Tzipori *et al*., 1995). Phillips and Frankel (2000) investigated tissue tropism of intimin using human intestinal organ cultures. Complementation of an *eae*-α mutant EPEC strain, resulted in colonization of the small intestine, but complementation with *eae*-γ, resulted in colonization of Peyer's patches (Phillips and Frankel, 2000). Intimin can also adhere to cell culture without Tir, and thus, can use host cell surface receptors to adhere (Farfan and Torres, 2012).

Fimbrial adhesins

Intimin is the primary adhesin in STEC, but fimbriae also contribute to adhesion. There are 16 fimbrial loci in *E. coli* O157:H7 (Gonyar and Kendall, 2014) and at least two others in non-O157 STEC (Wurpel *et al*., 2013). Long polar fimbria (Lpf), curli, *E. coli* common pilus (ECP) and hemorrhagic *E. coli* pilus (HCP) are the best characterized fimbrial proteins.

Lpf were found in STEC O157 strains to increase adherence to HeLa and MDCK cells (Torres *et al*., 2002). Two fimbrial loci, *lpf1* and *lpf2*, encode Lpf. They display long, fine structures based on transmission electron microscopy (McWilliams and Torres, 2014). In sheep and pigs inoculated with *lpf1*/*lpf2* double mutants, a significantly lower recovery rate was reported (Jordan *et al*., 2004). Lpf are most likely important for initial adhesion before intimate attachment is established (Farfan and Torres, 2012)

STEC have specialized fimbria that allow them to colonize plant leaves such as those of lettuce and spinach. *E. coli* K-12 strains fail to bind the plant, but successful attachment occurred when Jeter and Mattysse (2005) introduced curli into K-12 strains. Curli is an adhesin found in STEC and EPEC that binds to fibronectin to mediate attachment to animal cells, but has a dual role in binding plant surfaces when outside the host (Farfan and Torres, 2012).

ECP is found in most *E. coli* including STEC. In one study, expression of ECP was found in 121 of 169 *E. coli* strains covering diarrheagenic and extraintestinal strains (Rendón *et al*., 2007). High levels of expression for ECP are observed between 20°C to 26°C and a lack of expression is observed at 37°C (McWilliams and Torres, 2014). Using

Purified ECP, Saldaña *et al*., 2009 found ECO bound to HeLa cells, and anti-ECP antibodies blocked binding. This confirmed ECP role as an adhesin in STEC.

HCP was shown to be involved in binding to human intestinal and non-intestinal cell lines (McWilliams and Torres, 2014). HCP is a multipurpose fimbria involved in attachment, binding to extracellular matrix proteins, invasion of epithelial cells, interbacterial connections, hemagglutination of rabbit erythrocytes, and twitching motility (Xicohtencatl-Cortes *et al*., 2009b). Multiple functions of HCP contribute to virulence, survival, and transmission.

Non-fimbrial adhesins

Other adhesins contribute to intestinal colonization by STEC. EHEC factor for adherence (Efa-1) is a 365-kDa protein found in STEC and is known as lymphostatin (LifA) in EPEC (Farfan and Torres, 2012). Efa-1 is found on the chromosome and its homologue designated ToxB is found on pO157. ToxB is 28% identical to Efa-1 by amino acid identity (Tatsuno *et al*., 2001). Using transposon mutagenesis, Efa-1 was first identified as an important adhesin in EHEC O111, but was not required for A/E lesion formation (Nicholls *et al*., 2000). Efa-1 has been associated with inducing secretion of T3SS effectors, cell adherence and lymphostatin activity (Farfan and Torres, 2012).

Autotransporters are outer membrane proteins that facilitate their own transport across the bacterial cell membrane. A number of autotransporters have been identified in STEC. Three common autotransporters involved in adhesion are EHEC autotransporters (Eha), EhaA, EhaB, and EhaJ. EhaA and EhaB are more prevalent in STEC strains compared to EhaJ (Farfan and Torres, 2012). EhaA is involved in adherence to mucosal

epithelium of the bovine terminal rectum, which makes it a key target for reducing shedding of STEC in cattle (Farfan and Torres, 2012). EhaB and EhaJ bind extracellular matrix proteins (Wells *et al*., 2009; Easton *et al*., 2011).

Two other autotransporters, EspP and STEC autoagglutinating adhesion (*saa*), are important adherence factors. The gene for expression of EspP (*espP*) is carried on pO157. EspP forms structures called "ropes" that mediate adherence to epithelial cells (McWilliams and Torres, 2014). Deletion of *espP* in STEC O157 demonstrated reduced adherence to T84 intestinal epithelial cells (Puttamreddy *et al*., 2010) and primary bovine rectal cells (Dziva *et al*., 2007) compared to the wild-type strain. LEE-negative STEC need adhesion factors to be able to colonize the gastrointestinal tract. The adhesin, *saa*, is an autotransporter found in O113 STEC. It increased adherence 10-fold when introduced into a *saa*-negative strain (Paton *et al*., 2001).

Additional virulence factors

Catalase peroxidase (KatP)

KatP is an 82-kDa protein, encoded by a gene (*katP*) carried on pO157; KatP has a dual role of catalase and peroxidase (Croxen *et al*., 2013). It is thought to be involved in recovery from heat stress and protection from macrophage oxidative burst (Law, 2000). The distribution of this gene in STEC even among STEC of the same serogroup is variable (Bosilevac and Koohmaraie, 2011) and its role in causing disease is unclear.

EHEC-hemolysin

EHEC-hemolysin is a member of the repeats-in toxin (RTX) family and encoded by a gene (*ehxA*) on pO157 (Kaper *et al*., 2004). EHEC-hemolysin is released in its free form upon lysis of bacterial cells or in outer membrane vesicles (Bielaszewska *et al*., 2014). It produces thin rings of hemolysis after 18 to 24 h of incubation on blood agar unlike the better described alpha-hemolysin of UPEC strains, which produces large zones of clearing after 4 h of incubation on blood agar (Beutin and Fach, 2014). Most *eae*-positive STEC possess *ehxA*, but some *eae*-negative STEC may lack the gene (Verstraete *et al*., 2013). EHEC-hemolysin forms a pore in the cell membrane of erythrocytes and other eukaryotic cells. It was found to elicit a proinflammatory response by releasing interleukin-1β from human macrophages, which may amplify the effect of LPS and Stx (Zhang *et al*., 2012). In patients with HUS, antibodies are produced against the toxin (Schmidt *et al*., 1995).

Mitochondrial associated protein (Map)

Map is expressed by EPEC and EHEC and functions to stimulate filopodia formation and acts on mitochondria function (Kenny *et al*., 2002). Additionally, Map was shown to be coordinately regulated with Tir such that Map signaling inhibited pedestal formation (Kenny *et al*., 2002). Therefore, Map may act on filopodia before intimate adherence occurs.

Type III secretion system

The type III secretion system (T3SS) structural components were thought to have evolved from flagella, and based on structure comparison has similar structures to

flagella (Blocker *et al*., 2003). *E. coli*, in addition to other pathogens, such as *Chlamydia*, *Salmonella*, *Shigella*, and *Yersinia* use a T3SS to attach to host cells, inject virulence proteins, and/or invade into cells. The T3SS is includes proteins that form ring-like structures in the inner and outer cell membrane of the Gram-negative bacterium, and a channel to the exterior of the bacterial cell. In addition, this channel is continuous with other T3SS proteins that form a needle-like structure on the bacterial surface that is inserted into and through the host cell membrane. This allows for secretion of proteins from the bacterial cytoplasm directly into the host cell cytoplasm. The T3SS is a needle structure made up of the needle filament protein EspA and pore proteins EspD and EspB that allow effector proteins to be injected and secreted through the pore (Connolly *et al*., 2015).

Jarvis and Kaper (1996) demonstrated that EHEC secrete proteins by a T3SS, are recognized by antiserum raised against proteins secreted by EPEC, and suggested that they are similar to secreted proteins of EPEC. Translocators are proteins that help translocate proteins into the host cell cytoplasm. Translocators are typically conserved among different pathogens (Connolly *et al*., 2015). Those proteins injected into the host cytoplasm are known as effectors, since they effect changes in the host cells. Effectors act to inhibit immune system to avoid pathogen detection and inhibit cell death (Vossenkämper *et al*., 2011).

STEC strains not only use the T3SS to adhere and alter the host cell, the T3SS can induce epithelial barrier disruption, which can contribute to diarrhea (Coburn *et al*., 2007). To block the effects of the T3SS small molecule inhibitors have been designed to block secretion of effector proteins (Gu *et al*., 2015). This would be an attractive strategy

to treat infections caused by a broad range of Gram-negative pathogens that used the T3SS.

Pre-harvest interventions

Pre-harvest interventions are designed to reduce the carriage of STEC in live cattle and the environment. Interventions have addressed cleaning of equipment such as feed and water troughs, bedding, livestock trailers, reducing animal density, removal of cattle shedding high numbers of STEC O157, bacteriophage, direct-fed microbial (DFM), probiotics, and vaccines.

Direct fed microbials and prebiotics

Bacteria produce antimicrobial compounds called bacteriocins for the purpose of inhibiting the growth of other competing bacteria. Direct-fed microbials (DFM) are compounds that are fed to animals with the intended main purpose of reducing intestinal colonization by pathogenic bacteria, and probiotics are non-pathogenic bacteria added to the feed used for the same purpose. *Lactobacillus*, *Bacillus* and *Bifidobacterium* are common genera of probiotics (Uyeno *et al*., 2015).

Tabe *et al*. (2008) evaluated the effect of DFM on fecal shedding of STEC O157 in 138 feedlot cows. They found that cows treated with a DFM had a lower prevalence of STEC O157 compared to an untreated control group. *Lactobacillus acidophilus* has been shown to reduce intestinal epithelial colonization *in vitro* (Medellin-Peña and Griffiths, 2009) and in live cattle (Uyeno *et al*., 2015).

Vaccines

The use of vaccines against STEC O157:H7 have shown promising results for reducing STEC shedding in cattle (Smith, 2014b). Since STEC rarely cause recognizable disease in cattle, vaccines against STEC would be used primarily for public health purposes. Vaccines against STEC O157 have yet to be adopted by cattle producers, and most likely will not be adopted until their direct benefit to the producer is found to outweigh the cost. Most vaccines are serotype specific, which results in poor cross-protection of other serotypes (Cernicchiaro *et al*., 2014). Vaccines targeting various T3SS components including intimin, Tir, EspA, and EspB/EspD have had mixed results, but some formulations of the T3SS vaccines were effective in challenge studies and field studies (Smith, 2014b). The length of immunity with vaccines against STEC O157 is unknown, because most field studies only last 60 to 100 days (Smith, 2014b).

Models have shown a reduction in fecal shedding in summer months down to that of winter months with the use of a T3SS protein vaccine (Vogstad *et al*., 2014). Additional models have demonstrated the potential usefulness of vaccines against STEC O157 in cattle, which would reduce STEC O157 infections in humans (Smith, 2014b). Although vaccines can help to reduce shedding of STEC, it is unlikely that they will completely eradicate these organisms from cattle (Vande Walle *et al*., 2013).

Models for STEC infection

Animal and cell culture models have provided insight into the pathogenesis and virulence of STEC. Human derived cell lines, Caco-2, Hep-2, T84 cells, have been used to study adherence (Farfan and Torres, 2012). Currently, there are no bovine intestinal

epithelial cell lines that can be used to study STEC adherence. Human and cattle *in vitro* organ culture provide a more suitable environment to mimic host infection, but these tissue quickly undergo postmortem autolysis (Baehler and Moxley, 2000). Baboons, *Caenorhabditis elegans*, chickens, ferrets, greyhounds, monkeys, and rats have been used a limited number of times, but mice, pigs, and rabbits are the most commonly used animal models (Ritchie, 2014). Mice are resistant to colonization, but renal damage occurs with inoculation of Stx into the bloodstream (Tesh *et al*., 1993). Differences in renal damage between mice and humans due to Stx render this model of questionable relevance (Ritchie, 2014). The mouse pathogen *Citrobacter rodentium* shares many virulence factors including intimin with EHEC strains and has been used as a model system to study *in vivo* colonization. An approach using *C. rodentium,* which naturally colonizes mice, has been developed to study attachment in the mouse model. This has been further developed by Mallick *et al*. (2012) to include a *C. rodentium* strain that expresses Stx. Construction of human and bovine intestinal xenograft models in mice have improved standard models (Golan *et al*., 2011). When a STEC strain was injected into the lumen of intestinal xenografts, tissue damage in the colon but not small intestine was observed, consistent with previous findings that the colon is the main site of attachment (Kaper *et al*., 2004). A lack of normal food passage or peristalsis, and a lack of natural microflora are limitations of this model (Ritchie, 2014).

Rabbits vary in their susceptibility and usefulness as a model for STEC infection. When inoculated with STEC O157 young New Zealand White rabbits developed diarrhea, but diarrhea was not observed in rabbits older than 20 days (Farmer *et al*., 1983). Inoculation of STEC O157 did not result in signs of HUS likely due to a lack of

Gb3in the renal tubular epithelial cells, but deletion of *eae* in the same strain did result in reduced colonization supporting the importance of intimin for attachment (Ritchie *et al*., 2003).

Gnotobiotic piglets are delivered via cesarean secretion and raised in incubators as germ-free animals. They can be used to study the influence of a single organism without the influence of the normal microflora. In addition to intestinal colonization, A/E lesions, they developed central nervous system disease with evidence of vascular damage in the brain identical to what is seen in humans (Ritchie, 2014). This model has been helpful to study STEC pathogenesis (Francis *et al*., 1986; Tzipori *et al*., 1986).

Detection of STEC

STEC detection is important for diagnosis of clinical infections, outbreak investigations, environmental monitoring, and food safety. In order to know that STEC contamination has been prevented, e.g., through interventions or other process controls, effective methods must be established to detect these organisms. Due to these and other concerns, which include meeting regulatory requirements, a number of test methods have been developed to detect STEC. The USDA FSIS developed standardized methods for detection of EHEC-7 in meat products (USDA, 2014b). This relies on the screening of enriched samples by PCR for O-groups, *stx*, and *eae*. Potential positive samples are plated on chromogenic agar, and individual colonies are tested by PCR, latex agglutination, and biochemical testing is performed. These methods are laborious, expensive, and time consuming. A number of commercial products have been developed to reduce the time and cost for screening for STEC. Some products have received a letter

of no objection by the USDA FSIS, which means they can be used in place of the standard USDA FSIS protocol.

Enrichment

Enrichment of a sample is often needed to improve detection because STEC is found in such low numbers (Wang *et al*., 2013). Factors of an enrichment procedure are the base enrichment broth, added selective components, time of enrichment, and temperature. Generally the sample is diluted 1:10 in enrichment media to reduce inhibitors of STEC growth (Paddock *et al*., 2012). Evans *et al*. (2011) found an increased recovery of *E. coli* isolates from 1 g fecal samples enriched in 20 ml compared to 10 g of the same sample enriched in 225 ml, which indicates sample size in addition to dilution factor is important. Samples are typically enriched at 37°C to 42°C for 6 to 24 h. To enhance recovery, pre-enrichment media can be used before an enrichment step to enable growth of stressed bacterial cells (Wang *et al*., 2013). Buffered peptone water has been used as a pre-enrichment broth and optimized by supplementation with 8-hydroxyquinoline and sodium deoxycholate for *E. coli* growth in minced meat (Margot *et al*., 2015).

Selective components added to enrichment media are used to inhibit growth of background flora without reducing the growth of STEC. Acriflavin, bile salts, cefixime, cefsulodin, novobiocin, potassium tellurite, and vancomycin are commonly used alone or in combination as selective components (Hussein and Bollinger, 2008). Cefixime has been used to inhibit swarming growth of *Proteus* on agar plates. *Proteus* is found in feces and the swarming phenotype inhibits isolation of organisms (Chapman *et al*., 1991).

Cefsulodin inhibits *Aeromonas* spp., *Pseudomonas aeruginosa*, and *Vibrio cholerae* (Hussein and Bollinger, 2008). Novobiocin is known to inhibit Gram-positive organisms, but some STEC may grow poorly under concentrations of novobiocin commonly used in enrichment media (Baylis, 2008). Acriflavin, bile salts, and vancomycin are additional components that are selective against Gram-positive organisms (Hussein and Bollinger, 2008; Savoye *et al*., 2011). Potassium tellurite inhibits both Gram-positive and -negative organisms. Resistance to potassium tellurite is variable among STEC strains and *eae*-positive strains are more likely to grow in the presence of potassium tellurite (Fukushima *et al*., 2000).

Concentrations of antimicrobials are based on minimum inhibitory concentrations of strains, field studies, and inoculation experiments. Tellurite resistance is variable among EHEC-7 strains. In one study, the majority of EHEC-7 strains had a MIC above 50 mg/liter (Fukushima *et al*., 2000). However, they did not test any *E. coli* O45 strains. The absence of bile salts and novobiocin and the presence of yeast extract in TSB related to higher detection of STEC O26 and O157 in raw ewe's milk (Caro *et al*., 2011). Possé *et al*. (2008a) used TSB with 8.0 mg/liter of novobiocin, 16 mg/liter vancomycin, 2.0 mg/liter rifampicin, 1.5 mg/liter bile salts, and 1.0 mg/liter potassium tellurite to enrich inoculated cattle feces. This selective enrichment procedure did not recover all strains from inoculated cattle feces, which indicates that some strains may be inhibited at these concentrations. Most STEC O157 strains were recovered, but after selective enrichment there was poor recovery for non-O157 STEC (Possé *et al*., 2008a).

An enrichment time that is long enough to grow STEC to a detectable concentration is needed for recovery of STEC in complex matrices. Typical enrichment

times have been from 6 to 24 h depending on sample type, convenience, or follow-up analysis. When the follow-up analysis includes plating on agar media, longer enrichment times may cause overgrowth by other background organisms (Vimont *et al*., 2006). A short enrichment time (6 h) was compared to a longer enrichment time (24 h) for the recovery of EHEC-7 from inoculated cattle feces (Verstraete *et al*., 2010). The 6 h enrichment time compared to 24 h did not influence the recovery of EHEC-7, but two enrichment times with direct plating and immunomagnetic separation (IMS) may result in improved recovery. Improved recovery of EHEC O157 was observed with the inclusion of IMS, but recovery was not improved for other EHEC serogroups (Verstraete *et al*., 2010). Similarly, Paddock *et al*. (2012) found better detection limits when inoculated bovine feces were enriched for 6 h compared to 24 h. The use of multiple enrichment times and plating schemes increases the labor required per sample. Similarly, Durso and Keen (2007) investigated the effect of enrichment on recovery of STEC O157 from 116 naturally contaminated fecal samples. For STEC O157, 6 h enrichment improved detection. Enrichment tubes are typically incubated statically, but no studies have compared growth or detection of STEC in fecal samples incubated statically versus agitated. Using a metagenomics approach for STEC detection in spinach samples, Leonard *et al*. (2015) found that STEC were detected more robustly in statically enriched samples compared to samples that had been shaken during enrichment.

Simultaneous enrichment and detection is an appealing strategy to reduce the time to result for pathogen testing. This would reduce the time that foods are held before they can be sold to the consumer. This has been developed for STEC O157 using a biochip with antibodies specific for *E. coli* O157:H7 (Mondani *et al*., 2014). In contrast, the use

of multiple enrichment broths has resulted in improved recovery of STEC O157 in naturally contaminated fecal samples (Vidovic *et al*., 2007). The time and cost saved using one enrichment media for multiple pathogens compared to multiple enrichment media for one pathogen needs to be considered. A universal enrichment media that is selective for all STEC serogroups is unlikely due to the diversity observed between STEC strains.

IMS is a method that uses treatment of a culture with paramagnetic beats coated with antibodies directed against the O antigen of interest. Bacteria bound to beads are separated from the broth by the use of an external or intrasolution magnet and wash steps are used to reduce non-specific binding. The PickPen is an intrasolution magnetic particle transfer device that showed improved recovery of *E. coli* O157 from naturally contaminated carcass, hide, and fecal samples compared to an external magnet (Nou *et al*., 2006). IMS has improved the detection of STEC O157 in fecal samples (Chapman *et al*., 1994). Bead size and buffers have been optimized for the recovery of STEC O157 (Parham *et al*., 2003). Multiple IMS technologies are available for *E. coli* O157. Lau *et al*. (2012) compared the Pathatrix Auto and KingFisher Flex for recovery of *E. coli* O157 from inoculated food. After 24 h of enrichment, 100% recovery of *E. coli* O157 was achieved from ground beef, sprouts, and green onions by both platforms. IMS has been shown to improve recovery of STEC O26, O111 and O157 in inoculated foods, such as cabbage, carrots, lettuce, potatoes, and radishes (Safaríková and Safarík, 2001).

Simultaneous IMS for multiple serogroups would reduce the processing time and cost per sample. Kanki *et al*. (2014) evaluated the use of simultaneous IMS for O26, O111, and O157 added to enriched food samples. They found comparable results for

isolation of STEC O26 and O111, and similar but weaker results were observed for isolation of STEC O157 (Kanki *et al*., 2014). Currently there are commercial antibodies and IMS reagents for EHEC-7 serogroups (Medina *et al*., 2012). However, antibodies for some serogroups lack specificity to the O-groups targeted, and may target other conserved areas on the LPS antigen (Stromberg *et al*., 2015b). After IMS, the culture may undergo PCR, or plated on agar media for the isolation of STEC. Type of IMS bead may influence the recovery of organisms. Low affinities for O111 and O145 were observed by Verstraete *et al*. (2010) using Dynabeads.

Agar media

Agar media provides isolated colonies for follow-up detection tests, further investigation of virulence, and comparison to other isolates for outbreaks investigations. A distinct fermentation pattern belonging to STEC O157 has allowed for the development of agar media designed to screen for its unique phenotype. STEC O157 does not ferment sorbitol within 24 h, while other *E. coli* are sorbitol fermenters (Doyle and Schoeni, 1984). This led to replacing lactose in MacConkey agar with sorbitol (SMAC). STEC O157 cannot cleave 4-methylumbelliferyl-β-D-glucuronide (MUG), which produces a fluorescent product under UV light. MUG negative colonies are thus typical of STEC O157, while most non-O157 STEC are MUG positive (Thompson *et al*., 1990).

Commercial agar media are typically faster to prepare or come pre-poured, which reduces the labor needed. The USDA FSIS has implemented a modification of Rainbow Agar O157 in their testing of STEC in enriched beef samples. On this media, STEC O157

produce grey to black colonies, STEC O26 produce purple to magenta, STEC O111 are grey-green, and other non-O157 STEC are blue, purple, white, violet, or red (Verhaegen *et al*., 2015). The wide ranges of phenotypes exhibited within a STEC serogroup and within EHEC-7 make this agar difficult for use in screening. CHROMagar STEC is another commercial chromogenic agar for the detection of STEC. On CHROMagar STEC, all STEC strains are screened based on one phenotype, mauve (Verhaegen *et al*., 2015). STEC O157 colonies appear non-fluorescent under UV light while non-O157 STEC may be fluorescent or non-fluorescent. Some STEC are inhibited on this agar, and therefore, using it could give false-negative results (Verhaegen *et al*., 2015). Gouali *et al*. (2013) evaluated the performance of CHROMagar STEC for detection of STEC in human stool samples, and found that 82% of the Stx-positive stools based on PCR recovered a STEC isolate on CHROMagar STEC.

Most STEC O26 strains cannot ferment rhamnose and thus lactose in MacConkey agar was replaced with rhamnose in rhamnose-MacConkey agar (RMAC; Hiramatsu *et al*., 2002). Some strains of *Enterobacter cloacae*, *Shigella flexneri*, and *Shigella sonnei* mimicked the phenotype of STEC O26 on RMAC, which suggested a need for selective components to reduce background organisms. RMAC supplemented with potassium tellurite and cefixime at concentrations of 2.5 and 0.05 mg/liter, respectively (CT-RMAC), selected for STEC O26. CT-RMAC had improved recovery for STEC O26 in inoculated human stool samples when compared to RMAC (Hiramatsu *et al*., 2002).

In general, non-O157 STEC do not have biochemical features that distinguish them from generic *E. coli*. Most non-O157 STEC ferment sorbitol which means that SMAC developed for STEC O157 will not be useful for detection of non-O157 STEC.

The development of agar for isolation of non-O157 STEC has been more difficult and relied on a combination of carbohydrates, utilization of X-gal, and resistance to antimicrobials. A standardized method for isolation of non-O157 STEC from cattle matrices has not been developed. Possé *et al*. (2008b) formulated a chromogenic agar media for non-O157 STEC of serogroups O26, O103, O111, and O145 based on the fermentation of sucrose and sorbose, β-galactosidase activity and selective components. After plating on this agar, a second agar was used to identify each serogroup (Possé *et al*., 2008b). A limited number of strains within these four serogroups were tested, and a larger number of strains and serogroups would help evaluate the usefulness of this agar. Variations in use of chromogenic agar, such as incubation time and temperature may cause slight differences in typical colony color. Due to the lack of media to detect non-O157 STEC, current data largely underestimates the true incidence of STEC infection in humans (Gill *et al*., 2014; Verhaegen *et al*., 2015).

Agar media have been designed for detection of STEC based on the expression of their virulence factors. STEC heart infusion washed blood agar with mitomycin-C (SHIBAM) screens for STEC colonies based on the lysis of red blood cells by EHEC-hemolysin (Feng *et al*., 2011). Mitomycin C enhances the production of EHEC-hemolysin (Sugiyama *et al*., 2001). Screening for Stx by immunoblots on colony lifts from agar plates has also been used to detect STEC in culture (Hull *et al*., 1993), but may require screening a large number of colonies. Currently agar media developed for non-O157 STEC have not been completely investigated for their effectiveness in isolating non-O157 STEC from food or cattle matrices including fecal samples. The lack of standardized methods for detection of non-O157 STEC has led to few studies using the

same methodologies, which complicates comparing the ecology of these organisms across studies.

Acid treatment has been used to reduce background organisms before plating procedures (Tillman *et al*., 2012). Most *E. coli* strains are resistant to pH as low as 2.0, which enable them to survive the acidic environment of the stomach (Hong *et al*., 2012). Treatment of pure cultures found a reduction in growth for *Enterobacter cloacae*, *Citrobacter freundii*, *Klebsiella pneumoniae*, but not STEC O26, O45, O103, O111, O121 or O145 strains from pH 5.0 to 2.0 (Tillman *et al*., 2012). Tillman *et al*. (2012) also found improved recovery of STEC strains after acid treatment in inoculated ground beef samples.

A limitation of culture is the number of colonies that can be screened. Isolation of STEC and *E. coli* O157 from enriched carcass swabs on CT-SMAC, BCMO157 agar, and CHROMagar O157 found a lower number of samples positive compared to PCR for *stx* and a gene representing the O157 antigen (Ohtsuka *et al*., 2010). Culture routinely detects a lower number of positive samples compared to molecular tests (Wang *et al*., 2014b; Noll *et al*., 2015).

Multiple methods rely on the isolation of colonies on agar plates. A light scattering sensor, called Bacterial Rapid Detection using Optical scattering Technology (BARDOT) based on the scatter pattern generated by the colony characteristics such as O-antigen, peptidoglycan, or metabolites has been developed for detection of STEC-7 (Tang *et al*., 2014). The BARDOT relies on a library of scatter profiles and is limited to the existing library. It also relies on a defined growth time, because as colonies grow for a longer time their characteristics change (Tang *et al*., 2014). A rapid method for

identification of STEC-7 serogroups termed the EHEC colony check assay is a cloth-based hybridization array that test isolated colonies from agar media (Blais *et al*., 2014). Although these assays directly test colonies from agar plates, a single agar media for detection of all STEC has not been developed. It has been recommended that two agar media be used for screening STEC (Gill *et al*., 2014), one agar that is highly selective and one agar that is less selective.

PCR detection

After colonies have been isolated or if testing from enriched samples, further testing is may be required to determine if a STEC strain is present. Cytotoxic, immunological, DNA-based, and culture-based assays have been used for STEC detection (Beutin and Fach, 2014). PCR is a common nucleic acid detection assay for multiple pathogens. PCR can be performed directly from isolated colonies or colonies that have been lysed causing DNA to be released from cells. There is not a standard procedure for the number of colonies tested from an individual agar plate, and it can vary from one to hundreds depending on the study. The number of colonies tested is commonly based on cost and convenience. On enriched samples, a DNA purification step is included to extract DNA and remove potential PCR inhibitors, which are particularly problematic in fecal samples (Paddock *et al*., 2012). Multiplex PCR (mPCR) allows for the detection of multiple genes in a single assay. This technique allows for detection of multiple pathogens or multiple genes from a single pathogen or a combination of both. Despite the advantage of sensitivity and high-throughput capabilities, PCR cannot determine if genes representing an O-group, *eae*, and *stx* are being contributed from one

or multiple organisms. Detection of all three genes, commonly by PCR, in one organism is required by the USDA FSIS to identify meat contaminated with an EHEC-7 organism.

Bai *et al*. (2010) developed a mPCR to detect six genes in EHEC O157:H7. This mPCR assay had a sensitivity of 10^4 CFU/g in unenriched cattle fecal samples. Paddock *et al*. (2012) designed a mPCR assay to detect EHEC-7 serogroups and found a sensitivity of 4 x 10^5 CFU/g in unenriched fecal samples. These two multiplex reactions were further developed and modified into an 11-plex PCR that detected EHEC-7 serogroups, *stx*$_1$, *stx*$_2$, *eae* and *ehxA* (Bai *et al*., 2012).

Real-time PCR developed from traditional PCR is a method to detect genes during amplification, but also allows for quantification of gene sequences. Real-time PCR assays have been developed for detection of O157 (*rbfE*), *stx*$_1$ and *stx*$_2$ to estimate the prevalence of O157 from different cattle matrices. By a DNA-based real-time PCR, 17.5% of carcasses were potentially contaminated with STEC O157 (Breum and Boel, 2010).

Gene targets other than *stx* and *eae* have been used solely or in combination with *stx* and *eae* to detect EHEC. The *E. coli* attaching and effacing gene-positive conserved fragment (*ecf1*) is found on pO157 of EHEC. It has shown promising results as a single target for EHEC detection in beef (Livezey *et al*., 2015). However, *ecf1* is also found on EPEC strains lacking *bfp*; these may have been strains that were EHEC but lost the Stx phage. Using *ecf1* could result in an overestimation of EHEC positive samples. Non-LEE effector genes in combination with *eae* have been used to further discriminate between EPEC and EHEC (Beutin and Fach, 2014). Clustered regularly interspaced short palindromic repeat (CRISPR) regions of STEC have been used for serogroup specific

detection of STEC (Delannoy *et al*., 2012). However, Delannoy *et al*. (2012) found some STEC of different serogroups with the same H-type exhibited cross-activity. Recent advances in whole genome sequencing have led to the use of sequencing for outbreak investigations (Dallman *et al*., 2015). This allows for better resolution and determines if strains from different products or in different locations have a common source.

Commercial assays

To ensure that food and other products sold to consumers are safe, commercial assays have been developed for the detection of STEC. The goals for these assays are to be rapid, easy to use, and cheap. They have primarily been developed for detection of STEC in food matrices (Wang *et al*., 2013). Assays may be validated by an external laboratory for use in a particular matrix (Livezey *et al*., 2015).

PCR based commercial assays for detection of STEC in foods have been developed. The BioControl Assurance Genetic Detection System MPX Top 7 STEC, Qualicon BAX System STEC Suite, and GeneDisc array were compared for detection of STEC in inoculated beef trim enrichments (Wheeler *et al*., 2015). The Pall GeneDisc array is a microarray based system that identifies 12 O-groups, seven H-types, and 15 virulence genes. The NeoSEEK STEC Detection and Identification (NS) is a PCR/mass spectrometry based system and is similar to the GeneDisc array in that it identifies multiple targets. NS detects EHEC-7 based on the identification of over 80 targets. The primers or targets that are used for detection of STEC are typically not known for commercial assays, which make them difficult to evaluate.

Wasilenko *et al*. (2014) used the BAX system to test retail ground beef for EHEC-7 and confirmation was done by plating on modified Rainbow Agar O157. By BAX, 3.9% of samples were potentially positive. Culture could confirm only four of 14 that were predicted to be positive by BAX (Wasilenko *et al*., 2014). This disparity leaves questions on the reliability of culture and molecular tests for EHEC. Attempted verification of commercial molecular screens can be done by follow-up culture isolation, but may yield a sample positive by only one method. In veal calf hide samples, 93.9% were positive by NS analysis and by culture 53.0% of samples isolated an EHEC (Wang *et al*., 2014b).

Quantification of STEC

High concentrations of STEC in the feces and on hides pose a greater risk for carcass contamination during processing. Cattle shedding levels above 10^4 CFU/g of STEC ("super-shedders") account for the majority of contamination of hides and in turn pose the greatest risk to carcass contamination. It has been suggested that cattle shedding high levels of STEC should be separated or not sent to processing (Arthur *et al*., 2009). The concentration that STEC are shed could be due to the concentration of organisms ingested, virulence and adherence genes, growth rate of strain, or host factors (Chase-Topping *et al*., 2008). Williams *et al*. (2015) proposed that animals shedding $>10^4$ CFU/g be termed "super-shedding events" not "supper-shedding animals", because based on their findings super-shedding was transiently detected as opposed to a subset of animals shedding high levels continually.

Quantification of EHEC remains challenging due to the high numbers of background organisms present in fecal and hide samples. Direct plating of samples for quantification allows for the quantification of viable organisms. Ten-fold serial dilutions of fecal samples and spread plating on agar media was used to quantify EHEC O157 (Omisakin *et al*., 2003). Omisakin *et al*. (2003) found that 44 of 579 cattle fecal samples were positive for EHEC O157, and of those, 17 of 44 were quantifiable. These results are consistent with others reporting that the majority of cattle shed EHEC O157 at $<10^2$ CFU/g (Lahti *et al*., 2003; Pearce *et al*., 2004). In addition to direct plating, spiral plating has been used to reduce the number of dilutions needed. The spiral plate method uses a known volume dispensed in an Archimedean spiral on an agar plate. Spiral plating has been used to quantify EHEC O157 (Robinson *et al*., 2004), but not for non-O157 EHEC. In this study, the limit of quantification for spiral plating was 500 CFU/g. Spiral plate quantification for EHEC O157 in veal hide samples was plated on CHROMagar O157, but no hides samples were found quantifiable for EHEC O157 (Wang *et al*., 2014b). Although this study tested for non-O157 EHEC, they did not attempt to quantify by culture or a molecular assay.

Nalidixic acid-resistant EHEC O157 strains have been used to assess the sensitivity of quantification methods. Antimicrobial resistant strains are easily selected by inhibiting background organisms with the use of high levels of antimicrobials that only the target strain can tolerate. Using a nalidixic acid-resistant EHEC O157 strain, LeJeune *et al*. (2006) determined that direct plating methods could quantify concentrations >100 CFU/g in cattle feces.

The most-probable number (MPN) is a technique where samples are diluted and the most dilute sample that gives a positive result is used to determine the concentration. MPN has been used with multiplex PCR and IMS to determine concentration of EHEC O157 in inoculated cattle feces (Guy *et al*., 2014). This approach quantified samples inoculated with 10^2 to 10^4 CFU/g. MPN could theoretically allow for the quantification of as low as one CFU/g of feces. Although this technique allows for the quantification of low numbers of bacteria, it requires an enrichment time and many dilution tubes, which can be time consuming, labor intensive, and expensive.

Quantitative PCR (qPCR) is a rapid method that can quantify multiple genes in a single reaction. This method is high-throughput and methods have been developed to quantify specific serogroups and virulence gene from complex samples. Typically, DNA must be extracted from cattle samples (fecal, hide, carcass) due to potential PCR inhibitors present in the sample (Paddock *et al*., 2012; Noll *et al*., 2015). qPCR cannot rule out the possibility that multiple genes or the same gene came from multiple strains. To avoid this problem, Luedtke *et al*. (2014) developed a qPCR assay based on *ecf1*. This method quantifies total EHEC present in a sample and is not limited to an induvial serogroup. Luedtke and Bosilevac (2015) used this method to quantify EHEC from veal hides and carcasses. They found that the assay enumerated 32% of hide samples and 17% of carcass samples for EHEC, and found moderate agreement for enumeration between the qPCR assay and an MPN assay. However, no relation was observed between the qPCR assay and an MPN assay for enumeration of EHEC on carcass samples (Luedtke and Bosilevac, 2015). The low concentration of EHEC on carcass samples may not have allowed for a relationship to be observed between the qPCR and MPN assays.

Digital PCR (dPCR) is a method used to quantify nucleic acids by separating a sample into partitions. Each partition is read by a flow cytometer to determine the number of positive partitions. This allows for the detection of targets that are infrequently found in a sample. It has an advantage to other PCR methods in that it does not require a standard curve and is relatively insensitive to PCR inhibitors. Using dPCR, Luedtke and Bosilevac (2015) found that EHEC could be quantified from 10^3 to 10^7 CFU/ml. EHEC concentration had a higher estimated concentration in hide and carcass samples previously tested by qPCR and MPN assays.

Hypotheses derived from existing literature

Detection methods such as culture-based and PCR methods for STEC typically require an enrichment step. There is no defined enrichment media for all STEC, and it may be different depending on the matrix type. Enrichment media for STEC O157 have been supplemented with antibiotics to decrease background flora, or have been optimized for detection of multiple foodborne pathogens alongside STEC O157 (Suo and Wang, 2014). Vimont *et al*. (2006) reviewed enrichment broths for STEC over a wide range of matrices and found that trypticase soy broth (TSB) and *E. coli* EC broth were the most common. Paddock *et al*. (2012) showed that after enrichment in EC broth, PCR detected more *E. coli* serogroups and virulence genes in cattle fecal samples compared to TSB. Vimont *et al*. (2007) found the use of EC broth reduced the false negative results for detection of non-O157 STEC in cattle fecal samples. EC broth contains bile salts and lactose compared to the absence of bile salts and glucose potentially making EC more

selective. Thus, we hypothesize that that EC broth would yield greater growth of STEC compared to TSB.

The prevalence of EHEC-7 in cattle is poorly understood. Although many studies have estimated the prevalence of EHEC O157 on cattle hides and carcasses (Elder *et al*., 2000; Arthur *et al*., 2002; Barkocy-Gallagher *et al*., 2003; Nou *et al*., 2003; Rivera-Betancourt *et al*., 2004; Woerner *et al*., 2006; Brichta-Harhay *et al*., 2008), few studies have estimated the prevalence of EHEC-7 (Monaghan *et al*., 2012; Thomas *et al*., 2012). Based on data from the CDC, seven serogroups of EHEC are the most predominant in clinical infections (Brooks *et al*., 2005), which led us to hypothesize that these seven EHEC would be found on the hides and to a lesser extent, carcasses of feedlot cattle at harvest.

Production type and environment may influence the prevalence of STEC (Sargeant *et al*., 2004; Smith, 2014a). The age of cattle may also influence the status of STEC carriage in cattle (Mir *et al*., 2015). Older animals may be immunosuppressed, which would make them more likely to carry STEC (Hancock *et al*., 1994), and dairy cattle tend to be older than feedlot cattle at harvest on a percentage basis. We hypothesized that dairy cattle will be heavy colonized with EHEC-7.

Isolation of non-O157 STEC remains challenging on current agar media. Possé *et al*. (2008b) developed a media for screening four serogroups of STEC. Novobiocin and potassium tellurite are selective components of this agar, but some strains do not grow at higher concentrations of these antibiotics (Hussein and Bollinger, 2008). We hypothesized that reduction of novobiocin and potassium tellurite in this agar medium

would allow for a greater number of STEC-7 positive fecal samples detected compared to the original formulation.

Baehler and Moxley (2000, 2002) showed that EHEC O157 produces A/E lesions on colonic mucosal explants from cattle. Girard *et al*. (2007) found that some, but not all EHEC strains adhere to bovine colonic explants. We hypothesized that STEC-6 with different intimin subtypes (*eae*-β1, *eae*-ε, *eae*-γ1, and *eae*-γ2) and STEC O104:H4 would adhere, and strains with intimin would produce A/E lesions on cattle and human colonic epithelial cells.

CHAPTER 2

COMPARISON OF ENRICHMENT BROTHS FOR SUPPORTING GROWTH OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI*

(Current Microbiology, 71:214-219)

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Abstract

Detection of Shiga toxin-producing *Escherichia coli* (STEC) in complex sample matrices remains challenging. In an attempt to improve detection, nonselective and selective enrichment broths were compared as follows: (1) trypticase soy broth (TSB) was compared with TSB plus novobiocin, vancomycin, rifampicin, bile salts, and potassium tellurite (TSB-NVRBT) for supporting growth of STEC in pure culture; (2) *E. coli* broth (EC), TSB, and TSB plus bile salts (mTSB) were compared for enrichment of STEC O26, O45, O103, O104, O111, O121, O145, and O157 (STEC-8) in inoculated cattle fecal samples; (3) EC, TSB, and mTSB were compared for the detection of STEC-8 in inoculated cattle fecal samples. Fecal samples were inoculated with wild-type STEC-8 or nalidixic acid- or rifampicin-resistant derivatives of the same strains at 100, 1000, or 10,000 colony-forming units per gram (CFU/g) of feces. In pure culture, the mean STEC CFU/mL following enrichment in TSB was 1.17 $\log_{10}$ greater than that in TSB-NVRBT ($P < 0.05$). In inoculated fecal samples, EC enrichment yielded growth of STEC-8 (6.42 $\log_{10}$ CFU/g) that was significantly greater than in TSB (6.23 $\log_{10}$ CFU/g; $P < 0.05$), and numerically but not significantly greater than in mTSB (6.37 $\log_{10}$ CFU/g; $P = 0.60$). Wild-type STEC strains were detected in 43.8 % (21/48) of the samples enriched in EC and mTSB compared to 27.1 % (13/48) of the samples enriched in TSB ($P = 0.15$). Overall, STEC grew significantly better when enriched in EC compared to TSB. Modification of TSB by the addition of bile salts improved the growth and detection of STEC compared to TSB alone.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are the major cause of hemorrhagic colitis and hemolytic-uremic syndrome in human patients (Kaper *et al*., 2004; Brooks *et al*., 2005; Bettelheim, 2007) and commonly colonize the large intestines of cattle, sheep, and other ruminants, which serve as reservoirs (Bettelheim, 2007; Sánchez *et al*., 2009). These organisms are shed in ruminant feces, often in relatively low numbers, in conjunction with high numbers of commensal flora including nonpathogenic *E. coli* (Fegan *et al*., 2004; Bettelheim, 2007). The infectious dose of STEC O157:H7 in humans is very low, estimated to be <50 bacterial cells (Tilden *et al*., 1996). Due to the frequent presence of STEC at low concentrations in different sample matrices and given their low infectious dose, enrichment is usually necessary for detection (Tilden *et al*., 1996; Fegan *et al*., 2004; Barkocy-Gallagher *et al*., 2005).

The majority of illnesses due to STEC in the U.S. are caused by serogroups O26, O45, O103, O111, O121, O145, and O157 (STEC-7; Brooks *et al*., 2005); further, most of these STEC-7 organisms are intimin (*eae*)-positive and hence classified as enterohemorrhagic *E. coli* (EHEC) (Kaper *et al*., 2004). Additionally, the O104:H4 outbreak associated with fenugreek sprouts (Bucholz *et al*., 2011) has raised concern about STEC contaminating other food sources, and therefore, STEC-7 and STEC O104:H4 (collectively referred to herein as STEC-8) were addressed in this study.

Many culture-based and -independent methods for the detection of STEC in food and environmental samples include an enrichment step to enhance detection (Wang *et al*., 2013). However, a medium that gives optimal enrichment, collectively, for all STEC-7 or -8 has yet to be determined. Trypticase soy broth (TSB) and *E. coli* broth (EC) have been

used most commonly for enrichment in support of detection of STEC O157:H7 and non-O157 in studies spanning across all sample matrix types (Vimont *et al*., 2006). Strains enriched in TSB were more likely to yield false-negative results compared to strains enriched in EC (Vimont *et al*., 2007). Enrichment media have been modified by the addition of antibiotics and selective components depending on the matrix (Hussein and Bollinger, 2008). The addition of novobiocin (Vimont *et al*., 2007; Hussein *et al*., 2008) or a combination of vancomycin, cefsulodin, and cefixime (Baylis, 2008) has been reported to inhibit growth of some STEC. The inclusion of bile salts as a selective measure in media has previously been shown to delay the Jameson effect and was therefore interpreted to potentially promote STEC growth (Vimont *et al*., 2007). Enrichment time is another important consideration that increases STEC to a detectable level. An enrichment time of 24 h did not enhance the recovery of STEC compared to 6 h (Verstraete *et al*., 2010).

The objectives of this study were to (i) compare the growth of STEC-7 in pure culture using TSB and TSB plus novobiocin (8.0 mg/L), vancomycin (16.0 mg/L), rifampicin (2.0 mg/L), bile salts No. 3 (1.5 g/L), and potassium (K) tellurite (1.0 mg/L; TSB-NVRBT); (ii) evaluate the growth of STEC-8 in cattle fecal samples enriched in EC, TSB, and TSB with 1.5 g/L of bile salts No. 3 (modified TSB; mTSB) using spontaneous antibiotic-resistant mutant derivative strains; and (iii) compare EC, TSB, and mTSB for the detection of STEC-8 in inoculated cattle fecal samples using wild-type (WT) strains.

Materials and Methods

Bacterial strains, preparation of inoculum, and inoculation of fecal samples. All strains used in this study were STEC. Strains used for Experiment 1 were obtained from Dr. Shannon Manning (Michigan State University STEC Center) and Dr. David G. Renter (Kansas State University), or from our laboratory collection (Table 1). Spontaneous nalidixic acid-resistant mutant (Nal^R), spontaneous rifampicin-resistant (Rif^R) mutant, and WT parent strains used for Experiments 2 and 3 were obtained from Dr. John B. Luchansky (USDA, Agricultural Research Service, Eastern Regional Research Center) or were from our laboratory collection (Table 2).

Frozen (−80 °C) stock cultures of strains were streaked onto Luria–Bertani broth, Miller (LB; BD, Sparks, MD) agar plates without antibiotics or with 40 mg/L nalidixic acid (Fisher) or 100 mg/L rifampicin (Sigma-Aldrich) when appropriate and incubated at 37 °C for 24 h. A single colony was inoculated in LB with corresponding antibiotics for 24 h at 37 °C in a stationary culture.

Fresh feces were collected from beef cattle housed one per pen at the University of Nebraska–Lincoln. One gram (g) of cattle feces was suspended in 9 mL of EC (Oxoid Ltd., Hampshire, UK), TSB (BD, Sparks, MD) or mTSB and vortexed for 2 min. Serial tenfold dilutions of the inoculum were prepared in buffered peptone water (BPW) and used to inoculate fecal samples at a concentration of 100, 1000, or 10,000 colony-forming units per gram (CFU/g) of feces, with BPW used as a mock inoculation. Following 20 s of vortexing, samples were enriched for 6 h at 40 °C in a stationary culture, as described by Paddock *et al*. (2012).

Experiment 1: Comparison of TSB and TSB-NVRBT. Frozen stock cultures were streaked onto TSB agar and used to inoculate 5 mL of TSB. Stationary

cultures were incubated 24 h at 37 °C. The medium, TSB-NVRBT, was prepared according to Possé *et al*. (2008a). Duplicate stationary cultures of each strain were prepared in TSB and TSB-NVRBT. Following incubation for 18 h at 42 °C, serial tenfold dilutions in BPW were made from each culture and plated on TSB agar. Plates containing TSB agar were incubated for 18 h at 37 °C, and aerobic plate counts (APC) were performed according to the FDA *Bacteriological Analytical Manual* (BAM) (Maturin and Peeler, 2001).

Experiment 2: Evaluating growth of STEC-8 using $Nal^R$and Rif^R strains. Cattle fecal samples were inoculated with Nal^R or Rif^R STEC-8 strains at concentrations of 100, 1000, or 10,000 CFU/g to evaluate the effect of different enrichment broths on STEC growth in two independent experiments. After enrichment, samples were serially diluted and spread plated onto Possé differential agar (Possé *et al*., 2008b) modified by supplementation with nalidixic acid (40 mg/L) or rifampicin (100 mg/L) with reduced bile salts No. 3 (1.5 g/L) and containing no novobiocin or K tellurite (mPossé1); these plates were incubated 18 h at 37 °C. Plates were counted by standard methods (Maturin and Peeler, 2001), growth was determined as CFU/g, and these values were $\log_{10}$ transformed prior to statistical analysis. Colonies were confirmed to be the respective inoculum strain by PCR (Paddock *et al*., 2012).

Experiment 3: Detection of STEC-8 using WT strains. Cattle fecal samples inoculated with WT STEC at 100, 1000, and 10,000 CFU/g were subjected to immunomagnetic separation (IMS) for the corresponding O-antigen using a KingFisher™ Flex Magnetic Particle Processor (Thermo Scientific, Waltham, MA). Anti-O157 Dynabeads® (Invitrogen, Carlsbad, CA), and IMS beads for *E. coli* O26, O45, O103,

O104, O111, O121, and O145 (Abraxis LLC, Warminster, PA) were used for IMS. After IMS, samples were diluted and spread plated onto CHROMagar™ STEC (DRG International, Mountainside, NJ), Possé differential agar (Possé *et al*., 2008b), and Possé differential agar modified by having reduced concentrations of novobiocin (5 mg/L) and K tellurite (0.5 mg/L; mPossé2). Colonies were tested by PCR (Paddock *et al*., 2012) and recorded as detection or no detection. Two independent experiments were conducted for all STEC-8 WT strains.

Detection by PCR. Five colonies were picked from countable plates according to the methods described in the FDA BAM (Maturin and Peeler, 2001), put into 50 μL of ultrapure water, and heated at 95 °C for 10 min for use as DNA template. From these, a single colony was selected at random and tested by multiplex PCR for inoculum-specific O-type and Shiga toxin (*stx*) type as described by Paddock *et al*. (2012) to confirm that colonies on agar plates were that of the respective inoculum strain.

Statistical analysis. A paired *t* test was used to compare the growth of STEC in TSB to that in TSB-NVRBT (SAS 9.2, PROC GLIMMIX). The restricted maximum likelihood method was used to compare the growth of antibiotic-resistant STEC in inoculated fecal samples, and a χ^2 analysis was used to compare the detection of WT STEC in inoculated fecal samples (SAS 9.2, PROC MIXED, PROC GLIMMIX).

Results and Discussion

Antimicrobials have the potential to inhibit background organisms and allow STEC to grow to high numbers; however, it has been reported that some antimicrobials, e.g., novobiocin and K tellurite, can restrict the growth of some STEC (Vimont *et al*.,

2006; Vimont *et al*., 2007; Baylis, 2008; Kanki *et al*., 2011). In order to assess whether antimicrobials in an enrichment medium designed for STEC (Possé *et al*., 2008a) were actually selective against STEC growth, TSB was compared to TSB-NVRBT on a relatively large number of STEC-7 strains. There was a mean CFU/mL $\log_{10}$ reduction of 1.17 for strains grown in TSB-NVRBT compared to TSB and, in both media, strains within and among serogroups varied in their growth (Table 1). Overall, strains grown in TSB had significantly greater growth than strains grown in TSB-NVRBT ($P < 0.05$); this was true for all non-O157 STEC and STEC O157:H7, and growth of STEC O26, O103, and O121 strains was particularly reduced. The ranking of STEC serogroups in descending order of tolerance of the enrichment conditions were O45, O111, O145, O157, O121, O103, and O26. Two strains (one O26 and one O157:H7) exhibited poor growth in TSB with a minimal further reduction in growth in TSB-NVRBT. Since supplementation of TSB with NVRBT resulted in a significant reduction in growth of STEC, we did not test it further and chose instead to focus on TSB and EC for further experiments in bovine fecal samples. EC and TSB without additional antimicrobials (e.g., novobiocin) had been shown in previous studies to be less inhibitory to growth, and EC reportedly most suitable for supporting detection of non-O157 STEC in bovine feces, but these results were based on very few strains or strains of unidentified serogroups, and did not test the effects of bile salts per se (Vimont *et al*., 2007; Paddock *et al*., 2012). Since EC and TSB vary, respectively, by the presence of lactose versus glucose, and bile salts versus none, we sought to determine whether the addition of bile salts to TSB might make it comparable to EC in performance.

Strains that were Nal^R or Rif^R were used to accurately assess growth in a complex matrix since these antibiotics effectively eliminated the background microflora in fecal samples. All strains had detectable growth in all enrichment media. The APCs resulting from enrichment of fecal samples inoculated at different CFU/g concentrations were significantly different ($P < 0.05$); samples inoculated with 10,000 CFU had the highest APC, 1000 CFU the next highest, and 100 CFU the lowest. This indicated that the cultures had not become saturated by 6 h of enrichment. A longer enrichment time may increase the concentration of STEC in low abundance. Conversely, when the background microflora reaches its maximum level, STEC growth will stop (Vimont *et al*., 2007), and an extended time may not increase STEC in low abundance.

Strains in inoculated samples grew to 6.42 $\log_{10}$ CFU/g in EC, 6.23 $\log_{10}$ CFU/g in TSB, and 6.37 $\log_{10}$ CFU/g in mTSB. Although growth of STEC-8 in EC appeared only slightly higher compared to that in TSB, a significant difference was observed ($P < 0.05$), and means were numerically but not significantly greater in mTSB compared to TSB (Table 3). All strains isolated from the feces were confirmed to be that of the inoculum by PCR, and no STEC were isolated in non-inoculated samples. Higher growth of the STEC inoculum strains in EC suggests that lactose provided a selective advantage for these organisms, as expected, in contrast to glucose. Also, as expected, the bile salts in EC and mTSB suppressed growth of background microflora and allowed the STEC to grow to a higher level.

Cattle fecal samples were inoculated with WT STEC strains to compare the detected number of STEC positive samples enriched in EC, TSB, and mTSB. Strains inoculated at 10,000 CFU were detected more frequently than strains at 1000 and

100 CFU ($P < 0.05$). One STEC-8 WT strain was detected in 21 of 48 samples enriched in EC and mTSB and in 13 of 48 samples enriched in TSB (Table 4). Although STEC-8 WT strains were detected more frequently in samples enriched in EC and mTSB compared to TSB, a significant difference for broth type was not observed ($P = 0.15$). Increased growth of STEC in EC and mTSB may have led to more frequent detection compared to TSB. In a study comparing four enrichment broths for detection of non-O157 STEC in inoculated radish sprouts and beef, modified EC with novobiocin demonstrated a lower recovery rate compared to modified TSB with bile salts with or without novobiocin and universal pre-enrichment broth (Kanki *et al*., 2011). These findings suggest that modification of EC by adding novobiocin may be inhibitory to STEC detection in food compared to other enrichment broths.

There have been extensive improvements for enrichment of STEC O157:H7 in cattle feces (Sanderson *et al*., 1995; Fegan *et al*., 2004; Barkocy-Gallagher *et al*., 2005), but methods to improve enrichment of non-O157 STEC have yielded inconsistent results (Fukushima and Gomyoda, 1999; Vimont *et al*., 2006; Vimont *et al*., 2007; Hussein *et al*., 2008; Possé *et al*., 2008a, Verstraete *et al*., 2010; Kanki *et al*., 2011; Paddock *et al*., 2012; Wang *et al*., 2013). Our work suggests that lactose and bile salts in EC provide a more suitable environment compared to the presence of glucose and absence of bile salts as found in TSB for the enrichment of STEC-8.

Conclusion

The addition of several selective antimicrobials to TSB was found to inhibit growth of STEC in pure culture compared to TSB. EC broth yielded significantly greater STEC growth compared to TSB but not TSB plus bile salts in cattle fecal samples.

Acknowledgments

This project was supported by Agriculture and Food Research Initiative Competitive Grant No. 2012-68003-30155 from the USDA National Institute of Food and Agriculture. The authors thank Drs. Shannon Manning, David Renter, and John Luchansky for sharing strains.

Table 1. Comparison of growth for Shiga toxin-producing *Escherichia coli* strains under pure culture conditions.

Strain	Serotype	Source	*stx*$_1$	*stx*$_2$	*eae*	Mean $\log_{10}$ CFU/ml	
						TSB	TSB-NVRBT
DEC10B	O26:H11	Human	+	-	+	8.64	7.46
97-3250	O26:H11	Human	+	+	+	8.62	7.79
MT#10	O26:NT	Human	+	-	+	7.30	7.11
TB352A	O26:NM	Human	+	-	+	8.63	7.30
DA-10	O26:NM	Human	+	-	+	9.28	7.41
16272	O26:NT	Cattle	+	-	+	10.30	7.34
1577-88	O26:H11	Cattle	+	-	+	8.85	7.68
M103-19	O45:H2	Human	+	-	+	8.79	7.90
MI01-88	O45:H2	Human	+	-	+	9.04	7.87
MI05-14	O45:H2	Human	+	-	+	9.00	8.30
DA-21	O45:H2	Human	+	-	+	9.00	8.20
B8026-C1	O45:H2	Cattle	+	-	+	9.15	8.18
B8227-C8	O45:H2	Cattle	+	-	+	9.04	8.30
MT#80	O103:H2	Human	+	-	+	9.20	7.72
TB154A	O103:H2	Human	+	-	+	8.82	7.54
8419	O103:H25	Human	+	-	+	9.08	7.62
PT91-24	O103:NM	Human	+	-	+	8.92	7.60
6:38	O103:NM	Human	+	-	+	8.83	7.67
3720	O103:H2	Water	+	-	+	8.90	7.62

15612	O103:H11	Cattle	+	-	+	8.85	7.11
6708-87	O103:H2	Goat	+	-	+	9.11	7.91
RD8	O111:H10	Human	-	+	-	9.15	7.63
3215-99	O111:H8	Human	+	+	+	9.15	8.37
0201 9611	O111:H8	Human	+	-	+	8.93	7.81
3007-85	O111:H8	Human	+	+	+	9.18	8.04
95-3208	O111:NM	Human	+	+	+	8.92	8.08
7726	O111:NT	Cattle	+	+	+	9.36	8.18
8266	O111:NT	Cattle	+	+	+	9.34	8.26
10049	O111:H11	Cattle	+	-	+	8.97	7.68
MT#2	O121:H19	Human	-	+	+	8.81	6.98
MT#18	O121:NT	Human	-	+	+	8.95	7.85
DA-5	O121:H19	Human	-	+	+	8.91	7.85
DA-37	O121:H19	Human	-	+	+	8.59	7.63
3377-85	O121:H19	Human	-	+	+	8.85	8.02
1553	O121:H7	Cattle	+	-	-	8.97	7.02
4190	O121:NT	Cattle	+	-	-	9.15	7.96
DEC10I	O145:H16	Human	+	-	+	8.92	7.38
4865/96	O145:H28	Human	-	+	+	8.93	7.76
GS G5578620	O145:H28	Human	+	-	+	9.08	7.83
IH 16	O145:NM	Human	-	+	+	8.81	7.82
75-83	O145:NM	Human	-	+	+	9.11	7.98
1234	O145:H28	Cattle	+	+	+	9.11	8.15

7744	O145:NT	Cattle	+	-	+	9.08	8.08
933	O157:H7	Beef	+	+	+	8.60	7.68
86-24	O157:H7	Human	-	+	+	8.97	7.89
S2006 #1	O157:H7	Cattle	+	+	+	8.93	7.72
S2006 #2	O157:H7	Cattle	+	+	+	8.95	7.77
S2006 #3	O157:H7	Cattle	+	+	+	8.93	7.72
S2006 #4	O157:H7	Cattle	+	+	+	8.00	7.82
S2006 #5	O157:H7	Cattle	+	+	+	9.34	7.87
S2006 #6	O157:H7	Cattle	+	+	+	9.00	7.99

Strains were incubated in enrichment broth as stationary cultures for 18 h at 42°C.

NT not typed, TSB trypticase soy broth, TSB-NVRBT trypticase soy broth plus novobiocin, vancomycin, rifampicin, bile salts, and K tellurite.

Table 2. Shiga toxin-producing *Escherichia coli* strains used for inoculation of cattle fecal samples.

Strain	Serotype	Source	*stx*$_1$	*stx*$_2$	*eae*	Resistance
DA-10	O26:NM	Human	+	-	+	N
H30	O26:H11	Human	+	-	+	WT, R
B8227-C8	O45:H2	Cattle	+	-	+	N
CDC 96-3285	O45:H2	Human	+	-	+	WT, R
TB154A	O103:H2	Human	+	-	+	N
CDC 90-3128	O103:H2	Human	+	-	+	WT, R
TY-2482	O104:H4	Human	-	+	-	WT, R
0201 9611	O111:H8	Human	+	-	+	N
JB1-95	O111:NM	Human	+	+	+	WT, R
KDHE 55	O121:NT	Human	-	+	+	N
CDC 97-3068	O121:H19	Human	-	+	+	WT, R
IH 16	O145:NM	Human	-	+	+	N
83-75	O145:NM	Human	-	+	+	WT, R
933	O157:H7	Beef	+	+	+	WT, N
USDA FSIS 380-94	O157:H7	Salami	+	+	+	R

NT not typed, WT wild-type, N nalidixic acid-resistant, R rifampicin-resistant.

Table 3. Growth of antibiotic-resistant Shiga toxin-producing *Escherichia coli* in inoculated cattle fecal samples.

Enrichment broth	Mean $\log_{10}$ CFU/g
EC	6.42
TSB	6.23
mTSB	6.37

Means with different letters are significantly different ($P < 0.05$).

EC *E. coli* broth, TSB trypticase soy broth, mTSB trypticase soy broth plus bile salts.

Table 4. Number of samples (%) detected positive for Shiga toxin-producing *Escherichia coli* in 48 inoculated cattle fecal samples

Enrichment broth	Agar media			Total
	CHROMagar™ STEC	Possé	mPossé2	
EC	8 (16.7)	15 (31.3)	16 (33.3)	21 (43.8)
TSB	5 (10.4)	8 (16.7)	8 (16.7)	13 (27.1)
mTSB	9 (18.8)	13 (27.1)	15 (31.3)	21 (43.8)

EC *E. coli* broth, TSB trypticase soy broth, mTSB trypticase soy broth plus bile salts.

CHAPTER 3

PREVALENCE OF ENTEROHEMORRHAGIC *ESCHERICHIA COLI* O26, O45, O103, O111, O121, AND O157 ON HIDES AND PREINTERVENTION CARCASS SURFACES OF FEEDLOT CATTLE AT HARVEST

(Foodborne Pathogens and Disease, 12:631-638)

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Abstract

Cattle hides are a main source of enterohemorrhagic *Escherichia coli* (EHEC) contamination of beef carcasses. The objectives of this study were to (1) determine the prevalence of "top 6" non-O157 plus O157:H7 EHEC (EHEC-7) on feedlot cattle hides and their matched preintervention carcasses; (2) assess the agreement among detection methods for these matrices; and (3) conduct a molecular risk assessment of EHEC-7 isolates. Samples from 576 feedlot cattle were obtained at a commercial harvest facility and tested for EHEC-7 by a culture-based method and the polymerase chain reaction/mass spectrometry–based NeoSEEK™ STEC Detection and Identification test (NS). Prevalence data were analyzed with generalized linear mixed models. The cumulative prevalence of EHEC-7 in hide samples as detected by NS was 80.7%, with a distribution of 49.9%, O145; 37.1%, O45; 12.5%, O103; 11.0%, O157; 2.2%, O111; 2.0%, O121; and 0.2%, O26. In contrast, the cumulative prevalence of EHEC-7 in hide samples by culture was 1.2%, with a distribution of 0.6%, O157; 0.4%, O26; 0.2%, O145; and 0%, O45, O103, O111, and O121. The cumulative prevalence of EHEC-7 on matched preintervention carcasses as detected by NS was 6.0%, with a distribution of 2.8%, O157; 1.6%, O145; 1.2%, O103; 1.1%, O45; 0.2%, O26; and 0.0%, O111 and O121. Although the culture-based method detected fewer positive hide samples than NS, it detected EHEC in five hide samples that tested negative for the respective organism by NS. McNemar's chi-square tests indicated significant (p<0.05) disagreement between methods. All EHEC-7 isolates recovered from hides were seropathotype A or B, with compatible virulence gene content. This study indicates that "top 6" and O157:H7 EHEC are present on hides, and to a lesser extent, preintervention carcasses of feedlot cattle at

harvest. However, continued improvement in non-O157 detection methods is needed for accurate estimation of prevalence, given the discordant results across protocols.

Introduction

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) are major pathogens of humans and most commonly acquired through the consumption of contaminated food or water (Cole *et al.*, 2014; Luna-Gierke *et al.*, 2014). *E. coli* O157:H7 is the prototype of STEC further classified as enterohemorrhagic *E. coli* (EHEC), which by definition contain genes for Stx (*stx*) and products encoded on the locus of enterocyte effacement pathogenicity island, most notably intimin (*eae*) (Kaper *et al.*, 2004). All STEC not of the O157 O-antigen type are commonly called non-O157 (Bettelheim, 2007). From 1983 to 2002, 6 serogroups (viz., O26, O45, O103, O111, O121, and O145) accounted for 71% of the reported non-O157 STEC cases in the United States (Brooks *et al.*, 2005); STEC belonging to these serogroups have become known as the "top 6" (Hegde *et al.*, 2012; Conrad *et al.*, 2014). A total of 84% of the "top 6" isolates in the cases reported by Brooks *et al.* (2005) were EHEC.

Collectively, the "top 6" non-O157 plus O157:H7 (hereafter referred to as EHEC-7) were responsible for 92.3% of the human cases of STEC infection in the United States from 2000 to 2010 (Gould *et al.*, 2013). *E. coli* O157:H7 was declared an adulterant in raw ground beef in 1994 and in raw, nonintact beef in 1999 by the United States Department of Agriculture (USDA), Food Safety and Inspection Service (FSIS). The "top 6" non-O157 STEC were declared adulterants in raw, nonintact beef by the USDA-FSIS in 2011. Based on the requirement for *stx* and *eae* detection in a "top 6" isolate for qualification as an adulterant (USDA, 2014b), these organisms are also by definition EHEC.

STEC mainly contaminate beef through transfer from fecally contaminated hides to carcass surfaces during slaughter (Elder *et al.*, 2000; Arthur *et al.*, 2002; Keen and Elder, 2002; Barkocy-Gallagher *et al.*, 2003; Arthur *et al.*, 2004; Koohmaraie *et al.*, 2005; Schmidt *et al.*, 2012). The prevalence of O157 and non-O157 STEC on de-hided carcass surfaces has been reported, but little information is available on the "top 6." The objectives of this study were to (1) determine the prevalence of EHEC-7 on matched hides and de-hided preintervention carcass surface samples obtained from commercial feedlot cattle at harvest; (2) compare the performance capability and agreement of a culture-based method with that of a polymerase chain reaction (PCR)/mass spectrometry–based method for detection of EHEC-7 in enriched hide and carcass sponge samples, and a PCR/mass spectrometry–based method to a commercial immunomagnetic/PCR–based method for enriched carcass sponge samples; and (3) conduct a molecular risk assessment (MRA) of EHEC-7 isolates obtained from hides.

Materials and Methods

Study design and sample collection. Cattle from a commercial beef feedlot in the central United States were followed through harvest using a repeated cross-sectional study design. Matched samples from the hides and carcass surfaces of 576 cattle (24 pens, 24 cattle/pen, 2 pens/wk for 12 wks, from June to August 2013) were obtained at a processing plant using a modified protocol for *E. coli*O157:H7 sampling (USDA FSIS, 2005). While in lairage, cattle were sprayed with a commercial bacteriophage solution (Finalyse®; Elanco Animal Health, Greenfield, IN) that is lytic for O157:H7 (Sillankorva *et al.*, 2012). Stunned animals were sprayed with a high-pressure ambient

water rinse, shackled, and placed onto the overhead rail for exsanguination. Afterward, the hides were sampled immediately before the carcasses entered the first hide-on wash cabinet.

Hide samples were collected using 11.5×23.0-cm sponges (Speci-Sponge®; Nasco, Fort Atkinson, WI) premoistened with 35 mL of buffered peptone water (BPW). Sponges were used to sample an area of 1000 cm^2, 15 cm from the midline at the level of the diaphragm.

From each de-hided carcass, two surface samples were collected before the first carcass cabinet wash occurred. The first covered a 1000-cm^2 area in the brisket–short plate region using a Speci-Sponge® with 15 mL of BPW, following the procedure used for hides. The second was from a 3000-cm^2 area in the lateral hock and round-rump regions. These 2 sponges and their buffer volumes (representing a combined 4000 cm^2 area per carcass) were combined into a single Whirl-Pak® bag. Samples were shipped overnight on ice to the laboratories.

Culture of hide and carcass samples. Hide and carcass sponge samples were processed and analyzed within 24 h after collection. Ninety milliliters of *E. coli* broth (EC; Oxoid Ltd., Hampshire, UK) was added to each hide sponge sample, and these were incubated at 40°C for 6 h. After incubation, separate broth aliquots were subjected to immunomagnetic separation (IMS) using a KingFisher™Flex Magnetic Particle Processor (Thermo Scientific, Waltham, MA) with anti-O157 Dynabeads® (Invitrogen, Carlsbad, CA), and IMS beads for *E. coli* O26, O45, O103, O111, O121, and O145 (Abraxis LLC, Warminster, PA). Washed IMS beads were spread onto Possé differential agar (Possé *et al*., 2008b) modified (mPossé) by reducing the concentration of novobiocin and

potassium tellurite to 5.0 and 0.5 mg/L, respectively. mPossé plates were each spread-inoculated with 1 IMS treatment and incubated for 18 h at 37°C. Six or fewer colonies per mPossé plate were picked; on plates inoculated with O26, O45, O103, O111, or O157 IMS-treated cultures, red- or blue-purple colonies were picked and for O121 or O145 IMS-treated cultures, red- or blue-purple, and green colonies were picked. Colonies were streaked for isolation on 5% sheep blood agar (Remel, Lenexa, KS), and incubated for 15 h at 37°C. Isolated colonies were picked, suspended in 50 µL of ultrapure water, and boiled for 10 min for use as DNA template in PCR assays.

Eighty milliliters of EC broth was added to each carcass sponge sample, and incubated at 42°C for 8 h. Following incubation, a 1-mL aliquot of each enrichment broth sample was tested using the Biocontrol Assurance GDS™ MPX Top 7 STEC system (GDS) (Biocontrol Systems, Inc., Bellevue, WA) following the manufacturer's protocol. The GDS system consists of a proprietary IMS that targets the EHEC-7 serogroups (i.e., the seven O-group antigens) followed by PCR for detection of *eae*, stx_1, and stx_2, with EHEC-6 and EHEC O157 presumptive positive or negative results generated. An additional 1-mL aliquot of each enrichment broth sample was placed into 1.5-mL centrifuge tubes and stored at −80°C for later shipment to GeneSeek® Inc. (Lincoln, NE) for NeoSEEK™ analysis. Enrichment broths that tested negative by GDS were not cultured further. Those that tested presumptive positive by GDS were subjected to IMS and plated on mPossé agar as described above for the hide samples, with the exception that IMS was done manually. From each mPossé plate, ≤6 isolated red- or blue-purple, and green colonies were picked and subcultured onto trypticase soy agar (TSA) to ensure isolation. TSA-isolated colonies representing those from each mPossé plate were picked,

pooled, and tested by GDS. Any pools testing positive for EHEC-6 or EHEC O157 by GDS were to be tested by 11-plex PCR as described below for the hide samples.

Detection and characterization of EHEC isolates from hide samples. From each hide sample culture, ≤6 colonies from mPossé plates were pooled and tested by multiplex PCR for EHEC-7 serogroups. Isolates in positive pools were tested individually by 11-plex PCR for genes representing each of the EHEC-7 serogroups (*wzx, wbq,* or *rfbE*), plus stx_1, stx_2, *eae*, and EHEC-hemolysin (*ehxA*) using methods previously described (Bai *et al.*, 2010; Paddock *et al.*, 2012). H-antigen typing (*fliC*) was conducted using the methods of Wang *et al*. (2003). Serogroups for EHEC-7-positive isolates were confirmed by latex agglutination tests (Abraxis LLC, Warminster, PA). Isolates were further characterized by MRA PCR for *nleA, nleB, nleB2, nleC, nleD, nleE, nleF, nleG, nleG2-1, nleG2-3, nleG5-2, nleG6-2, nleG9, nleH1-1, nleH1-2,* and *ent* genes, using primers and conditions as previously described (Coombes *et al.*, 2008). In addition, isolates were tested for *eae* subtypes and putative virulence factor genes, viz., Z4321 (*Salmonella enterica* serovar Typhimurium [*pagC*]), Z4326 (*Shigella flexneri* enterotoxin 2 [*sen*]), and Z4332 and Z4333 (EHEC factor for adherence [*efa1*]) as previously described (Karmali *et al.*, 2003; Blanco *et al.*, 2004). In the respective PCR assays, water was used as a negative control. *E. coli* strain 933 (O157:H7) was used as a positive control for *nle, pagC, sen*, *efa1*, and *fliC*. For the 11-plex PCR, the following strains were used for the respective O group: DEC10B (O26:H11); B8227-C8 (O45:H2); 15612 (O103:H11); 7726 (O111:NT); 4190 (O121:NT); 1234 (O145:H28); and 86-24 (O157:H7).

Detection of EHEC by the NeoSEEK™ STEC Detection and Identification (NS) test. Enriched hide and carcass sponge sample aliquots stored at −80°C were shipped overnight or directly delivered for EHEC-7 testing by the NS (Neogen® Corp., Lansing, MI) test, a PCR/mass spectrometry–based analytical method at GeneSeek® Inc. NS results were reported by the company as confirmed positive or negative. Based on NS data, a positive result for EHEC was inferred by the concurrent detection of *stx*, targeted O-group single nucleotide polymorphism, specific *eae* subtypes, and several other virulence markers using a total of 70 independent markers. Identifying combinations of *eae* subtypes and EHEC-7 O group markers were as follows: *eae*-β with O26; *eae*-ε with O45, O103, or O121; *eae*-γ2 with O111; and *eae*-γ1 with O145 or O157.

Statistical analysis. Adjusted sample-level prevalence estimates for each EHEC serogroup, using the definitions for a positive test result described above, were obtained from model intercepts for each detection protocol (i.e., culture and NS for hide, and GDS and NS for carcass samples). Generalized linear mixed models were fitted using restricted pseudo-likelihood estimation, Newton-Raphson and Ridging optimization, binary distribution, logit link, and Kenward-Rogers degrees of freedom (PROC GLIMMIX, SAS 9.3, SAS Institute Inc., Cary, NC). Random effects of pens nested within sampling week were used to adjust prevalence estimates for the lack of independence resulting from collecting multiple samples from each study pen across multiple sampling weeks.

The overall agreement on detection of EHEC groups between the culture-based method and NS on hide samples, and between GDS and NS on carcasses, beyond that due to chance, was determined by computing the Cohen's κ coefficient and the

McNemar's chi-square test (Dohoo *et al.*, 2009). The degree of agreement was interpreted based on the scale proposed by Landis and Koch (1977). In addition, a test of proportions, based on *Z* tests, was used to compare the proportion of EHEC-7 positives obtained from hide and carcass surface samples based on NS.

Results

Detection of EHEC in hide sponge samples by culture-based and multiplex PCR assays. Of the 576 hide sponge samples cultured, 476 were tested by multiplex PCR, whereas 100 were not tested due to inadequate DNA concentration. At least 1 targeted O-group positive organism (which included both STEC and non-STEC) was isolated from 248 of the 476 hide samples (52.1%) as tested by 11-plex PCR on individual colonies. In descending order, the proportion of hide samples testing positive for the 7 targeted O-group genes (which included both STEC and non-STEC) by PCR following enrichment broth culture was 138/476 (29.0%), O103; 124/476 (26.1%), O157; 52/476 (10.9%), O26; 18/476 (3.8%), O45; 8/476 (1.7%), O111; and 3/476 (0.6%) each, O121 and O145. The proportion of hide samples in which the targeted virulence genes were identified by PCR in these cultures was 25/476 (5.3%), *stx*; 19/476 (4.0%), *eae*; and 51/476 (10.7%), *ehxA* (Table 1). Eight EHEC-7 isolates were recovered by the culture-based protocol from 6/476 (1.3%) of the samples. These isolates included 2 O26:[H11], 1 O145:[H28], and 5 O157:H7 (Table 2). No EHEC-6 or EHEC O157 isolates were obtained from the carcass samples by the culture-based method.

Characterization of EHEC-7 hide isolates. The results of molecularly serotyping and testing for virulence genes and *eae* subtype in EHEC-7 isolates from hides

are shown in Table 2. All EHEC O157:H7 isolates were positive for 15 or 16 *nle* genes in the MRA, whereas EHEC non-O157 isolates were positive for 7–15 of these genes.

Detection of EHEC on hides and carcasses by NS. The proportion of hide sponge samples testing positive for EHEC-7 by NS was 451/576 (78.3%, Table 3). In descending order, the proportions of these samples testing positive for the different EHEC were 282/576 (49.0%), O145; 227/576 (39.4%), O45; 107/576 (18.6%), O103; 83/576 (14.4%), O157; 14/576 (2.4%), O121; 13/576 (2.3%), O111; and 3/576 (0.5%), O26. Although the NS test detected a higher proportion of positive samples than the culture-based method, five hide samples from which EHEC isolates were obtained by culture tested negative for the respective serogroup by NS. NS detected EHEC O145 from the remaining hide sample from which an EHEC O145 organism was isolated (Table 2). By interpreting in-parallel the combined results of NS and the culture-based method, 78.5% of the hide samples (452/576) were positive for EHEC-7.

The proportion of preintervention de-hided carcass sponge samples testing positive by NS for EHEC-7 was 36/576 (6.3%, Table 4). In descending order, the proportions of these samples testing positive for the different EHEC serogroups was 16/576 (2.8%), O157; 11/576 (1.9%), O145; 10/576 (1.7%), O103; 8/576 (1.4%), O45; 2/576 (0.3%), O26; 0/576 (0.0%) each, for O111 and O121. Based on GDS, 165/576 (28.6%) and 86/576 (14.9%) of the carcass samples were presumptive positive for the "top 6" EHEC and EHEC O157, respectively. Based on NS, the proportion of EHEC-7 positives in hide sponge samples was significantly higher ($p<0.05$) than in carcass surface samples.

Model-adjusted cumulative prevalence estimates of EHEC on hides and carcasses. The model-adjusted cumulative prevalence estimates of EHEC-7 in hide sponge samples based on the culture-based method and NS are shown in Table 3. The model-adjusted cumulative prevalence estimate of EHEC-7 in hide sponge samples as detected by NS was 80.7%, with a distribution of 49.9%, O145; 37.1%, O45; 12.5%, O103; 11.0%, O157; 2.2%, O111; 2.0%, O121; and 0.2%, O26. In contrast, the model-adjusted cumulative prevalence estimate of EHEC-7 in hide samples based on culture was 1.2%, with a distribution of 0.6%, O157; 0.4%, O26; 0.2%, O145; and 0% for the remaining serogroups.

The model-adjusted cumulative prevalence estimate of EHEC-6 and EHEC O157 in carcass sponge samples based on GDS was 24.8% and 13.1%, respectively (Table 4). In contrast, the model-adjusted cumulative prevalence estimates of EHEC-6 and EHEC-7 in carcass sponge samples as detected by NS were 4.0% and 6.0%, respectively, with a distribution of 2.8%, O157; 1.6%, O145; 1.2%, O103; 1.1%, O45; 0.2%, O26; and 0.0%, O111 and O121.

Comparison of culture-based method versus NS for EHEC-7 detection in hide samples and GDS versus NS for carcass samples. The McNemar's chi-square tests comparing the culture-based method and NS results on hide samples were statistically significant ($p<0.05$) for all EHEC groups, indicating a serious disagreement between the tests, except for EHEC O26. The κ coefficient for the comparison of the culture-based method and NS for EHEC O26 ($\kappa=-0.005$; 95% CI=−0.0099, −0.0003) indicated a poor agreement between the tests. The McNemar's chi-square tests comparing

GDS and NS on carcass samples for detection of EHEC O157 and EHEC-6 were statistically significant ($p<0.05$), indicating disagreement between the tests.

Discussion

Although the results of this study were derived from only one plant, one feedlot, and one period, they suggest that EHEC-7, as detected by NS, are ubiquitous on hides of commercial feedlot cattle harvested in the summer (>80% positive), with EHEC O145, O45, O103, and O157, in descending order, the most prevalent. The results also suggest that at least 6 per 100 carcasses, prior to evisceration and interventions, are contaminated with EHEC-7, based on NS, with EHEC O157, O145, O103, and O45, in descending order, also the most prevalent.

Few studies have estimated the prevalence specifically of EHEC-6 and EHEC-7 on hides and carcasses, as compared to that for *E. coli* O157:H7 and non-O157 STEC. In one recent study, hide and carcass surface samples were cultured for EHEC-6 and −7, but no isolates were obtained from the hides; in addition, it was unclear whether carcass isolates were EHEC-7 (Svoboda *et al*., 2013), and the methods yielded only presumptive positive results. In our study, GDS yielded a higher proportion of presumptive positives for EHEC-6 on carcasses than the proportion of confirmed positive samples obtained using NS, and no isolates were obtained from these samples. Prevalence estimates made solely on the detection of O-group plus *stx* and *eae* in an enrichment broth sample (presumptive positive) will likely be higher due to false-positive results generated by background organisms containing some, but not all of the gene targets. NS results are reported as confirmed positive or negative; however, recovery of fully characterized

bacterial isolates provides the most definitive evidence of a true positive sample. In the present study, GDS testing was not conducted on the hide samples due to the unavailability of the test system in the lab handling these samples.

In contrast to non-O157 EHEC, a relatively large number of studies have reported on the prevalence of EHEC O157:H7 on hides and pre-evisceration beef carcass surfaces. A summarization of the results from these studies is provided in Table 5. Varying methods, media, geographical locations, and seasons undoubtedly contribute to differences in reported prevalence. In the present study, only 6 colonies per plate for each of the 7 IMS serogroups (i.e., 42 colonies per sample) were picked, but the chromogenic phenotype was shared by many of the background flora; hence this protocol is relatively insensitive. Interestingly, the culture-based protocol used in the study by Arthur *et al.* (2002) yielded similar reported EHEC-6 prevalence levels.

E. coli O157:H7 was isolated from fewer hide samples in this study than in previous ones (Elder *et al*., 2000; Brichta-Harhay *et al*., 2008). This may have been the result of using mPossé agar, which in its original formulation was intended for detection of *E. coli* O26, O103, O111, and O145, but not O157:H7 (Possé *et al.*, 2008b). We chose this medium because we had found in preliminary experiments with pure cultures that all seven EHEC serogroups could be screened for in three colony colors. A potential contributing factor to the lower proportion of EHEC O157 detected could have been the prior treatment of cattle with a commercial bacteriophage solution that is lytic for *E. coli* O157:H7. Improvements in culture-based methods are likely needed for isolation of EHEC, given the disparities we found between detection protocols. However, it should be noted that our culture method detected EHEC-7 in five hide samples that were negative

for the respective organism by NS. In these samples, the signals targeted in the NS test were below the level of detection (E. Hosking, personal communication). In addition to providing backup for more sensitive methods, culture-based tests remain important for trace-back of contaminated foods, the identification of novel genes, and other uses as they produce isolates that can be further confirmed and characterized.

Using the MRA developed by Coombes *et al.* (2008), all EHEC isolates from hide samples in the present study were seropathotype A or B, and had the virulence gene content (e.g., *nle* and other) expected for this classification. One isolate (serotype O145:[H28]) would have been less likely to cause a disease outbreak, since it lacked complete O-Islands 36, 57, 71, and 122, and contained only 7 of the 16 genes tested for in the MRA. In general, the MRA tests for genes on these four O-Islands that encode effectors that reduce pro-inflammatory signaling (viz., NleB, NleC, NleD, NleH1, and NleH2) and sustain colonization by preventing bacterial detachment and death of infected host epithelial cells (viz., NleD, NleH1, and NleH2; Vossenkämper *et al.*, 2011). In addition, we tested for three other genes on O-Island 122 (*efa1*, *sen*, and *pagC*; Karmali *et al*., 2003). Since all isolates had *efa1* and *sen*, and all O157:H7, but none of the non-O157 isolates had *pagC*, we hypothesize that isolates expressing the products of these genes might have increased virulence through enhancement of intestinal colonization (Efa1), water loss (Sen), and enhanced survival in macrophages (PagC; Karmali *et al*., 2003; Kitagawa *et al*., 2010).

Conclusions

The prevalence of enterohemorrhagic *E. coli* (EHEC) O26, O45, O103, O111, O121, O145, and O157 (EHEC-7) on hides and preintervention de-hided carcasses was estimated from a population of commercial feedlot cattle at harvest. The prevalence of EHEC-7 was 1.2% on hides based on culture, but 80.7% on hides and 6.0% on carcasses based on the NeoSEEK™ STEC Detection and Identification Test (NS). The prevalence of EHEC-7 on hides was significantly greater than that on carcasses, based on these NS results. This study provides evidence that "top 6" non-O157 and O157:H7 EHEC are present on the hides and carcasses of commercial feedlot cattle at harvest; however, continued improvement of methods is needed for accurate detection and estimation of non-O157 EHEC prevalence, given the discordant results across protocols.

Acknowledgements

We thank David Hanks, Megan Spencer, Diana Dewsbury, Donka Milke, Anteelah Phebus, and Rachael Henderson for technical assistance, Dr. Mick Bosilevac for comments on the data, and Dr. T.G. Nagaraja and Xiaorong Shi for assistance with molecular analysis of samples and isolates. This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2012-68003-30155 from the USDA National Institute of Food and Agriculture.

Table 1. Detection of enterohemorrhagic *Escherichia coli* (EHEC) O26, O45, O103, O111, O121, O145, and O157 in cattle hide samples by a culture-based protocol and NeoSEEK™ STEC Detection and Identification Test (NS)

Detection method	No. (%) of samples					
	Total[a]	O-group positive[b]	*stx* positive[c]	*eae* positive	*ehxA* positive	EHEC-7 positive
Culture	476 (100)	248 (52.1)	25 (5.3)	19 (4.0)	51 (10.7)	6 (1.3)
NS	576 (100)	576 (100)	574 (99.7)	576 (100)	NA	451 (78.3)

[a]A total of 100 hide samples had inadequate DNA concentration for 11-plex polymerase chain reaction; hence, the culture-based protocol could only be completed on 476 samples.

[b]Samples that isolated one or more O-group positive colonies from serogroups O26, O45, O103, O111, O121, O145 and O157.

[c]Samples positive for stx1 and/or stx2.

NA, not applicable.

Table 2. Molecular risk assessment of enterohemorrhagic *Escherichia coli* (EHEC) hide isolates by polymerase chain reaction assay

Isolate	Sample no.	Serotype	*stx*[a]	*eae*[b]	No. of detected *nle* genes[c]	*pagC*[d]	*sen*[e]	*efa1*[f]
3-O157-3	3	O157:[H7]	2	γ1	16	+	+	+
1379D	293	O157:[H7]	2	γ1	16	+	+	+
1568B	320	O157:[H7]	2	γ1	16	+	+	+
1379B	293	O157:[H7]	2	γ1	15	+	+	+
3-8G	320	O157:[H7]	2	γ1	15	+	+	+
1394E	296	O26:[H11]	1	β1	15	-	+	+
400D	154	O26:[H11]	1	β1	12	-	+	+
860B	219	O145:[H28]	2	γ1	7	-	+	+

[a]Shiga toxin type.

[b]Intimin type and subtype.

[c]Nonlocus of enterocyte effacement genes.

[d]Detection of putative virulence gene Z4321, homologue of Salmonella enterica serovar Typhimurium (*pagC*), originally reported on pathogenicity island OI-122 of EHEC O157:H7 strain EDL 933.

[e]Detection of putative virulence gene Z4326, homologue of Shigella flexneri enterotoxin 2 (*sen*), originally reported on pathogenicity island OI-122 of EHEC O157:H7 strain EDL 933.

[f]Detection of putative virulence genes Z4332 and Z4333 (EHEC factor for adherence [*efa1*]), originally reported on pathogenicity island OI-122 of EHEC O157:H7 strain EDL 933.

Table 3. Crude proportions and model-adjusted cumulative prevalence estimates of enterohemorrhagic *Escherichia coli* (EHEC) O26, O45, O103, O111, O121, O145, and O157 in cattle hide samples as detected by culture-based protocol and NeoSEEK™ STEC Detection and Identification Test (NS)

	Culture			NS		
	Total n = 476[a]			Total n = 576		
Serogroup	No. samples positive (%)	Mean prevalence, %	95% CI	No. samples positive (%)	Mean prevalence, %	95% CI
EHEC O26	2 (0.42)	0.42	0.11-1.66	3 (0.52)	0.21	0.03-1.48
EHEC O45	0 (0.00)	0.00	0.00-0.00	227 (39.41)	37.09	25.24-50.73
EHEC O103	0 (0.00)	0.00	0.00-0.00	107 (18.58)	12.48	6.87-21.59
EHEC O111	0 (0.00)	0.00	0.00-0.00	13 (2.26)	2.23	1.43-3.45
EHEC O121	0 (0.00)	0.00	0.00-0.00	14 (2.43)	1.98	0.98-3.99
EHEC O145	1 (0.21)	0.21	0.03-1.48	282 (48.96)	49.88	38.39-61.39
EHEC O157	3 (0.63)	0.63	0.20-1.94	83 (14.41)	11.02	6.73-17.52
EHEC-6	3 (0.63)	0.63	0.20-1.93	429 (74.48)	77.53	68.47-84.58
EHEC-7	6 (1.26)	1.24	0.54-2.84	451 (78.30)	80.71	73.27-86.46

[a]A total of 100 hide samples had inadequate DNA concentration for 11-plex polymerase chain reaction; hence, the culture-based protocol could be completed on only 476 samples.

Table 4. Crude proportions and model-adjusted cumulative prevalence estimates of enterohemorrhagic *Escherichia coli* (EHEC) O26, O45, O103, O111, O121, O145, and O157 in preintervention carcass samples by Biocontrol Assurance GDS™ MPX Top 7 STEC System (GDS) and NeoSEEK™ STEC Detection and Identification Test (NS)

	GDS			NS		
	Total n = 576			Total n = 576		
Serogroup or virulence gene	No. samples positive (%)	Mean prevalence, %	95% CI	No. samples positive (%)	Mean prevalence, %	95% CI
EHEC O26	NA	NA	NA	2 (0.35)	0.21	0.03-1.40
EHEC O45	NA	NA	NA	8 (1.39)	1.13	0.47-2.71
EHEC O103	NA	NA	NA	10 (1.74)	1.23	0.50-2.99
EHEC O111	NA	NA	NA	0.00	0.00	0.00-0.00
EHEC O121	NA	NA	NA	0.00	0.00	0.00-0.00
EHEC O145	NA	NA	NA	11 (1.91)	1.58	0.74-3.36
EHEC O157	86 (14.93)	13.13	9.02-18.73	16 (2.78)	2.78	1.71-4.49
EHEC-6	165 (28.65)	24.83	16.72-35.21	24 (4.17)	3.96	2.46-6.31
EHEC-7	NA	NA	NA	36 (6.25)	5.98	4.02-8.80
stx	NA	NA	NA	349 (60.59)	66.37	43.14-83.70
stx_1	118 (20.49)	15.69	9.55-24.71	NA	NA	NA
stx_2	138 (23.96)	21.89	15.91-29.33			
eae	385 (66.84)	71.69	58.02-82.27	398 (69.10)	74.52	60.17-84-99

NA, not applicable; STEC, Shiga toxin–producing *Escherichia coli*.

Table 5. Previous studies on the prevalence of enterohemorrhagic *Escherichia coli* O157:H7 and "Top 6" non-O157 on hides and de-hided pre-evisceration carcasses as detected by culture-based methods

Prevalence								
O157 %	*"Top 6" non-O157 %*	*Season*	*Sample area*	*Sample type*	*Enrichment*	*Immunomagnetic separation*	*Plating*	*Reference*
13.0	NA	Summer	450 cm^2	Hide	2% BGB, 37°C 6 h	O157	CT-SMAC	Elder *et al.*,
43.4	NA	Summer	4000 cm^2	Carcass	2% BGB, 37°C 10 h	O157	CT-SMAC and BCM	2000
NA	1.2	Summer	4000 cm^2	Carcass	2% BGB, 37°C 10 h	No	TSA, colony hybridization for *stx* followed by SMAC	Arthur *et al.*, 2002
60.6	ND	Year-round	1700 cm^2	Hide	TSB, 25°C 2 h, 42°C 6 h	O157	CT-SMAC and NT-Rainbow	Barkocy-Gallagher *et al.*,
73.8	ND	Spring	1700 cm^2	Hide	TSB, 25°C 2 h, 42°C 6 h	O157	CT-SMAC and NT-Rainbow	2003
73.5	ND	Summer	1700 cm^2	Hide	TSB, 25°C 2 h, 42°C 6 h	O157	CT-SMAC and NT-Rainbow	
67.2	ND	Fall	1700 cm^2	Hide	TSB, 25°C 2 h, 42°C 6 h	O157	CT-SMAC and NT-Rainbow	
29.4	ND	Winter	1700 cm^2	Hide	TSB, 25°C 2 h, 42°C 6 h	O157	CT-SMAC and NT-Rainbow	
26.7	ND	Year-round	Unknown	Carcass	TSB, 25°C 2 h, 42°C 6 h	O157	CT-SMAC and NT-Rainbow	

30.3	ND	Spring	Unknown	Carcass	TSB, 25°C 2 h, 42°C 6 h	O157	CT-SMAC and NT-Rainbow	
31.9	ND	Summer	Unknown	Carcass	TSB, 25°C 2 h, 42°C 6 h	O157	CT-SMAC and NT-Rainbow	
33.0	ND	Fall	Unknown	Carcass	TSB, 25°C 2 h, 42°C 6 h	O157	CT-SMAC and NT-Rainbow	
32.9	ND	Winter	Unknown	Carcass	TSB, 25°C 2 h, 42°C 6 h	O157	CT-SMAC and NT-Rainbow	
88.3	NA	Summer	2000 cm^2	Hide	TSB, 25°C 3 h, 42°C 6 h	O157	CT-SMAC and N-Rainbow	Nou *et al*., 2003
50.0	NA	Summer	8000 cm^2	Carcass	TSB, 25°C 3 h, 42°C 6 h	O157	CT-SMAC and N-Rainbow	
75.7	NA	Fall	100 cm^2	Hide	TSB, 25°C 2 h, 42°C 6 h	O157	CT-SMAC and NT-Rainbow	Arthur *et al*.,
14.7	NA	Fall	8000 cm^2	Carcass	TSB, 25°C 2 h, 42°C 6 h	O157	CT-SMAC and NT-Rainbow	2004
61.9	NA	Spring to Fall	1700 cm^2	Hide	TSB, 25°C 2-3 h, 42°C 6 h	O157	CT-SMAC and NT-Rainbow	Rivera-Betancourt *et al*., 2004
7.0	NA	Spring to Fall	4000 cm^2	Carcass	TSB, 25°C 2-3 h, 42°C 6 h	O157	CT-SMAC and NT-Rainbow	
14.7	NA	Unknown	300 cm^2	Hide	BGB, 37°C 6 h	O157	CT-SMAC and T-CO157	Woerner *et al*.,
10.1	NA	Unknown	300 cm^2	Carcass	BGB, 37°C 6 h	O157	CT-SMAC and T-CO157	2006
46.9	ND	Year-round	1000 cm^2	Hide	TSB, 25°C 2 h, 42°C 6h	O157	CT-SMAC and NT-CO157	Brichta-Harhay *et al*., 2008
43.2	ND	Spring	1000 cm^2	Hide	TSB, 25°C 2 h, 42°C 6h	O157	CT-SMAC and NT-CO157	
55.7	ND	Summer	1000 cm^2	Hide	TSB, 25°C 2 h, 42°C 6h	O157	CT-SMAC and NT-CO157	

38.7	ND	Fall	1000 cm^2	Hide	TSB, 25°C 2 h, 42°C 6h	O157	CT-SMAC and NT-CO157	
50.4	ND	Winter	1000 cm^2	Hide	TSB, 25°C 2 h, 42°C 6h	O157	CT-SMAC and NT-CO157	
16.7	ND	Year-round	8000 cm^2	Carcass	TSB, 25°C 2 h, 42°C 6h	O157	CT-SMAC and NT-CO157	
14.2	ND	Spring	8000 cm^2	Carcass	TSB, 25°C 2 h, 42°C 6h	O157	CT-SMAC and NT-CO157	
18.3	ND	Summer	8000 cm^2	Carcass	TSB, 25°C 2 h, 42°C 6h	O157	CT-SMAC and NT-CO157	
14.9	ND	Fall	8000 cm^2	Carcass	TSB, 25°C 2 h, 42°C 6h	O157	CT-SMAC and NT-CO157	
19.5	ND	Winter	8000 cm^2	Carcass	TSB, 25°C 2 h, 42°C 6h	O157	CT-SMAC and NT-CO157	
NA	0.0	Year-round	400 cm^2	Hide	mTSB+N, 41.5°C 24 h	O157	CT-SMAC	Wieczorek *et al.*, 2011
NA	0.0	Year-round	400 cm^2	Carcass	mTSB+N, 41.5°C 24 h	O157	CT-SMAC	
NA	0.0	Year-round	100 cm^2	Hide	TSB+V, 37°C overnight	No	TBX+SS	Monaghan *et al.*, 2012
NA	0.4	Year-round	Whole carcass	Carcass	TSB+V, 37°C overnight	No	TBX+SS	

17.9	0.2	Year-round	100 cm^2	Hide	mTSB+CV, 37°C 6 h	O157, O26, O103, O111 and O145	CT-SMAC and C-CCV, followed by EMB agar	Thomas *et al*., 2012
0.7	0.0	Year-round	100 cm^2	Carcass	mTSB, 37°C 6 h	O157, O26, O103, O111 and O145	CT-SMAC and C-CCV, followed by EMB agar	

BGB, brilliant green bile broth; TSB, trypticase soy broth; mTSB, trypticase soy broth modified with the addition of bile salts No. 3; N, novobiocin; V, vancomycin; C, cefixime; NA, not applicable; ND, not determined; CT-SMAC, MacConkey agar with sorbitol, cefixime and potassium tellurite; BCM, Biosynth chromogenic media; TSA, trypticase soy agar; SMAC, MacConkey agar with sorbitol; NT-Rainbow, Rainbow agar with novobiocin and potassium tellurite; N-Rainbow, Rainbow agar with novobiocin; T-CO157, CHROMagar O157 with potassium tellurite; NT-CO157, CHROMagar O157 with novobiocin and potassium tellurite; TBX + SS, tryptone bile x-glucuronide agar with streptomycin sulphate and sulfamethazine; C-CCV, Chromocult coliform agar with cefixime, cefsulodin sodium salt, and vancomycin; EMB, eosin methylene blue agar.

CHAPTER 4

Prevalence and Concentration of Enterohemorrhagic *Escherichia coli* in Culled Dairy Cows at Harvest

(Submitted to Journal of Food Protection)

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Abstract

The primary objective of this study was to determine the prevalence and concentration of enterohemorrhagic *Escherichia coli* (EHEC) O26, O45, O103, O111, O121, O145, and O157 in fecal, hide, and pre-intervention carcass surface samples from culled dairy cows. Matched samples (n = 300) were collected from 100 cows at harvest and tested by a culture-based method and 2 molecular-based methods, NeoSEEK STEC (NS), and Atlas STEC EG2 Combo (Atlas). Both culture and NS tested for presence of each of the 7 EHEC types, from which the cumulative (EHEC-7) prevalence was inferred, whereas Atlas only tested for presence of non-O157 EHEC without discrimination of serogroup and EHEC O157. The EHEC-7 prevalence in samples of feces, hides, and carcass surface, respectively, was 6.5%, 15.6%, and 1.0% by culture, and 25.9%, 64.9%, and 7.0% by NS. By Atlas, the prevalence of non-O157 EHEC was 29.1%, 38.3%, and 28.0% and that of EHEC O157 was 29.1%, 57.0%, and 3.0% for feces, hides, and carcasses, respectively. Only 2 samples originating from different cows, a hide and fecal sample contained quantifiable EHEC. In both samples these were EHEC O157, with 6.0 log colony-forming units (CFU)/1,000 cm^2 in the hide and 3.9 log CFU/g in the fecal sample. Moderate agreement was observed between culture and NS for detection of EHEC O26 (Kappa = 0.58, $P < 0.001$), EHEC O121 (Kappa = 0.50, $P < 0.001$), and EHEC O157 (Kappa = 0.40, $P < 0.001$). No significant agreement was observed between NS and Atlas or between culture and Atlas. Detection of an EHEC serogroup in fecal samples was significantly associated with detection of the same EHEC serogroup in hide samples for EHEC O26 ($P = 0.001$), EHEC O111 ($P = 0.002$), EHEC O121 ($P < 0.001$), and EHEC-6 ($P = 0.029$) based on NS detection and for EHEC O121

($P < 0.001$) based on detection by culture. This study provides evidence that non-O157 EHEC are ubiquitous on hides of culled dairy cattle, and feces are an important source of non-O157 EHEC hide contamination.

Introduction

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) cause severe illness in humans, including hemorrhagic colitis and hemolytic uremic syndrome (HUS) (Croxen *et al*., 2013). These organisms are naturally found in the intestines of ruminants, shed in the feces, and transmitted to humans through the ingestion of contaminated food or water, or by direct contact (Gyles, 2007; Cole *et al*., 2014; Luna-Gierke *et al*., 2014). Enterohemorrhagic *E. coli* (EHEC) is a subset of STEC and generally defined as *E. coli* that contain genes for Stx (*stx*) and locus of enterocyte effacement (LEE) proteins, e.g., intimin (*eae*), but may also include LEE-negative *stx*-positive *E. coli* strains that cause hemorrhagic colitis and HUS in human patients (Croxen *et al*., 2013). EHEC of the serogroups O26, O45, O103, O111, O121, and O145 (EHEC-6) caused 71% of the human STEC cases in the U.S. from 1983 to 2002 (Brooks *et al*., 2005). Cumulatively, EHEC-6 with EHEC O157:H7 (hereafter referred to as EHEC-7) caused >90% of the human STEC cases in the U.S. from 2000-2010 (Gould *et al*., 2013). Thus, the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) declared EHEC-7 as adulterants in raw, non-intact beef (USDA, 2014a).

The clonality and rather unique biochemical features of EHEC O157:H7 allowed for the development of sensitive and specific culture methods for this organism (Moxley, 2003; Beutin and Fach, 2014); in contrast, culture methods for non-O157 EHEC adulterants developed to date have lacked sensitivity and specificity due to the multiplicity of organisms needing to be targeted, the lack of clonality of these organisms, and, other than having Stx and intimin, these organisms lack characteristics that distinguish them from generic *E. coli* (Wang *et al*., 2013; Gill *et al*., 2014; Kase *et al*.,

2015; Stromberg *et al*., 2015a). USDA-FSIS methods for detection and isolation of EHEC from meat products, which involve PCR screening, immunomagnetic separation, cultural isolation with the use of a chromogenic agar, and confirmatory PCR and agglutination have been improved through a number of modifications, but are still not optimal (Wang *et al*., 2013; USDA, 2014b). Molecular methods that detect nucleic acids from EHEC organisms have been used in place of culture-based methods or as an initial screen before culture-based detection of EHEC in foods, but also lack specificity because gene targets can be contributed by background organisms leading to false positive results (Wang *et al*., 2013). In an attempt to increase sensitivity and specificity, the NeoSEEK STEC Detection and Identification test (NS; Neogen, Lansing, MI) and Atlas STEC EG2 Combo Detection Assay (Atlas; Roka Bioscience, Warren, NJ) have been used. The NS test includes a proprietary set of genetic markers, is approved by the USDA-FSIS as a confirmation test for detection of EHEC adulterants in beef trim, and has been used to detect EHEC in veal calf hide samples (Wang *et al*., 2014a) and feedlot cattle hide and carcass samples (Stromberg *et al*., 2015a). In addition, the Atlas test has been used on cattle fecal samples (Bosilevac *et al*., 2013).

EHEC pose a threat to food safety as they contaminate carcass surfaces during the removal of hides (Elder *et al*., 2000; Arthur *et al*., 2002; Keen and Elder, 2002; Barkocy-Gallagher *et al*., 2003; Koohmaraie *et al*., 2005; Arthur *et al*., 2007; Brichta-Harhay *et al*., 2008; Schmidt *et al*., 2012), and the predictive virulence of EHEC isolates is a question of major importance. In one study, based on NS test results, the prevalence of EHEC-7 in beef feedlot cattle was 80.7% on hides and 6.0% on pre-intervention carcass surfaces (Stromberg *et al*., 2015a). In addition to feedlot cattle, culled dairy cows are a

significant source of beef. In 2014, 9.5% of cattle slaughtered were classified as dairy cows (USDA, 2015c). In fecal samples from dairy cows tested by culture-based methods, the prevalence of EHEC O157:H7 ranged from 0.2% to 48.8% and that of non-O157 STEC ranged from 0.4% to 74.0% (Hussein and Sakuma, 2005). Coombes *et al*. (2008) developed a molecular risk assessment (MRA) protocol for assessing the predictive virulence or public health risk of non-O157 STEC strains based on the detection of specific virulence genes. The identification of more highly virulent strains is important since these are the ones most likely to cause outbreaks of disease and case progression to HUS (Karmali *et al*., 2003; Coombes *et al*., 2008).

Many factors have been implicated in the intestinal colonization of cattle with EHEC, including management practices (Renter *et al*., 2005, Smith, 2014a; Smith, 2014b). Production systems vary between beef feedlot cattle and dairy cattle, which may cause differences in EHEC prevalence. Culled dairy cows (6-8 years old) are typically older than feedlot steers and heifers (<2 years old). Cray and Moon (1995) reported that pre-weaned (3- to 14-week-old) calves were more susceptible to infection with STEC O157:H7 following experimental inoculation than adult (1- to 3-year-old) cattle; however, Mir *et al*. (2015) reported that cows (≥2 years old) had a higher natural prevalence of STEC compared to heifers (1-2 years old).

In addition to prevalence, concentration is an important contributor to risk of EHEC infection (Ekong *et al*., 2015). Concentrations of *E. coli* O157:H7 $>10^4$ CFU/g are associated with hide contamination (Arthur *et al*., 2009), and even higher concentrations in the environment may lead to more human exposure (Chase-Topping *et al*., 2008). The objectives of this cross-sectional study were to: (1) determine the prevalence and

concentration of EHEC-7 in fecal, hide, and pre-intervention carcass surface samples from culled dairy cows at a commercial processing plant; (2) conduct a MRA on recovered EHEC isolates; (3) compare the applicability of a culture-based method, NS, Atlas for detection of EHEC; and (4) determine the association between detection of an EHEC serogroup in fecal samples with that on hides.

Materials and Methods

Study design and sample collection. Matched fecal, hide, and carcass surface samples (n = 300) were collected from 100 dairy cows (10-30 cows/week for 5 weeks) at a small (60 animals/h) western U.S. commercial processing plant from June to July 2014 using a modified protocol for *E. coli* O157:H7 sampling (USDA, 2005). Samples of rectal lumen contents (as a surrogate for and hereafter referred to as feces) were obtained at the viscera table after the cattle were slaughtered and eviscerated. Hides were rinsed with water by plant employees as part of the routine process before the research team collected samples. Hide and carcass surface samples were collected using wet (35 ml buffered peptone water) Speci-sponges (Nasco, Ft Atkinson WI) according to methods previously described (Stromberg *et al*., 2015a). Hide samples were collected by sampling an area of approximately 1,000 cm^2 (32 cm × 32 cm), 15 cm from the ventral midline near the diaphragm. Two carcass surface samples per animal were collected: the first was obtained from an area of approximately 1,000 cm^2 in the brisket-short plate region, and the second was from an area of approximately 3,000 cm^2 in the lateral hock and round-rump regions. The 2 carcass sponges and their buffer volumes, representing a combined

4,000 cm^2 area per carcass, were combined into a single Whirl-Pak® bag. Samples were shipped overnight on ice to the laboratory.

Isolation and confirmation of EHEC using culture and PCR. Fecal, hide, and carcass samples (n = 300) were processed within 24 h after collection (Fig. 1). One g of feces was suspended in 9 ml of *E. coli* broth (EC; Oxoid Ltd., Basingstoke, UK) and vortexed for 1 min. Ninety ml of EC at room temperature (RT) was added to each hide sponge sample and 80 ml of EC at RT was added to each carcass sponge sample, and all samples were incubated at 40°C for 6 h. Following incubation, broth culture was inoculated onto a CHROMagar STEC plate (DRG Int., Springfield, NJ) for isolation, and incubated overnight at 37°C. Four or fewer pink to mauve colonies (2 with and 2 without UV fluorescing halos when present) were picked and inoculated into 500 µl trypticase soy broth and incubated overnight at 37°C. Genomic DNA was prepared from overnight cultures by centrifuging a 200-µl aliquot at 2,000 × *g* for 20 min, replacing the media with 200 µl PBS, vortexing to resuspend the cells, incubating at 95°C for 20 min, centrifuging again at 2,000 × *g* for 20 min, and collecting the supernatant. One µl of DNA was used in a 20-µl PCR reaction containing 1.3 × final concentration Bullseye HS Taq Buffer II (with *balanced ammonium/potassium*), 29.5 mM $MgCl_2$, 10 mM each dNTPs, 1.5 U Bullseye HS Taq (MidSci, St Louis, MO), and primers shown in Table 1. PCR amplification was performed as previously described (Paton and Paton, 1998).

Primers for the amplification of the type III secreted effector EspK (*espK*) were generated from the *E. coli* O157:H7 EDL933 genome (GenBank accession no. AE005174). The *espK* (Z1829) sequence was loaded into Geneious version 7.1.8 and Primer 3 software to design PCR primers with acceptable Tm and that generated a 200-bp

product. Primers EspK_F1 and EspK_R1 were validated against 86 *espK*-positive STEC strains. This included the U.S. Meat Animal Research Center *E. coli* O157:H7 molecular diversity panel, a set of 50 unique *E. coli* O157:H7 isolated from across North America, each with an individual pulsed-field gel electrophoresis restriction digest pattern, and 6 STEC strains each of the O26, O45, O103, O111, O121, and O145 serogroups. Negative controls for *espK* included the 72 commensal *E. coli* that make up the *E. coli* collection of reference (ECOR) strains (Ochman and Selander, 1984).

Additionally after enrichment, 3 separate 490-µl broth aliquots were diluted with 490 µl of PBS with 0.05% Tween-20 (PBS-Tween) and each aliquot was subjected to 1 of 3 immunomagnetic separation (IMS) treatments using a KingFisher Flex Magnetic Particle Processor (Thermo Scientific, Waltham, MA) (Fig. 1): i) 20 µl of anti-O157 Dynabeads (Invitrogen, Carlsbad, CA); ii) a pool of 20 µl of IMS beads (6.3 µl each) for *E. coli* O26, O45, and O121 (Abraxis LLC, Warminster, PA); and iii) a pool of 20 µl of IMS beads (6.3 µl each) for *E. coli* O103, O111, and O145 (Abraxis). Fifty µl of recovered beads from the *E. coli* O157 IMS treatment were spread onto CHROMagar O157 (DRG Int.) with cefixime trihydrate (0.025 mg/liter), cefsulodin (5.0 mg/liter), and potassium tellurite (2.5 mg/liter; CCT-CHROMagar O157) and incubated for 18 h at 37°C. Fifty µl of recovered beads from the remaining 2 IMS treatments were spread onto STEC heart infusion washed blood agar with mitomycin C (0.5 µg/ml; SHIBAM) and incubated for 18 h at 37°C. SHIBAM was prepared with 4% defibrinated sheep blood (Quad Five, Ryegate, MT), according to the methods of Feng *et al*. (2011). Five or fewer mauve colonies per CCT-CHROMagar O157 plate were picked and ≤20 enterohemolytic phenotype colonies per SHIBAM plate were picked. All colonies picked from CCT-

CHROMagar O157 and SHIBAM plates were streaked for isolation on 5% sheep blood agar (Remel, Lenexa, KS) and incubated for 15 h at 37°C. Isolated colonies were picked from blood agar, suspended in 50 µl of ultrapure water, and heated at 95°C for 10 min for use as DNA template in PCR assays. Individual DNA preparations from isolated colonies (n = 6) were pooled and the pooled DNA was tested by single-plex PCR for *stx* (Monday *et al*., 2007). If a pool was positive for *stx*, the DNA preparations from each of the isolate making up the pool were individually tested by 11-plex PCR. The 11-plex PCR included genes representing each of the EHEC-7 serogroups (*wzx, wbq,* or *rfbE*), plus stx_1, stx_2, and EHEC-hemolysin (*ehxA*) (Bai *et al*., 2012) which was modified by the use of primers for *eae* as described by Blanco *et al*. (2004), and primers for the *wzx* gene of O111 as described by Noll *et al*. (2015).

Additional aliquots of broth enrichment culture of each fecal, hide and carcass surface sample were obtained and held at 4°C (24-96 h) until molecular screening assays (Atlas and NS) were completed (Fig. 1). Broths identified as positive for *E. coli* O157:H7 by Atlas testing were subjected to O157 IMS by adding 20 µl of beads (Pickpen® IMS, BioControl, Bellevue, WA) to broth culture, shaking at 900 rpm on a bench top plate shaker and then separated using a KingFisher (Thermo Scientific). The IMS steps included 2 wash steps in PBS-Tween, and the final captured beads from the IMS-broth treatment were suspended in 200 µl PBS-Tween. The concentrated beads (50 µl and 1 µl) were spread plated onto 2 CHROMagar O157 plates supplemented with novobiocin (5.0 mg/liter) and potassium tellurite (1.0 mg/liter). Plates were incubated overnight at 37ºC and mauve colonies were picked and tested for *E. coli* O157:H7 by multiplex PCR as described by Hu *et al*. (1999).

A broth culture was suspected of containing an EHEC-6 if it was found positive for non-O157 EHEC by Atlas and/or NS. Based on those results, IMS for each particular suspect-positive serogroup was conducted; hence an enrichment broth culture was subjected to 1-6 IMS treatments depending on the screening results. One ml of each EHEC-6 suspect broth culture was subjected to IMS using individual specific O group IMS beads (Romer Laboratories, Union, MO) with captured beads from an IMS-broth treatment diluted 1:10 and 1:100 and spiral-plated using an Autoplate 4000 (Advanced Instruments Inc, Norwood, MA) onto STEC differentiation agar (SDA) (Kalchayanand *et al*., 2013) and washed blood agar with 0.5 µg/ml mitomycin C (WBAM) (Sugiyama *et al*., 2001), respectively. Plates were incubated overnight at 37°C. Suspect colonies on SDA plates were O-group specific shades of green and blue and on WBAM presented with an enterohemolytic phenotype. Four or fewer colonies per plate were picked and screened by multiplex PCR for *stx*, *eae*, *espK*, non-locus of enterocyte effacement genes (*nleB* and *nleF*), and subtilase (*subA*; Table 1). All *E. coli* isolates from SDA and WBAM that were positive for both *stx* and *eae* were serogrouped using multiplex PCR that identified all EHEC-6 serogroups (Bosilevac and Koohmaraie, 2011; Fig. 1).

Detection of EHEC by NS. Enriched sample aliquots were tested for EHEC-7 by NS (Neogen Corp., Lansing, MI), a PCR coupled with mass spectrometry-based analytical method. Based on NS data, a positive result for EHEC was defined by the concurrent detection of *stx*, targeted O-group single nucleotide polymorphisms, and specific *eae* subtype in conjunction with the O group marker. Identifying combinations of *eae* subtypes and EHEC-7 O group markers were as follows: *eae*-β with O26; *eae*-ε with O45, O103, or O121; *eae*-γ2 with O111; and *eae*-γ1 with O145 or O157.

Detection of EHEC by Atlas. Enriched sample aliquots were tested for EHEC O157 and non-O157 EHEC by Atlas (Roka Bioscience, Warren, NJ). After enrichment, 1.2 ml was transferred to a Roka Bioscience G2 RT lysis tube and placed in the Atlas System instrument for analysis using the STEC EG2 Combo Detection Assay (Roka Bioscience). Atlas identified samples suspected to be positive for EHEC O157 or non-O157 EHEC.

Quantification of EHEC-7. Pre-enriched 1 ml sample aliquots were frozen in 500 µl of brain heart infusion broth with 50% glycerol at -80°C. Samples to be quantified were identified based on NS or culture positive results from post-enriched samples. Pre-enriched samples were removed from -80°C and allowed to recover at room temperature for 2 h. Using an Eddy Jet 2 spiral plater (IUL Instruments, Königswinter, Germany), 50 µl was spiral plated on Possé agar modified by reducing novobiocin (5.0 mg/liter) and potassium tellurite (0.5 mg/liter; mPossé) as previously described (Stromberg *et al*., 2015) and incubated at 37°C for 18 h. Based on NS results, blue-purple and red-purple colonies were counted for samples positive for EHEC O26, O45, O103, O111, or O157 and blue-purple, red-purple, and green colonies were counted for those positive for EHEC O121 or O145. Additionally, EHEC O157 culture positive samples were spiral-plated on CCT-CHROMagar O157 and mauve colonies were enumerated. Up to 10 target colonies were picked per plate and heated at 95°C in 50 µl of water for use as DNA template. Colonies were tested by an 11-plex PCR as described above (Blanco *et al*., 2004; Bai *et al*., 2012; Noll *et al*., 2015). Those colonies positive for one of the EHEC-7 serogroup genes, plus *stx* and *eae* were confirmed as an EHEC-7 and counted as positive.

Concentration was determined based on the proportion of target colonies on an individual plate that were confirmed EHEC-7 positive by the 11-plex PCR assay.

Characterization of EHEC isolates. H-antigen typing (*fliC*) for H2, H7, H8, H11, and H28 was done as previously described (Luedtke *et al*., 2014). All other H-antigen typing was done at the Pennsylvania State *E. coli* Reference Center (Machado *et al*., 2000) or using the methods of Wang *et al*. (2003). Serogroups for EHEC-7 positive isolates were confirmed by latex agglutination tests (Abraxis LLC, Warminster, PA). If EHEC isolates were not an EHEC-7 serogroup, they were O:H serotyped at the Pennsylvania State University *E. coli* Reference Center using the methods of Ørskov *et al*. (1977), and Machado *et al*. (2000), or O-serogrouped by agglutination testing with pooled and specific Statens Serum Institute antisera (Cedarlane Labs, Burlington, NC) according to the manufacturer's instructions. Isolates were tested by MRA PCR for genes associated with HUS and foodborne outbreaks. The MRA PCR tested for the presence of *nleA, nleB, nleB2, nleC, nleD, nleE, nleF, nleG, nleG2-1, nleG2-3, nleG5-2, nleG6-2, nleG9, nleH1-1, nleH1-2,* and *ent*, using primers and conditions as previously described (Coombes *et al*., 2008). In addition, isolates were tested for putative virulence factor genes (*pagC, sen,* and *efa1*) and *eae* subtyped as previously described (Karmali *et al*., 2003; Blanco *et al*., 2004). The FDA *E. coli* identification array (FDA-ECID) (Jackson *et al*., 2011) was used to determine if isolates from different sample types of the same animal were the same strain.

Statistical analysis. The overall agreement on detection of EHEC groups, beyond that due to chance, between the culture-based method, NS, and Atlas, independent of sample type was determined by computing the Cohen's κ coefficient and the McNemar's

Chi-square test (Dohoo *et al*., 2009). The κ coefficient was interpreted on the scale of <0, 0 to 0.2, 0.21 to 0.4, 0.41 to 0.6, 0.61 to 0.8 or 0.81 to 1.0 as poor, slight, fair, moderate, substantial, or almost perfect agreement, respectively (Landis and Koch, 1977).

Associations between the detection of an EHEC serogroup or virulence gene (presence or absence of a gene) in fecal samples (explanatory variables) with the presence of an EHEC serogroup or virulence genes on hide samples (dependent variables) were evaluated, using generalized linear mixed models (GLMMs) in SAS (SAS 9.3, SAS Institute Inc., Cary, NC). Similar models were fitted to estimate associations between EHEC serogroup or virulence gene detected in hide samples with their presence in carcass surface samples. An independent model was fitted for each EHEC serogroup and detection method. Mixed models were fitted using a residual pseudo-likelihood estimation, binary distribution, logit link, Kenward-Roger degrees of freedom approximation, and a random intercept for sampling date to account for the clustering effect of samples nested within date of sample collection. Mean probability estimates and 95% confidence intervals were computed. *P*-values <0.05 were deemed significant.

Model-adjusted sample-level prevalence estimates and their 95% confidence intervals were computed for all EHEC serogroup and virulence genes, based on each detection protocol, using GLMMs. Prevalence estimates were calculated from model intercepts using the following formulae: $e^{\beta^\circ} / (1 + e^{\beta^\circ})$, where β° is the coefficient of the model intercept.

Results

Detection of EHEC in fecal, hide, and carcass samples by culture-based methods, NS, and Atlas. One hundred matched fecal, hide, and carcass samples were tested for EHEC by culture, NS, and Atlas. By culture, *stx*-positive isolates were detected in 20%, *eae*-positive isolates in 31%, and EHEC-7 in 7% of the fecal samples. EHEC-7 were detected in 26% of the fecal samples by NS. In descending order, the proportions of fecal samples testing positive for the different EHEC serogroups by NS were 11%, O45; 7%, O103; 7%, O111; 7%, O145; 4%, O157; 1%, O26; and 0%, O121. By Atlas, 29% of fecal samples tested positive for non-O157 EHEC and 29% tested positive for EHEC O157 (Table 2).

By culture, *stx*-positive isolates were detected in 24%, *eae*-positive isolates in 38%, and EHEC-7 in 16% of the hide samples. EHEC-7 were detected in 65% of the hide samples by NS. In descending order, the proportions of these samples testing positive for the different EHEC serogroups were 36%, O45; 23%, O145; 15%, O111; 10%, O103; 7%, O26; 7%, O157; and 3%, O121. By Atlas, 46% of the hide samples tested positive for non-O157 EHEC and 51% tested positive for EHEC O157 (Table 3).

By culture, *stx*-positive isolates were detected in 3%, *eae*-positive isolates in 12%, and EHEC-7 in 1% of the carcass samples. EHEC-7 were detected in 7% of the carcass samples by NS. In descending order, the proportions of these samples testing positive by NS for the different EHEC serogroups were 4%, O103; 3%, O26; 2%, O145; 1%, O157; and 0%, O45, O111 and O121. By Atlas, 28% of the carcass samples tested positive for non-O157 EHEC and 3% were EHEC O157 positive (Table 4).

Model-adjusted prevalence estimates of EHEC in fecal, hide, and carcass samples. The model-adjusted prevalence estimates for EHEC serogroups and virulence genes as detected by culture, NS, and Atlas are shown in Tables 2-4.

Quantification of EHEC-7. Pre-enriched aliquots from 26 fecal, 67 hide, and 7 carcass samples that were NS- or culture-positive were spiral-plated on mPossé to quantify EHEC-7, and 6 fecal, 9 hide, and 1 carcass samples were spiral-plated on CCT-CHROMagar O157 to quantify EHEC O157. No EHEC-7 were quantifiable from the carcass samples. One hide sample was positive for EHEC O26 and 1 fecal sample and 1 hide sample were positive for EHEC O157 as detected by spiral plating, and of these, the EHEC O157 samples were quantifiable. EHEC O157 was estimated to be 3.9 log CFU/g in 1 fecal sample and 5.0 log CFU/100 cm^2 on 1 hide sample. EHEC isolates that were of a serogroup other than EHEC-7 were quantified in 1 fecal and 3 hide samples. O-non-typeable EHEC were estimated to be 4.2 log CFU/g in 1 fecal sample. In hide samples, 2 EHEC O177 isolates were quantified at 5.0 log CFU/100 cm^2 and 4.1 log CFU/100 cm^2, and 1 EHEC O101 was quantified at 4.9 log CFU/100 cm^2.

Characterization of EHEC-7 isolates. The results of molecular serotyping and testing for virulence genes in EHEC isolates are shown in Table 5. EHEC O157 isolates tested positive for all 16 genes in the MRA, while non-O157 EHEC isolates tested positive for 6 to 13 out of 16 genes. Only EHEC-7 isolates tested positive for the EHEC factor for adherence (*efa1*).

Comparison of methods for the detection of EHEC-7. The McNemar's Chi-squared tests comparing the culture-based method and NS results, independent of sample type were statistically significant ($P < 0.05$) or not applicable (at least one of the tests did

not detect any positives). For EHEC O45, O103, O111, and O145 the McNemar's Chi-squared test was not applicable because at least 1 of the tests did not detect any positives. The κ coefficient was calculated for the comparison of the culture-based method and NS for EHEC O26 (Kappa = 0.58, $P < 0.001$), EHEC O121 (Kappa = 0.50, $P < 0.001$), and EHEC O157 (Kappa = 0.40, $P < 0.001$) indicating fair to moderate agreement (Table 6). The McNemar's Chi-squared test indicated disagreement ($P < 0.05$) between NS and Atlas or between culture and Atlas and no significant agreements were observed.

Associations of EHEC detection. Two EHEC O98 isolates from 1 fecal sample and 1 carcass sample of the same animal were inferred to be the same strain by the FDA-ECID. Similarly, 2 EHEC O157 isolates from 1 fecal sample and 1 carcass sample of the same animal were inferred to be the same strain. Detection of an EHEC serogroup in fecal samples was significantly associated with the detection of the corresponding EHEC serogroup on hide samples for EHEC O26 ($P < 0.001$), EHEC O111 ($P = 0.002$), EHEC O121 ($P < 0.001$), and EHEC-6 ($P = 0.029$) based on NS detection, and for EHEC O121 ($P < 0.001$) based on detection by culture (Table 7). No significant associations were found between detection of an EHEC serogroup on hide samples with the detection of an EHEC serogroup on carcass samples.

Discussion

Management practices and cattle type may play a role in the prevalence of STEC in animals and their production environments (Hancock *et al*., 1994; Cobbold *et al*., 2004; Callaway, 2010; Besser *et al*., 2014; Smith, 2014a; Smith, 2014b; USDA, 2014a).

In one study, a lower prevalence of STEC was detected in feedlot cattle compared to dairy and range cattle (Cobbold *et al*., 2004). In the present study, 65% of culled dairy cattle hides were positive for EHEC-7 by NS, compared to a previous study which found >80% of feedlot cattle hides positive by the same method (Stromberg *et al*., 2015a). Interventions such as high pressure water washes containing sanitizing agents (e.g., 1% cetylpyridinium chloride) that reduce bacterial concentrations on hides have been effective in reducing transfer of *E. coli* O157 onto carcasses during processing (Arthur *et al*., 2002; Bosilevac *et al*., 2004; Moxley and Acuff, 2014). EHEC-7 prevalence on pre-intervention carcasses surfaces of culled dairy cows was 7% by NS, which was similar to that of feedlot beef cattle (Stromberg *et al*., 2015a). However, these studies were done in different locations and different years making it difficult to make reliable comparisons.

Other than our recent publication in beef feedlot cattle at harvest (Stromberg *et al*., 2015a), we are aware of only 4 other publications that reported EHEC-6 data on cattle hides and carcasses based on culture-based protocols. In those 4 studies, prevalence estimates were based on proportion of samples positive instead of model-adjusted estimates. Two studies conducted at a beef export abattoir in Ireland, neither indicating the source of the cattle (i.e., beef or dairy) and both sampling 100 cm^2 of hides and carcasses, detected very low EHEC-6 prevalence. One of the studies, which did not include IMS in their protocol, detected only EHEC O26, and that in only 2 of 450 (0.4%) of carcasses and 0 of 450 hides (0.0%) (Monaghan *et al*., 2012). The other study from Ireland used IMS for O26, O103, O111, and O145 and of 402 hides and carcasses cultured, detected 1 of 402 hides positive for O26 (0.2%) and no other EHEC-6 serogroup on hides or carcasses (Thomas *et al*., 2012). One study conducted on cattle at

an abattoir in Poland, not specifying whether of beef or dairy origin, sampling 400 cm^2 of hide and carcass surface area and not including non-O157 IMS, did not isolate any EHEC-6 from any sample (Wieczorek *et al*., 2011). However, Arthur *et al.* (2002), who cultured samples from 4,000 cm^2 of carcass surface from beef feedlot cattle in the Midwest U.S. and used a protocol that involved enrichment followed by a colony blot screen for *stx* on agar plates, detected EHEC-6 in 4 of 334 carcass samples (1.2%). These isolates included EHEC of serogroups O103, O121 and O145. These results suggest that based on culture, EHEC-6 is relatively low in prevalence, but increased sampling area and use of a protocol that includes serogroup-specific IMS increases the prevalence estimates.

In addition to adequate sampling area and IMS, many other factors, such as variability in carbohydrate source for fermentation and resistance to antimicrobials affect the detection of non-O157 STEC or EHEC. Based on the variability of these organisms, the use of multiple agar media as conducted in our study are recommended for their isolation (Hussein *et al*., 2008; Gill *et al*., 2014). In the present study, EHEC O157 was the most common serogroup isolated by culture either because it is truly the most prevalent or it is more easily detected because of the greater specificity of media. Approximately half of the samples that tested positive for EHEC O157:H7 by NS were confirmed by culture, and significant agreement ($P < 0.001$) was observed between NS and culture for detection of EHEC O157:H7. However, most samples that tested positive for non-O157 EHEC by NS or Atlas did not test positive by culture. This finding is consistent with our previous observations (Stromberg *et al*., 2015a) in that it appears there is a need to improve agar media for detection of non-O157 EHEC. It is also

possible that the molecular screening methods result in detection of false positives in some samples, in which case improved specificity is needed. Discrepancies between molecular- and culture-based methods for detection of EHEC-7 have been previously reported. Wang *et al*. (2014a) reported that 93.9% of veal calf hide samples were positive for EHEC-7 by NS, but by culture only 53.0% were positive. Wasilenko *et al*. (2012) used another molecular screen (BAX) and culture to test retail ground beef for EHEC-7. By BAX, 14 of 308 samples were positive for *stx*, *eae* and at least one EHEC-7 serogroup, but culture could confirm only 4 of these (Wasilenko *et al*. 2012). Since the NS test sample consists of a DNA extract from an aliquot of an enrichment broth culture, it is able to concurrently test thousands if not millions of bacterial cells, in contrast to our culture-based method which would have tested approximately 50 bacterial colonies from the same sample (Fig. 1). This would provide one explanation why the NS test might be more sensitive than culture. An advantage of culture, however, is the recovery of isolates, which may include other EHEC serogroups that may prove useful in subsequent studies. For example, in the present study, EHEC O84, O98, O109 and O177 were isolated by culture. These EHEC serogroups have previously been isolated from cattle (Renter *et al*., 2005; Mekata *et al*., 2014), and caused disease in humans (Bettelheim, 2007).

Only two samples had EHEC-7 concentrations that were quantifiable by the methods used. In both samples, the organisms were EHEC O157 and in concentrations nearly 10,000 CFU per unit or greater. One was a fecal sample containing 7,900 CFU/g and one was a hide sample 100,000 CFU/100 cm^2. EHEC-7 were not detected in most pre-enrichment samples suggesting they were in low concentrations in most samples, indicating the importance of enrichment for their detection. Current methods to quantify

EHEC have relied upon real-time PCR assays for *eae* or *stx* and may overestimate the concentration of EHEC due the contribution of these genes from multiple different organisms (Conrad *et al*., 2014). Recently, *E. coli* attaching and effacing gene-positive conserved fragment 1 (*ecf1*) has been used as a gene target for detection of STEC (Livezey *et al*., 2015), and *ecf1* and *eae* have been used as targets to enumerate EHEC directly in cattle feces (Luedtke *et al*., 2014). Although this assay allows for enumeration of EHEC, it quantifies the total EHEC load and not specifically EHEC-7.

Previously, for most EHEC-7 serogroups, no significant agreement was observed between a culture-based method and NS (Stromberg *et al*., 2015a). In this study, significant agreement was observed with 3 of 7 EHEC-7 serogroups between the culture-based method and NS; however, there was no significant agreement beyond that due to chance between culture and Atlas or NS and Atlas. Atlas detects all EHEC compared to NS which only detects EHEC-7 and the culture-based method used in this study targeted EHEC-7 by using IMS beads directed against those 7 serogroups. Therefore, Atlas results only for EHEC O157 could be compared to NS and culture.

Hides can be contaminated by multiple sources, and with feces from one animal or multiple animals in lairage (Arthur *et al*., 2007). Significant association between the detection of an EHEC in fecal samples with the detection of an EHEC on hide samples was determined for EHEC O26, O111, O121, and EHEC-6 and supports the hypothesis that feces are a major source of hide contamination. Effective pre-harvest interventions that reduce carriage of EHEC in the intestines e.g., vaccines (Besser *et al*., 2014; Smith, 2014a; Smith, 2014b) may reduce prevalence on hides.

In summary, these data were collected to determine the prevalence of EHEC-7 in culled dairy cattle using multiple detection methods. Molecular screening assays detected more EHEC than the culture-based method. Although there was significant agreement for some EHEC-7 serogroups between the NS and culture-based method, continued improvement is needed for accurate detection and isolation of EHEC-7 in the matrices tested. Significant associations between EHEC detection in fecal samples with EHEC detection on hide samples supports the need to identify effective pre-harvest interventions to reduce EHEC contaminated hides. Prevalence and concentration estimates will be further used to populate a quantitative microbial risk assessment model.

Acknowledgements

The authors thank Isha Patel for conducting the FDA-ECID, the owners and management of the participating processing plant and Paul Rossitto, Dr. Deniece Williams, Jennifer Crook, Arianna Villarreal, Hannah Frolli, Greg Smith, and Lawnie Leudtke for technical assistance. This material is based upon work that is supported by the National Institute of Food and Agriculture, U.S. Department of Agriculture, under award number 2012-68003-30155. No competing financial interests exist. Trade names are necessary to accurately report experimental results. The use of trade names by a USDA author implies no approval of the product to the exclusion of others that may also be suitable, further the USDA neither guarantees nor warrants the standard of any products mentioned.

Table 1. List of primers for screening enterohemorrhagic *E. coli*

Gene	Primer	Sequence (5'→3')	Conc. (nM)	Amplicon Size (bp)	Reference
nleF	nleF F	ATGTTACCAACAAGTGGTTCTTC	250	567	Coombes *et al*., 2008
	nleF R	ATCCACATTGTAAAGATCCTTTGTT	250		
subA	SubHCDF	TATGGCTTCCCTCATTGCC	300	556	Paton and Paton, 2005
	SubSCDR	TATAGCTGTTGCTTCTGACG	300		
eae	eaeAF	GACCCGGCACAAGCATAAGC	150	384	Paton and Paton, 1998
	eaeAR	CCACCTGCAGCAACAAGAGG	150		
nleB	nleB F	GGAAGTTTGTTTACAGAGACG	500	297	Coombes *et al*., 2008
	nleB R	AAAATGCCGCTTGATACC	500		
espK1	EspK_F1	ATCAAAAGCGAAATCACACC	500	200	This report
	EspK_R1	TGTAATTTTTCACAGTTAATGACG	500		
stx[a]	Stx1/2-F	TTTGTYACTGTSACAGCWGAAGCYTTACG	1,000	132	Wasilenko *et al*., 2012
	Stx1/2-R	CCCCAGTTCARWGTRAGRTCMACDTC	1,000		

[a]Degenerate nucleotide codes: Y (C,T), W (A,T), R (A,G), M (A,C), D (A,G,T), S (C,G).

Table 2. Number of positive samples and model-adjusted prevalence estimates of enterohemorrhagic *E.coli* (EHEC) in fecal samples from culled dairy cows as detected by culture isolation, NeoSEEK STEC Detection and Identification test (NS), and the Atlas STEC EG2 Combo Detection Assay (Atlas)

	n = 100								
	Culture			NS			Atlas		
Serogroup or virulence gene	No. of positive samples	% Mean prevalence	Prevalence 95% CI	No. of positive samples	% Mean prevalence	Prevalence 95% CI	No. of positive samples	% Mean prevalence	Prevalence 95% CI
EHEC O26	1	1.01	0.09-9.90	1	1.01	0.09-9.90	NA[a]	NA	NA
EHEC O45	0	0.00	0.00-0.00	11	11.00	6.20-18.78	NA	NA	NA
EHEC O103	0	0.00	0.00-0.00	7	7.00	3.37-13.96	NA	NA	NA
EHEC O111	1	1.00	0.12-7.94	7	4.90	1.20-17.96	NA	NA	NA
EHEC O121	0	0.00	0.00-0.00	0	0.00	0.00-0.00	NA	NA	NA
EHEC O145	0	0.00	0.00-0.00	7	6.74	2.47-17.05	NA	NA	NA
EHEC O157	6	6.13	2.22-15.84	4	4.00	1.51-10.18	29	29.10	20.23-39.92
Non-O157 EHEC	NA	NA	NA	NA	NA	NA	29	29.11	18.27-43.00
EHEC-6	2	2.10	0.45-9.26	24	23.59	13.86-37.21	NA	NA	NA
EHEC-7	7	6.54	2.40-16.63	26	25.90	16.08-38.94	NA	NA	NA

stx	20	20.42	9.50-38.54	90	90.00	82.40-94.53	NA	NA	NA
eae	31	31.40	22.06-42.53	83	83.00	74.34-89.16	NA	NA	NA

[a]NA: not applicable.

Model-adjusted prevalence estimates for all EHEC serogroup and virulence genes for each diagnostic protocol were obtained from mixed-effects models that incorporated a random intercept for sampling date.

Table 3. Number of positive samples and model-adjusted prevalence estimates of enterohemorrhagic *E. coli* (EHEC) on hide samples from culled dairy cows as detected by culture isolation, NeoSEEK STEC Detection and Identification test (NS), and the Atlas STEC EG2 Combo Detection Assay (Atlas)

	n = 100								
	Culture			NS			Atlas		
Serogroup or virulence gene	No. of positive samples	% Mean prevalence	Prevalence 95% CI	No. of positive samples	% Mean prevalence	Prevalence 95% CI	No. of positive samples	% Mean prevalence	Prevalence 95% CI
EHEC O26	5	3.19	0.50-17.59	7	1.53	0.06-28.70	NA[a]	NA	NA
EHEC O45	0	0.00	0.00-0.00	36	27.10	10.77-53.36	NA	NA	NA
EHEC O103	0	0.00	0.00-0.00	10	10.00	5.47-17.60	NA	NA	NA
EHEC O111	1	1.00	0.14-6.75	15	12.03	4.76-27.26	NA	NA	NA
EHEC O121	1	1.01	0.09-9.90	3	1.71	0.14-17.99	NA	NA	NA
EHEC O145	0	0.00	0.00-0.00	23	18.59	7.71-38.45	NA	NA	NA
EHEC O157	9	6.65	1.85-21.22	7	6.04	2.05-16.51	51	57.03	32.43-78.59
Non-O157 EHEC	NA	NA	NA	NA	NA	NA	46	38.29	17.27-64.83
EHEC-6	7	6.68	2.39-17.31	62	61.37	46.84-74.13	NA	NA	NA
EHEC-7	16	15.56	8.35-27.16	65	64.94	49.44-77.83	NA	NA	NA

stx	24	24.11	16.08-34.52	98	98.00	92.36-99.50	NA	NA	NA
eae	38	39.34	21.39-60.72	98	98.00	92.36-99.50	NA	NA	NA

[a]NA: not applicable.

Model-adjusted prevalence estimates for all EHEC serogroup and virulence genes for each diagnostic protocol were obtained from mixed-effects models that incorporated a random intercept for sampling date.

Table 4. Number of positive samples and model-adjusted prevalence estimates of enterohemorrhagic *E. coli* (EHEC) on pre-intervention carcass surface samples from culled dairy cows as detected by culture isolation, NeoSEEK STEC Detection and Identification test (NS), and the Atlas STEC EG2 Combo Detection Assay (Atlas)

	n = 100								
	Culture			NS			Atlas		
Serogroup or virulence gene	No. of positive samples	% Mean prevalence	Prevalence 95% CI	No. of positive samples	% Mean prevalence	Prevalence 95% CI	No. of positive samples	% Mean prevalence	Prevalence 95% CI
EHEC O26	0	0.00	0.00-0.00	3	1.39	0.09-17.35	NA[a]	NA	NA
EHEC O45	0	0.00	0.00-0.00	0	0.00	0.00-0.00	NA	NA	NA
EHEC O103	0	0.00	0.00-0.00	4	4.00	1.51-10.18	NA	NA	NA
EHEC O111	0	0.00	0.00-0.00	0	0.00	0.00	NA	NA	NA
EHEC O121	0	0.00	0.00-0.00	0	0.00	0.00	NA	NA	NA
EHEC O145	0	0.00	0.00-0.00	2	2.00	0.50-7.64	NA	NA	NA
EHEC O157	1	1.00	0.12-7.93	1	1.00	0.14-6.75	3	3.00	0.97-8.89
Non-O157 EHEC	NA	NA	NA	NA	NA	NA	28	28.01	19.48-38.50
EHEC-6	0	0.00	0.00-0.00	7	7.00	3.37-13.96	NA	NA	NA
EHEC-7	1	1.00	0.12-7.94	7	7.00	3.37-13.96	NA	NA	NA

stx	3	2.80	0.61-11.90	45	41.61	24.78-60.65	NA	NA	NA
eae	12	12.00	6.94-19.95	50	50.06	32.25-67.86	NA	NA	NA

[a]NA: not applicable.

Model-adjusted prevalence estimates for all EHEC serogroup and virulence genes for each diagnostic protocol were obtained from mixed-effects models that incorporated a random intercept for sampling date.

Table 5. Distribution of virulence genes in Enterohemorrhagic *E. coli* isolates recovered from post-enrichment cultures

Serotype	Virulence genes	No. of detected MRA[a] genes	*pagC*	*sen*	*efa1*	No. of isolates			
						Feces	Hide	Carcass	Total
O26:[H11]	*stx*$_1$, *eae*-β1, *ehxA*	13	-	+	+	1	4	0	5
O26:[H11]	*stx*$_1$, *eae*-β1, *ehxA*	6	-	+	+	0	1	0	1
O84:[H2]	*stx*$_1$, *eae*-ζ, *ehxA*	6	+	+	-	0	0	1	1
O98:NM	*stx*$_1$, *eae*-ζ, *ehxA*	8	+	+	-	6	0	1	7
O109:[H10]	*stx*$_2$, *eae*-ι, *ehxA*	6	-	-	-	1	0	0	1
O111:[H8]	*stx*$_1$, *eae*-γ2, *ehxA*	9	+	+	+	1	1	0	2
O121:[H19]	*stx*$_2$, *eae*-ε, *ehxA*	7	+	+	+	0	1	0	1
O157:[H7]	*stx*$_2$, *eae*-γ1, *ehxA*	16	+	+	+	5	2	0	7
O157:[H7]	*stx*$_1$, *stx*$_2$, *eae*-γ1, *ehxA*	16	+	+	+	5	23	5	33
O177:[H25]	*stx*$_2$, *eae*-β1, *ehxA*	12	-	+	-	3	8	0	11

[a]MRA: molecular risk assessment.

Table 6. Cohen's kappa coefficient and McNemar's χ^2 analysis for agreement between a culture-based method and NeoSEEK STEC Detection and Identification test, independent of sample type

Serogroup	Kappa	*P*-value	McNemar's χ^2 *P*-value
EHEC O26	0.58	<0.0001	0.059
EHEC O45	NA[a]	NA	NA
EHEC O103	NA	NA	NA
EHEC O111	0.16	<0.0001	<0.0001
EHEC O121	0.50	<0.0001	0.157
EHEC O145	NA	NA	NA
EHEC O157	0.40	<0.0001	0.317

[a]NA: not applicable (at least one of the tests did not detect any positives).

Table 7. P-values of associations between the detection of an enterohemorrhagic *E. coli* (EHEC) serogroup or virulence gene in fecal samples with the presence of an EHEC serogroup or virulence gene on hide samples using generalized linear mixed models

	EHEC serogroup or virulence gene on hide samples		
EHEC serogroup or virulence gene in fecal samples	Detection method		
	Culture *P*-value	NS[a] *P*-value	Atlas[b] *P*-value
EHEC O26	0.989	<0.001	NA[c]
EHEC O45	NA	0.973	NA
EHEC O103	NA	0.764	NA
EHEC O111	0.989	0.002	NA
EHEC O121	<0.001	<0.001	NA
EHEC O145	NA	0.139	NA
EHEC O157	0.418	0.323	0.262
Non-O157 EHEC	NA	NA	0.519
EHEC-6	0.985	0.029	NA
EHEC-7	0.952	0.251	NA
stx	0.625	0.998	NA
eae	0.373	0.242	NA

[a]NS: NeoSEEK Detection and Identification test.

[b]Atlas: Atlas STEC EG2 Combo Detection Assay.

[c]NA: not applicable.

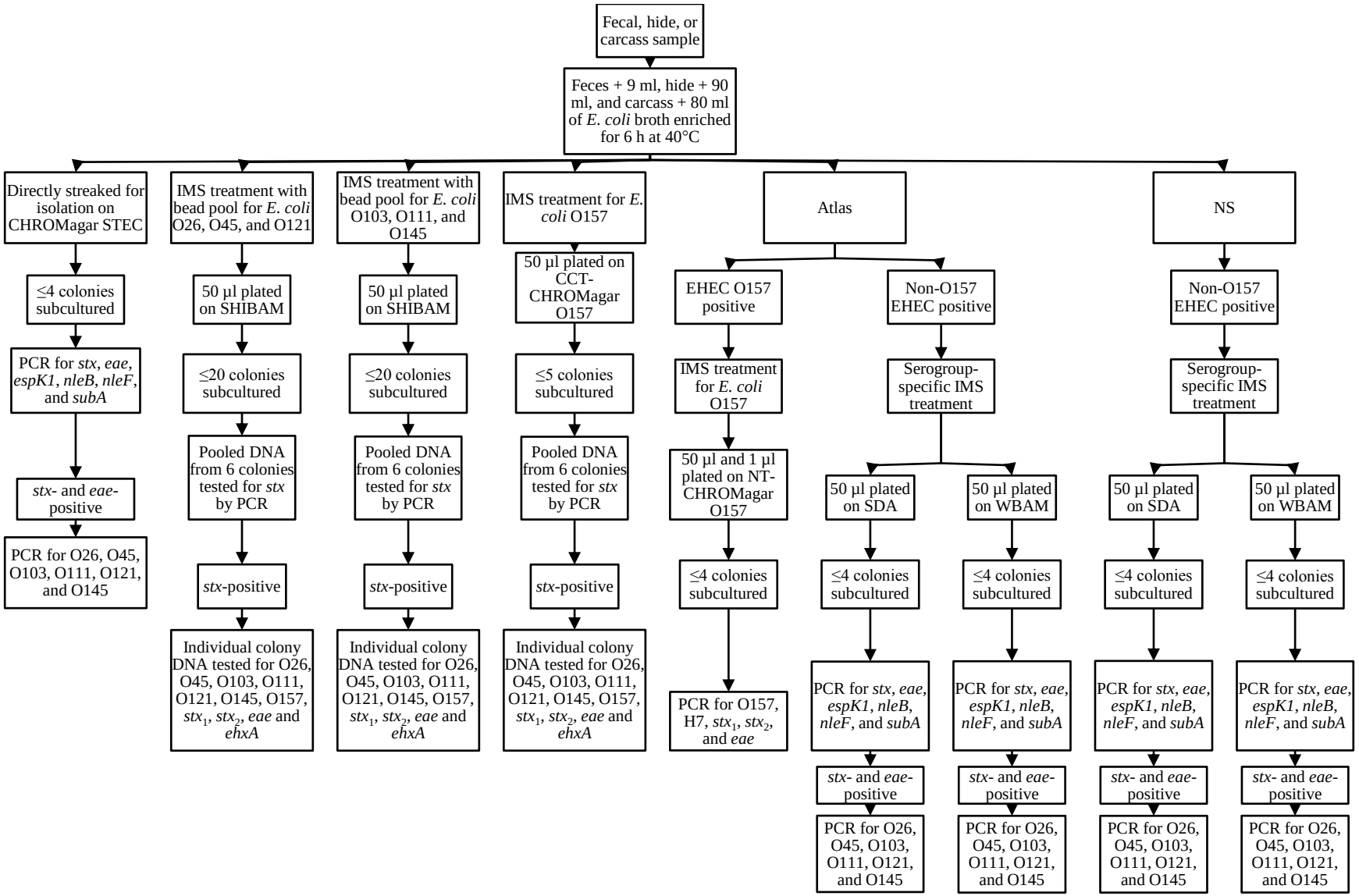
Fecal, hide, or carcass sample
Feces + 9 ml, hide + 90 ml, and carcass + 80 ml of *E. coli* broth enriched for 6 h at 40°C
Directly streaked for isolation on CHROMagar STEC
≤4 colonies subcultured
PCR for *stx*, *eae*, *espK1*, *nleB*, *nleF*, and *subA*
stx- and *eae*-positive
PCR for O26, O45, O103, O111, O121, and O145
IMS treatment with bead pool for *E. coli* O26, O45, and O121
50 µl plated on SHIBAM
≤20 colonies subcultured
Pooled DNA from 6 colonies tested for *stx* by PCR
stx-positive
Individual colony DNA tested for O26, O45, O103, O111, O121, O145, O157, stx_1, stx_2, *eae* and *ehxA*
IMS treatment with bead pool for *E. coli* O103, O111, and O145
50 µl plated on SHIBAM
≤20 colonies subcultured
Pooled DNA from 6 colonies tested for *stx* by PCR
stx-positive
Individual colony DNA tested for O26, O45, O103, O111, O121, O145, O157, stx_1, stx_2, *eae* and *ehxA*
IMS treatment for *E. coli* O157
50 µl plated on CCT-CHROMagar O157
≤5 colonies subcultured
Pooled DNA from 6 colonies tested for *stx* by PCR
stx-positive
Individual colony DNA tested for O26, O45, O103, O111, O121, O145, O157, stx_1, stx_2, *eae* and *ehxA*
Atlas
EHEC O157 positive
IMS treatment for *E. coli* O157
50 µl and 1 µl plated on NT-CHROMagar O157
≤4 colonies subcultured
PCR for O157, H7, stx_1, stx_2, and *eae*
Non-O157 EHEC positive
Serogroup-specific IMS treatment
50 µl plated on SDA
≤4 colonies subcultured
PCR for *stx*, *eae*, *espK1*, *nleB*, *nleF*, and *subA*
stx- and *eae*-positive
PCR for O26, O45, O103, O111, O121, and O145
50 µl plated on WBAM
≤4 colonies subcultured
PCR for *stx*, *eae*, *espK1*, *nleB*, *nleF*, and *subA*
stx- and *eae*-positive
PCR for O26, O45, O103, O111, O121, and O145
NS
Non-O157 EHEC positive
Serogroup-specific IMS treatment
50 µl plated on SDA
≤4 colonies subcultured
PCR for *stx*, *eae*, *espK1*, *nleB*, *nleF*, and *subA*
stx- and *eae*-positive
PCR for O26, O45, O103, O111, O121, and O145
50 µl plated on WBAM
≤4 colonies subcultured
PCR for *stx*, *eae*, *espK1*, *nleB*, *nleF*, and *subA*
stx- and *eae*-positive
PCR for O26, O45, O103, O111, O121, and O145

Figure 1. Flowchart of culture- and molecular-based methods performed for detection of enterohemorrhagic *E. coli*. Atlas, Atlas STEC EG2 Combo Detection Assay; CCT-CHROMagar O157, CHROMagar O157 containing cefixime, cefsulodin, and potassium tellurite; IMS, immunomagnetic separation; NS, NeoSEEK STEC Detection and Identification; NT-CHROMagar O157, CHROMagar O157 containing novobiocin and potassium tellurite; SHIBAM, STEC heart infusion washed blood agar with mitomycin C; SDA, STEC differentiation agar; WBAM, washed blood agar with mitomycin C.

CHAPTER 5

COMPARISON OF AGAR MEDIA FOR THE DETECTION AND QUANTIFICATION OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* O26, O45, O103, O111, O121, O145, AND O157 IN CATTLE FECES

(Submitted to Journal of Food Protection)

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Abstract

The isolation and quantification of Shiga toxin-producing *Escherichia coli* (STEC) from cattle fecal samples remains difficult. The objective of this study was to compare agar media for the detection and quantification of STEC in cattle feces. Comparison studies were performed using CHROMagar^{TM} STEC, Possé differential agar (Possé), Possé modified by the reduction or addition of antimicrobials, STEC heart infusion washed blood agar with mitomycin C (SHIBAM), and SHIBAM modified by the addition of antimicrobials. Fourteen STEC strains, two each belonging to serogroups O26, O45, O103, O111, O121, O145, and O157, were used to test their detection in inoculated fecal samples at concentrations of 10^2 or 10^3 colony-forming units (CFU)/g. One STEC strain from each of these six non-O157 serogroups was used to estimate the concentration of recovered STEC in feces inoculated at 10^3, 10^4, or 10^5 CFU/g. Significantly ($P < 0.05$) more samples were positive for STEC when plated on Possé containing reduced concentrations of novobiocin and potassium tellurite compared to SHIBAM, but not SHIBAM modified by containing these same antimicrobials at the same concentrations. Numerically, more samples were positive for STEC using this same form of modified Possé compared to Possé, but this difference was not statistically significant. More samples were positive for STEC cultured on CHROMagar STEC compared to Possé, ($P < 0.05$) and modified Possé ($P = 0.05$). Most inoculated fecal samples below 10^4 CFU/g of feces were underestimated for the concentration of STEC, or were not quantifiable using CHROMagar STEC or modified Possé. These results suggest that CHROMagar STEC performs better than Possé or SHIBAM for detection of

STEC in bovine feces, but adjustments in the concentrations of novobiocin and potassium tellurite in the latter two media result in significant improvements in their performance.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are enteric pathogens of humans that cause hemorrhagic colitis and hemolytic-uremic syndrome (Thorpe, 2004). STEC are naturally found in the intestinal tract of ruminants and these organisms are shed in ruminant feces (Karmali *et al*., 2010). STEC are commonly transmitted to humans through the ingestion of contaminated food (Erickson and Doyle 2007), particularly beef products (Duffy *et al*., 2014). Thus, the U.S. Department of Agriculture Food Safety and Inspection Service declared seven serogroups of intimin-positive STEC that account for the majority of disease due to STEC in the U.S. as adulterants in raw, non-intact beef (USDA 2012).

Detection of STEC is critical for food safety, clinical diagnosis of STEC infection, and determination of environmental prevalence. Epidemiological studies (Wells *et al*., 1991) and clinical laboratories (March and Ratnam 1986; Krishnan *et al*., 1987; Simor *et al*., 1990) have used sorbitol-MacConkey agar to detect sorbitol-negative *E. coli* O157:H7, which lack the ability to ferment sorbitol within 24 h. The addition of antimicrobials such as cefixime and potassium tellurite to agar media has further improved detection of *E. coli* O157:H7 (Sanderson *et al*., 1995). However, most non-O157 STEC ferment sorbitol and have varying levels of resistance to these antimicrobials (Hussein *et al*., 2008). Therefore, sorbitol-MacConkey agar is not a suitable agar media to detect non-O157 STEC, and would result in undetected non-O157 STEC (Klein *et al*., 2002).

Non-O157 STEC are estimated to account for approximately 64% of foodborne illness caused by STEC in the U.S. (Scallan *et al*., 2011), and their detection in clinical

patients has increased in recent years (Gould *et al*., 2013). STEC O26, O45, O103, O111, O121, and O145 (STEC-6) caused 71% of the cases of non-O157 STEC infection in the US from 1983 to 2002 (Brooks *et al*., 2005). However, detection of non-O157 STEC remains challenging due to their diversity (Gill *et al*., 2014). Agar media designed to detect non-O157 STEC have relied on fermentation of specific carbohydrates, β-galactosidase activity, and resistance to antimicrobials (Possé *et al*., 2008; Kalchayanand *et al*., 2013). Organism isolation remains important for confirmational assays such as hyperspectral imaging (Windham *et al*., 2013), latex agglutination (Medina *et al*., 2012), PCR (Bai *et al*., 2012), and other nucleic acid detection methods (Blais *et al*., 2012).

Concentration estimates of STEC are important for assessing the risk of STEC in the food chain (Ekong *et al*., 2015). Concentration has been determined by use of spiral plate methods for STEC O157:H7 (Robinson *et al*., 2004; Berry and Wells 2008) or real-time PCR assays that quantify total STEC load or specific serogroups of STEC (Conrad *et al*., 2014; Luedtke *et al*., 2014; Ahmed *et al.*, 2015). Real-time assays with multiple targets may detect genes contributed by multiple organisms. Since there is no single target for STEC-7, organism isolation by spiral plating could be used to determine if the O-group, Shiga toxin (*stx*) and intimin (*eae*) genes are from one organism.

The purpose of this study was to assess the performance of agar media for the detection of STEC-6 and STEC O157:H7 (STEC-7) and quantification of STEC-6 in cattle feces. This study compared CHROMagar^{TM} STEC, Possé differential agar (Possé; Possé *et al*., 2008), modifications of Possé, STEC heart infusion washed blood agar with mitomycin C (SHIBAM), and a modification of SHIBAM for the detection of STEC-7 in inoculated cattle fecal samples. Additionally, CHROMagar STEC and a modification of

Possé were used to establish a quantification method for STEC-6 in inoculated cattle fecal samples by spiral plating.

Materials and Methods

Bacterial strains and preparation of inoculum. All strains used in this study were STEC, and were obtained either from Dr. Shannon Manning (Michigan State University), Dr. David Renter (Kansas State University), Dr. John Luchansky (USDA, Agricultural Research Service, Eastern Regional Research Center), or our laboratory collection. Frozen stock cultures (-80°C) of strains were streaked onto Luria-Bertani broth, Miller (LB; BD, Sparks, MD) agar plates without antibiotics or with 100 mg/liter rifampicin (Sigma) when appropriate and incubated at 37°C for 24 h. A single colony was inoculated in LB with corresponding antibiotics for 24 h at 37°C in a stationary culture. Serial ten-fold dilutions of the inoculum were prepared in buffered peptone water (BPW), and used to inoculate fecal samples at a concentration of 10^2 or 10^3 colony-forming units (CFU)/g of feces for detection studies and 10^3, 10^4, or 10^5 CFU/g of feces for quantification studies. BPW was used as a mock inoculation.

Sample collection. Fresh feces were collected from steers housed one per pen at the University of Nebraska-Lincoln, and processed as previously described (Stromberg *et al*., 2015a). Briefly, one gram of cattle feces was suspended in 9 ml of *E. coli* broth (EC; Oxoid Ltd., Hampshire, UK) and vortexed for 1 min. After inoculation, samples were vortexed for 20 s and enriched for 6 h at 40°C in a static culture.

Agar media. Agar media was prepared according to the manufacturers' instructions or as published with minor modifications. CHROMagar STEC (DRG Int.,

Springfield, NJ) was prepared according to the manufacturer's instructions and CHROMagar STEC with rifampicin was prepared by adding rifampicin at 100 mg/liter and without adding the CHROMagar STEC supplement. Possé differential medium was prepared according to the published instructions (Possé *et al*., 2008). The first modified form of Possé differential medium was made by reducing the concentrations of novobiocin (5.0 mg/liter; Sigma) and potassium tellurite (0.15 mg/liter; Sigma) and by adding cefixime trihydrate (Sigma) at 0.05 mg/liter (mPossé1). A second modified form of Possé as described by Stromberg *et al*. (2015b) was made by reducing novobiocin (5.0 mg/liter) and potassium tellurite (0.5 mg/liter; mPossé2). Modified Possé with rifampicin was prepared by adding rifampicin to Possé differential agar at 100 mg/liter, with reduced bile salts (1.5 g/liter), and containing no novobiocin or potassium tellurite. SHIBAM was prepared according to the FDA Bacteriological Analytical Manual (Feng *et al*., 2011) and modified by adding novobiocin at 5.0 mg/liter and potassium tellurite at 0.5 mg/liter (mSHIBAM).

Detection of STEC-7 on mPossé2 and SHIBAM. Two independent experiments were performed to compare mPossé2 and SHIBAM for the detection of STEC. After fecal samples were inoculated and enriched in EC for 6 h, 490-µl aliquots were diluted in 490 µl of PBS with 0.05% Tween-20 and subjected to three separate immunomagnetic separation (IMS) treatments using a KingFisher™ Flex Magnetic Particle Processor (Thermo Scientific, Waltham, MA). The first treatment consisted of 20 µl of anti-*E. coli* O157 IMS Dynabeads® (Invitrogen, Carlsbad, CA), the second treatment used a 20-µl pool of O103, O111 and O145 IMS beads (Abraxis LLC, Warminster, PA), and the third treatment used a 20-µl pool of O26, O45 and O121 IMS beads (Abraxis). After IMS, the

beads were dropped into 1.0 ml of BPW. The bead suspension was serial diluted in BPW and 50 µl of each bead suspension diluted 100-fold was spread plated onto mPossé2 and SHIBAM and incubated at 37°C for 18 h. On mPossé2, ≤20 red/blue-purple and green colonies were picked and ≤20 enterohemolytic colonies were picked from SHIBAM. Colonies were suspended in 50 µl of water and heated for 10 min at 95°C for use as DNA template in PCR reactions. Five colonies were pooled and tested by PCR for *stx* according to Monday *et al*. (2007). Individual colonies from PCR positive pools were tested by 11-plex PCR for genes representing EHEC-7 serogroups, *stx*$_1$, *stx*$_2$, EHEC-hemolysin (*ehxA*) (Bai *et al*., 2012) modified with primers for *eae* (Blanco *et al*., 2004) and primers for O111 as described by Noll *et al*. (2015) to confirm the inoculum.

Detection of STEC-7 on mPossé2 and mSHIBAM. Three independent experiments were performed to compare mPossé2 and mSHIBAM for the detection of STEC. Fecal samples were processed and colonies were tested as described for the comparison between mPossé2 and SHIBAM with a minor modification. After IMS, the bead suspension was dropped into 1.0 ml of BPW and serial diluted in BPW. Fifty µl of non-diluted or 10-fold diluted bead suspension was spread plated onto mPossé2 and 50 µl of 100-fold bead suspension was spread plated onto mSHIBAM and incubated at 37°C for 18 h. On mPossé2, ≤20 red/blue-purple and green colonies were picked and ≤20 enterohemolytic colonies were picked from mSHIBAM. Colonies were tested by 11-plex PCR as described above (Blanco *et al*., 2004; Bai *et al*., 2012; Noll *et al*., 2015).

Detection of STEC-7 on CHROMagar STEC, Possé, mPossé1, and mPossé2. Replicate experiments were performed to compare CHROMagar STEC, Possé, mPossé1 and mPossé2 for the detection of STEC. After enrichment, separate aliquots were

subjected IMS treatments with 20 µl of each IMS bead type (O26, O45, O103, O111, O121, O145, and O157). After IMS, the beads were dropped into 1.0 ml of BPW and diluted 10-fold and 100-fold in BPW. Fifty µl of each bead suspension was spread plated onto each agar and incubated at 37°C for 18 h. Colonies were picked from plates with 25 to 250 colonies. On Possé, mPossé1, and mPossé2 plates inoculated with O26, O45, O103, O111, or O157 IMS beads, ≤six red/blue-purple colonies were picked and for O121 or O145 IMS beads, ≤six red/blue-purple and green colonies were picked. On CHROMagar STEC, ≤six mauve colonies were picked per plate. Colonies were suspended in 50 µl of water and heated for 10 min at 95°C for use as DNA template in PCR reactions. Individual colonies were tested by 11-plex PCR as described above (Blanco *et al*., 2004; Bai *et al*., 2012; Noll *et al*., 2015).

Quantification of STEC-6 strains in inoculated cattle fecal samples.

Rifampicin-resistant strains were used to estimate the concentration of STEC recovered on CHROMagar STEC and mPossé2 from inoculated fecal samples from one animal. Samples were inoculated at a concentration of approximately 10^3, 10^4, or 10^5 CFU/g of feces in nine ml of EC broth, and 50 µl was spiral plated onto CHROMagar STEC, CHROMagar STEC with rifampicin, mPossé2 and mPossé with rifampicin using an Eddy Jet 2 spiral plater (Neu-tec, Farmingdale, NY). Red/blue-purple and green colonies were counted on mPossé2 and mPossé with rifampicin plates and mauve colonies were counted on CHROMagar STEC and CHROMagar STEC with rifampicin. A total of ≤10 colonies per plate of the target phenotype were picked and each colony was suspended in 50 µl of water each. The suspended colonies were heated for 10 min at 95°C and tested by 11-plex PCR (Blanco *et al*., 2004; Bai *et al*., 2012; Noll *et al*., 2015). Concentration

was determined by the proportion of colonies that tested positive for O-group, *stx*, and *eae* that matched that of the inoculum.

Statistical analysis. Detection of STEC on different agar media was compared using a binary distribution in SAS v 9.3 (PROC GLIMMIX, SAS Institute INC., Cary, NC). Statistically significant results were represented by *P*-values <0.05.

Results

Agar comparison studies using inoculated cattle fecal samples. Two independent experiments were performed for the comparison between mPossé2 and SHIBAM for the recovery of STEC in inoculated fecal samples. Of the strains tested, six of 14 were detected on both agars, seven of 14 were detected only on mPossé2, and one of 14 was not detected. STEC strains were detected in 11 of 28 on mPossé2 and two of 28 samples on SHIBAM when inoculated with 10^2 CFU/g, and 17 of 28 on mPossé2 and four of 28 samples on SHIBAM when inoculated with 10^3 CFU/g. In total, STEC strains were detected in 28 of 56 when plated on mPossé2 compared to six of 56 samples when plated on SHIBAM (TABLE 1). mPossé2 detected significantly ($P < 0.05$) more STEC positive samples compared to SHIBAM.

In three independent experiments, mPossé2 and mSHIBAM were compared for the recovery of STEC in inoculated fecal samples. Of the strains tested, four of 14 were recovered on mPossé2 alone, and 10 of 14 were not recovered on either agar. STEC strains were detected in two of 42 on mPossé2 and zero of 42 samples on mSHIBAM when inoculated with 10^2 CFU/g, and four of 42 on mPossé2 and zero of 42 samples on SHIBAM when inoculated with 10^3 CFU/g. In total, STEC strains were detected in six of

84 samples plated on mPossé2, however, no STEC were recovered when plated on mSHIBAM (TABLE 2). mPossé2 detected fewer STEC positive samples than in the previous comparison, and did not detect significantly more STEC compared to mSHIBAM.

In two independent experiments, CHROMagar STEC, Possé, mPossé1, and mPossé2 were compared for the recovery of STEC in inoculated cattle feces. No strains were detected on all media, three of 14 were recovered on CHROMagar STEC only, three of 14 were recovered on all except mPossé1, two of 14 were recovered on CHROMagar STEC and mPossé2 only, one of 14 were recovered on CHROMagar STEC and Possé only, one of 14 were recovered on all except Possé, and four of 14 were not recovered. STEC strains were detected in nine of 28 on CHROMagar STEC, four of 28 on Possé, one of 28 on mPossé1, and four of 28 samples on mPossé2 when inoculated with 10^2 CFU/g. When inoculated with 10^3 CFU/g, STEC were detected in eight of 28 on CHROMagar STEC, two of 28 on Possé, zero of 28 on mPossé1, and five of 28 samples on mPossé2. In total, STEC were recovered from 17 of 56 plated on CHROMagar STEC, nine of 56 on mPossé2, six of 56 on Possé, and one of 56 samples on mPossé1 (TABLE 3). Significantly ($P < 0.05$) more STEC positive samples were detected by plating on CHROMagar STEC compared to Possé and mPossé1, and approached significance ($P = 0.05$) compared to mPossé2. Possé was not significantly different compared to mPossé1 or mPossé2. However, mPossé2 detected significantly ($P <0.05$) more STEC positive samples compared to mPossé1.

Quantification of STEC using inoculated cattle fecal samples. Rifampicin resistant STEC strains were spiral plated on CHROMagar STEC and mPossé2 to

determine whether STEC-6 strains could be quantified on these media. CHROMagar STEC and mPossé2 were also supplemented with rifampicin to suppress background organisms and allow for optimal recovery of STEC. No strains were quantifiable in samples inoculated with STEC at 6.2 x 10^2 to 1.0 x 10^3 CFU/g. However, when inoculated at 6.2 x 10^3 to 1.0 x 10^4 CFU/g, two of six strains were quantifiable on CHROMagar STEC and CHROMagar STEC supplemented with rifampicin, one of six strains were quantifiable on mPossé2, and 0 of six strains were quantifiable on mPossé2 with rifampicin. When inoculated at 6.2 x 10^4 to 1 x 10^5 CFU/g, five of six strains were quantifiable on CHROMagar STEC with rifampicin and mPossé2 with rifampicin, four of six strains were quantifiable on CHROMagar STEC, and three of six strains were quantifiable on mPossé2 (TABLE 4).

Discussion

Successful detection of STEC in a complex background requires an agar media that suppresses the growth of background organisms with minimal suppression of the growth of STEC. In addition to reducing background organisms for detection of STEC, other factors such as cost, time to prepare the media, shelf life, and ease of interpreting phenotypes are important in agar selection (Kase *et al*., 2015). In general, commercial agars are more expensive but quicker to prepare compared to published agars such as Possé and SHIBAM. STEC are screened based on one phenotype on CHROMagar STEC and SHIBAM. STEC on CHROMagar STEC can vary in their shade of mauve from purple/pink to brown. For SHIBAM, it can be difficult to determine the enterohemolytic phenotype, and other *E. coli* may present as α-hemolytic, which can obscure the target

phenotype. On Possé and modifications of Possé, STEC are screened based on three phenotypes (blue-purple, red-purple, and green). It can be difficult to discriminate between blue-purple and red-purple on Possé and modifications of Possé in the fecal background.

The matrix is also important when evaluating an agar media. Cattle feces will likely have a higher concentration of non-pathogenic *E. coli* and other background organisms compared to foods. The FDA recommends using Levine's Eosin-Methylene Blue and SHIBAM for isolating non-O157 STEC (Feng *et al*., 2011). Lin *et al*. (2012) found that SHIBAM was useful for isolating non-O157 STEC from romaine lettuce and tomatoes. Although SHIBAM may be suitable for food products with low numbers of background organisms, this study found that SHIBAM is not a suitable agar for detection of STEC in cattle feces. A significant difference was not found when mSHIBAM was compared with mPossé2 even though no STEC were detected on mSHIBAM. A high number of background organisms in these fecal samples may have reduced the ability to recover STEC.

Agar media designed to detect *E. coli* O157 use potassium tellurite as a means to select against non-pathogenic *E. coli* and other enteric organisms (Sanderson *et al*., 1995). Verhaegen *et al*. (2015) compared the isolation of a variety of non-O157 STEC including those of the O26, O45, O103, O111, and O145 serogroups on multiple agar media in pure culture. There was a correlation between growth of the STEC strain on Possé and CHROMagar STEC with the presence of the tellurite resistance gene *terB* (Verhaegen *et al*., 2015).The agars tested in this study had a range of potassium tellurite concentrations from 0.15 mg/liter to 2.50 mg/liter or an unknown concentration in

CHROMagar STEC. mPossé2, which had higher levels of potassium tellurite, detected significantly more STEC positive fecal samples compared to mPossé1. mPossé1 had concentrations of potassium tellurite, novobiocin, and cefixime as recommended by the Microbiology Laboratory Guidebook for detection and isolation of non-O157 STEC on modified Rainbow agar (USDA 2014). A higher level of potassium tellurite may increase the detection rate of STEC and suppress the growth of non-target organisms.

CHROMagar STEC has been evaluated on human stool samples (Gouali *et al*., 2013) and in pure cultures and beef (Gill *et al*., 2012). In stool samples, 82% of samples that were found to be Shiga toxin-positive resulted in recovery of a STEC on CHROMagar STEC (Gouali *et al*., 2013). Gill *et al*. (2012) found lower recovery concentrations on CHROMagar STEC in pure culture and in inoculated beef compared to brain heart infusion agar. These results suggest that some STEC may be inhibited on CHROMagar STEC. This study found that some STEC could not be recovered from enriched fecal samples on CHROMagar STEC or on other agars tested.

CHROMagar O157 has been used in spiral plate quantification of STEC O157 (Robinson *et al*., 2004; Berry and Wells, 2008), and mPossé2 has been used to quantify STEC in feces, hide and carcass samples (Stromberg *et al*., 2015c). To our knowledge this is the first report of the use of CHROMagar STEC for spiral plate quantification of STEC-6. For most samples, quantification by spiral plating underestimated the concentration of STEC or did not quantify STEC in the fecal sample. Even when rifampicin was supplemented in the agar media to reduce background organisms, STEC were infrequently enumerated at their true values. Most samples could be enumerated when concentrations were above 10^4 CFU/g. These results suggest that some strains can

be quantified by spiral plating on CHROMagar STEC or mPossé2, but their enumeration may be underestimated.

In conclusion, there is no single optimal agar media for detection of STEC-7 in cattle feces. Although, this study suggests that CHROMagar STEC and mPossé2 detect more STEC positive samples compared to other agar media tested, some STEC strains remained undetected when using these media. Additionally, this study established that CHROMagar STEC and mPossé2 could be used to quantify STEC-6 in cattle feces. Further studies are needed to determine the optimum antimicrobial concentrations in agar media for detection of STEC-7.

Acknowledgements

The authors thank Drs. John Luchansky, Shannon Manning and David Renter for sharing strains. This material is based upon work that is supported by the National Institute of Food and Agriculture, U.S. Department of Agriculture, under award number 2012-68003-30155.

Table 1. Comparison between Possé differential agar with reduced novobiocin and potassium tellurite (mPossé2) and STEC heart infusion washed blood agar with mitomycin C (SHIBAM) for the detection of Shiga toxin-producing *E. coli* in inoculated cattle fecal samples

						Positive samples/No. tested at 10^2 CFU/g		Positive samples/No. tested at 10^3 CFU/g	
Strain	Serotype[a]	Source	*stx*	*eae*	*ehxA*	mPossé2[b]	SHIBAM	mPossé2	SHIBAM
DEC10B	O26:H11	Human	*stx*$_1$	+	+	1/2	1/2	1/2	0/2
16272	O26:H11	Bovine	*stx*$_1$	+	+	1/2	0/2	1/2	0/2
B8227-C8	O45:H2	Bovine	*stx*$_1$	+	-	1/2	0/2	1/2	0/2
D88-28058	O45:NM	Bovine	*stx*$_1$	+	+	1/2	0/2	2/2	1/2
MT#80	O103:H2	Human	*stx*$_1$	+	+	1/2	0/2	1/2	0/2
236-5	O103:NT	Bovine	*stx*$_1$ and *stx*$_2$	+	+	1/2	0/2	1/2	1/2
8266-1	O111:NT	Bovine	*stx*$_1$ and *stx*$_2$	+	+	1/2	0/2	1/2	0/2
10049	O111:H11	Bovine	*stx*$_1$ and *stx*$_2$	+	+	1/2	1/2	2/2	0/2
DA-37	O121:H19	Human	*stx*$_2$	+	+	0/2	0/2	0/2	0/2

8-084	O121:H19	Bovine	*stx*$_2$	+	-	0/2	0/2	2/2	0/2
GS G5578620	O145:H28	Human	*stx*$_1$	+	+	1/2	0/2	1/2	1/2
1234	O145:H28	Bovine	*stx*$_1$ and *stx*$_2$	+	-	1/2	0/2	2/2	1/2
933	O157:H7	Beef	*stx*$_1$ and *stx*$_2$	+	+	1/2	0/2	1/2	0/2
S2006#1	O157:H7	Bovine	*stx*$_1$ and *stx*$_2$	+	+	0/2	0/2	1/2	0/2
Total						11/28	2/28	17/28	4/28

[a]NM, non-motile. NT, not typed.

[b]Possé differential agar with 5.0 mg/liter novobiocin and 0.5 mg/liter potassium tellurite.

Table 2. Comparison between Possé differential agar with reduced novobiocin and potassium tellurite (mPossé2) and STEC heart infusion washed blood agar with mitomycin C, novobiocin and potassium tellurite (mSHIBAM) for the detection of Shiga toxin-producing *E. coli* in inoculated cattle fecal samples

						Positive samples/No. tested at 10^2 CFU/g		Positive samples/No. tested at 10^3 CFU/g	
Strain	Serotype[a]	Source	*stx*	*eae*	*ehxA*	mPossé2[b]	mSHIBAM[c]	mPossé2	mSHIBAM
DEC10B	O26:H11	Human	*stx*$_1$	+	+	0/3	0/3	2/3	0/3
16272	O26:H11	Bovine	*stx*$_1$	+	+	0/3	0/3	1/3	0/3
B8227-C8	O45:H2	Bovine	*stx*$_1$	+	-	0/3	0/3	0/3	0/3
D88-28058	O45:NM	Bovine	*stx*$_1$	+	+	0/3	0/3	0/3	0/3
MT#80	O103:H2	Human	*stx*$_1$	+	+	0/3	0/3	0/3	0/3
236-5	O103:NT	Bovine	*stx*$_1$ and *stx*$_2$	+	+	1/3	0/3	0/3	0/3
8266-1	O111:NT	Bovine	*stx*$_1$ and *stx*$_2$	+	+	0/3	0/3	0/3	0/3
10049	O111:H11	Bovine	*stx*$_1$ and *stx*$_2$	+	+	0/3	0/3	0/3	0/3
DA-37	O121:H19	Human	*stx*$_2$	+	+	0/3	0/3	0/3	0/3

8-084	O121:H19	Bovine	*stx*$_2$	+	-	0/3	0/3	0/3	0/3
GS G5578620	O145:H28	Human	*stx*$_1$	+	+	1/3	0/3	1/3	0/3
1234	O145:H28	Bovine	*stx*$_1$ and *stx*$_2$	+	-	0/3	0/3	0/3	0/3
933	O157:H7	Beef	*stx*$_1$ and *stx*$_2$	+	+	0/3	0/3	0/3	0/3
S2006#1	O157:H7	Bovine	*stx*$_1$ and *stx*$_2$	+	+	0/3	0/3	0/3	0/3
Total						2/42	0/42	4/42	0/42

[a]NM, non-motile. NT, not-typed.

[b]Possé differential agar with 5.0 mg/liter novobiocin and 0.5 mg/liter potassium tellurite.

[c]STEC heart infusion washed blood agar with mitomycin C, 5.0 mg/liter novobiocin and 0.5 mg/liter potassium tellurite.

Table 3. Comparison between CHROMagar STEC, Possé differential agar, Possé differential agar with novobiocin, cefixime trihydrate, and potassium tellurite (mPossé1) and Possé differential agar with reduced novobiocin and potassium tellurite (mPossé2) for the detection of Shiga toxin-producing *E. coli* in inoculated cattle fecal samples

					Positive samples/No. tested at 10^2 CFU/g				Positive samples/No. tested at 10^3 CFU/g			
					CHROMagar				CHROMagar			
Strain	Serotype[a]	Source	*stx*	*eae*	STEC	Possé	mPossé1[b]	mPossé2[c]	STEC	Possé	mPossé1	mPossé2
16272	O26:NT	Bovine	stx_1	+	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
DEC10E	O26:H11	Bovine	stx_1	+	1/2	1/2	0/2	0/2	1/2	0/2	0/2	1/2
B8227-C8	O45:H2	Bovine	stx_1	+	1/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
D88-28058	O45:NM	Bovine	stx_1	+	1/2	0/2	0/2	0/2	1/2	0/2	0/2	0/2
236-5	O103:NT	Bovine	stx_1 and stx_2	+	1/2	1/2	0/2	0/2	1/2	1/2	0/2	1/2

RW1372	O103:H2	Bovine	*stx*$_1$	+	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
8266-1	O111:NT	Bovine	*stx*$_1$ and *stx*$_2$	+	1/2	1/2	0/2	0/2	1/2	1/2	0/2	0/2
10049	O111:H11	Bovine	*stx*$_1$ and *stx*$_2$	+	1/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
DA-37	O121:H19	Human	*stx*$_2$	+	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
8-084	O121:H19	Bovine	*stx*$_2$	+	1/2	0/2	0/2	1/2	1/2	0/2	0/2	0/2
1234	O145:H28	Bovine	*stx*$_1$ and *stx*$_2$	+	0/2	0/2	0/2	1/2	1/2	0/2	0/2	1/2
B6820-C1	O145:NM	Bovine	*stx*$_2$	+	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2

S2006#1	O157:H7	Bovine	*stx*$_1$ and *stx*$_2$	+	1/2	0/2	1/2	1/2	1/2	0/2	0/2	1/2
S2006#2	O157:H7	Bovine	*stx*$_1$ and *stx*$_2$	+	1/2	1/2	0/2	1/2	1/2	0/2	0/2	1/2
Total					9/28	4/28	1/28	4/28	8/28	2/28	0/28	5/28

[a]NM, non-motile. NT, not-typed.

[b]Possé differential agar with 5.0 mg/liter novobiocin, 0.05 mg/liter cefixime trihydrate, and 0.15 mg/liter potassium tellurite.

[c]Possé differential agar with 5.0 mg/liter novobiocin and 0.5 mg/liter potassium tellurite.

Table 4. Quantification of Shiga toxin-producing *E. coli* in inoculated cattle feces by a spiral plate method

							Estimated CFU/g			
Strain	Serotype[a]	Source	*stx*	*eae*	Resistance	Inoculum CFU/g	CHROMagar STEC	CHROMagar STEC + Rif[b]	mPossé2[c]	mPossé + Rif[d]
H30	O26:H11	Human	stx_1	+	Rifampicin	1.0×10^3	NQ[e]	NQ	NQ	NQ
						1.0×10^4	NQ	NQ	NQ	NQ
						1.0×10^5	NQ	8.2×10^3	NQ	1.1×10^4
CDC 96-3285	O45:H2	Human	stx_1	+	Rifampicin	8.8×10^2	NQ	NQ	NQ	NQ
						8.8×10^3	6.2×10^3	9.1×10^3	NQ	NQ
						8.8×10^4	8.0×10^4	1.1×10^5	NQ	1.2×10^4 [f]
CDC 90-3128	O103:H2	Human	stx_1	+	Rifampicin	7.7×10^2	NQ	NQ	NQ	NQ
						7.7×10^3	5.2×10^3 [f]	8.1×10^3	NQ	NQ
						7.7×10^4	1.1×10^5	1.1×10^5	6.1×10^4	4.7×10^4
JB1-95	O111:NM	Human	stx_1 and stx_2	+	Rifampicin	6.2×10^2	NQ	NQ	NQ	NQ

						6.2×10^3	NQ	NQ	NQ	NQ
						6.2×10^4	NQ	NQ	NQ	NQ
CDC 97-	O121:H19	Human	*stx*$_2$	+	Rifampicin	8.9×10^2	NQ	NQ	NQ	NQ
3068						8.9×10^3	NQ	NQ	NQ	NQ
						8.9×10^4	9.7×10^3	9.6×10^4	3.1×10^4	4.8×10^4
83-75	O145:NM	Human	*stx*$_2$	+	Rifampicin	9.0×10^2	NQ	NQ	NQ	NQ
						9.0×10^3	NQ	NQ	1.3×10^4	NQ
						9.0×10^4	5.8×10^3 [f]	8.1×10^3	6.8×10^3	6.6×10^3

[a]NM, non-motile.

[b]CHROMagar STEC without the supplement and with 100 mg/liter rifampicin.

[c]Possé differential agar with 5.0 mg/liter novobiocin and 0.5 mg/liter potassium tellurite.

[d]Possé differential agar without novobiocin and potassium tellurite and with 100 mg/liter rifampicin.

[e]NQ, not quantified.

[f]Quantified from one plate.

CHAPTER 6

PRELIMINARY EXPERIMENTS INVOLVING NON-O157 SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* ADHERENCE TO BOVINE AND HUMAN COLONIC EPITHELIAL CELLS

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Abstract

Shiga toxin-producing *Escherichia coli* (STEC) are foodborne pathogens that naturally colonize the intestines of ruminants and cause hemorrhagic colitis and hemolytic uremic syndrome in humans. In order to colonize the intestine STEC must produce adherence factors. In this study, representative strains of STEC O26, O45, O103, O104, O111, O121, and O145 were used to test the ability of these organisms to adhere to intestinal epithelial cells. Strains were tested by polymerase chain reaction for intimin, flagella, fimbria, and other adhesin genes and tested for adherence to cattle colonic explants and Caco-2 cells. Quantitative adherence and invasion assays were performed using Caco-2 cells. Non-O157 STEC strains were positive for 8 to 10 of 18 fimbrial genes tested. All non-O157 STEC adhered to colonic explants from cattle and intimin-positive STEC caused attaching-effacing lesions on Caco-2 cells. One strain, MT#80, was found to invade into cattle colonic mucosal epithelial cells, which was confirmed using an invasion assay with Caco-2 cells. In addition, this study found that a STEC O104 outbreak strain can attach to bovine colonic mucosal epithelial cells and had significantly higher levels of adherence on Caco-2 cells compared to other STEC. This research suggests that interventions which block the effects of intimin and fimbria in non-O157 STEC may be effective for pre-harvest control.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are foodborne pathogens that pose a threat to public health. Ruminants, specifically cattle are a known reservoir for STEC (Gyles, 2007). Clinically evident disease caused by STEC infections in cattle is usually limited to nursing calves <5 weeks old, whereas disease in human patients is often seen at any age (Moxley and Smith, 2010; Croxen *et al*., 2013). STEC serogroups other than O157 are termed non-O157 STEC (Bettleheim, 2007). Scallan *et al*. (2011) estimated that non-O157 STEC account for approximately 64% of foodborne illness caused by STEC in the U.S. Over 400 serotypes of STEC have been isolated from clinical disease (Blanco *et al*., 2004), however, the most frequently isolated non-O157 STEC from clinical patients fall within 6 serogroups, O26, O45, O103, O111, O121, and O145 (Brooks *et al*., 2005).

In cattle and humans, STEC cause attaching-effacing (A/E) lesions through production of the adherence factor intimin and secretion of the translocated intimin receptor (Tir) along with effector proteins through a type III secretion system encoded on the locus of enterocyte effacement (McDaniel *et al*., 1995). Other adhesion factors have been described for STEC, including flagella (Mahajan *et al*., 2009) and fimbria (Gonyar and Kendall, 2014). Fimbriae serve as initial adherence factors before intimate adherence (Farfan and Torres, 2012)

STEC of different serogroups may vary with regard to their preferred site of localization in the intestinal tract. STEC O157 has been found along the entire gastrointestinal tract in cattle (Keen *et al*., 2010). Girard *et al*. (2007) found that a STEC O26 strain adhered more extensively to the terminal ileum and rectum compared to the

terminal colon. Lewis *et al*. (2015) showed that STEC colonize and form A/E lesions using *in vitro* organ culture of human colonic biopsy samples. This suggests that in humans the colon is a major site of attachment.

The overall goal of this study was to compare the abilities of STEC O26, O45, O103, O111, O121, and O145 to colonize cattle and human colonic epithelial cells. The specific objectives were to compare STEC O26, O45, O103, O111, O121, and O145 isolates of varying flagellar and intimin type composition for adherence and A/E lesions in mucosal explants and primary epithelial cell cultures.

Materials and Methods

Bacterial strains. Strains were obtained from Dr. Shannon Manning (STEC Center at Michigan State University), Dr. John B. Luchansky (USDA, Agricultural Research Service, Eastern Regional Research Center), or from our collection representing STEC serogroups O26, O45, O103, O104, O111, O121 and O145. STEC O157:H7 strains 86-24 and 933 were used as positive controls for virulence genes. Enteroinvasive *E. coli* strain 43893 was used as a positive control for invasiveness in gentamicin protection assays. *E. coli* G58-1 was used as a negative control for adherence for explant experiments. *E. coli* K-12 strain DH5α was used as a negative control for virulence genes, adherence, and invasion of Caco-2 cells.

Molecular determination of O-antigen, Shiga toxin, EHEC-hemolysin, flagellin, fimbria, and intimin type. Bacterial strains were streaked for isolation onto blood agar and incubated 24 h at 37°C. A single colony was picked, placed into 50 µl of water, heated for 10 min at 100°C, centrifuged for 3 min at 16,000 x g, and the

supernatant was transferred to a fresh tube for use as DNA template. Strains were tested for the presence of genes specific for O-antigen type (*wbqEF* or *wzx*), Shiga toxin (stx_1 and stx_2), enterohemolysin (*ehxA*), flagellin (*fliC*), and intimin (*eae*) by PCR using primers and conditions previously described (Wang *et al*., 2003; Blanco *et al*., 2004; Bai *et al*., 2012). Additionally, strains were tested for *irgA* homologue adhesin (*iha*), *E. coli* factor for adherence (*efa1*), and 18 fimbrial loci (Wu *et al*., 2010; Wurpel *et al*., 2013; Gonyar and Kendall, 2014).

Flagella detection. Strains were tested for flagella by motility media, staining for flagella, and agglutination. Strains were streaked on trypticase soy broth (TSB) agar and incubated 24 h at 37°C. A single colony was stabbed with a needle down the center of a tube of Motility Test Medium w/ TTC Indicator (MTM; Remel, Lenexa, KS) and incubated 24 h at 37°C. MTM tubes were examined for motility and if the test was negative, it was re-passaged a maximum of 10 times in MTM before the strain was recorded as non-motile.

STEC strains were stained for flagella according to the manufacturer's instructions (Presque Isle Cultures, Erie, PA). After staining, slides were examined by light microscopy for detection of flagella. For H-antigen agglutination reactions, MTM tubes were inoculated with a single colony and incubated at 37°C for 24 h. Using a Pasteur pipet, aspirated "core" samples from the most motile area of the MTM tubes were used to inoculate 10 ml Nutrient Broth (Oxoid) and incubated for 6 h at 37°C. The surfaces of blood agar plates were swabbed with the broth culture and incubated overnight at 37°C. Bacteria were transferred from approximately one-quarter of the plate's surface to 10 ml Nutrient Broth. Cultures were incubated for 6 h at 37°C, and then

135.1 µl of 37% formaldehyde solution was added and incubated at room temperature for 30 min. One hundred µl of this killed bacterial suspension was added to an equal volume of the appropriate H antisera in each microtiter plate well, and plates were incubated for 1.5 h in a humidified chamber at 50-52°C. Microtiter reactions were evaluated with an inverted light microscope and scored as – for no agglutination or 1+ to 4+ for increasing levels of agglutination, with either 3+ or 4+ reactions considered a positive result.

Bovine colonic mucosal explants. Bovine colonic mucosal explants and bacterial strains were prepared and cultured by methods previously described (Baehler and Moxley, 2000, 2002) with minor modifications. Bacterial strains were grown statically overnight in TSB at 37°C and then transferred 1:10 into fresh TSB and for 6 h.

Mucosal tissues from the colon were taken at the junction of the centrifugal and centripetal coils of the spiral colon. These tissues were obtained from three steers at slaughter with each animal representing a single experiment. The time lapse between death and initiation of explant cultures was approximately 2 h. Upon collection, the mucosal surfaces of these tissues were washed with cold water, rinsed with chilled 0.9% saline, immersed in chilled RPMI 1640 medium, and transported to the laboratory on ice. Tissues were cut into 4 x 8 mm explants and 3 explants were placed mucosal side up onto biopsy foam pads (Simport, Montreal, Canada), with one foam pad per well in six-well polystyrene tissue culture plates. Explants were inoculated with 2 $x10^7$ CFU, which was confirmed by serial dilution and plating on TSB agar. Five ml of RPMI 1640 complete media (RPMI 1640 supplemented with 10% FBS, 0.25% lactalbumin hydrolysate, 0.10 µg/ml human insulin, and 1% D-+-mannose) was added to each well, and plates were incubated at 37°C in 5% CO_2 on a rocker (16 cycles/min). Media was changed once per h

starting at the second h of incubation. After 8 h incubation, the explants were harvested for sample collection. One explant per well was collected for histopathology, scanning electron microscopy, and confirmational bacterial culture. Samples for confirmational bacterial culture were homogenized, serially diluted and plated on differential Possé media without novobiocin and potassium tellurite (Possé *et al*., 2008b).

Histopathology and immunohistochemistry (IHC). Samples for histopathology were fixed in 10% neutral buffered formalin, dehydrated through a graded alcohol and xylene series, embedded in paraffin, sectioned 5 μm thick, stained with H&E, and examined in detail by light microscopy. H&E stained tissue sections were used to examine explants for evidence of enterocyte necrosis and renewal, and bacterial adherence. IHC was used to specifically identify the bacteria that were adherent to enterocytes in explants. Sections of paraffin-embedded explant tissues were stained with rabbit anti-O26, -O45, -O103, -O104, -O111, -O121, and -O145 as the primary antisera to detect the corresponding O-antigen inoculum strains. Alkaline phosphatase-labeled goat anti-rabbit serum, Fast Red, and hematoxylin counterstain were used in the IHC staining procedures.

Scanning electron microscopy (SEM). Explants for SEM were fixed in 2.5% glutaraldehyde, dehydrated in a graded alcohol series, critical-point dried, sputter coated, and examined with a Hitachi S-3000N SEM.

Transmission electron microscopy (TEM). Paraffin blocks containing epithelial-adherent STEC were deparaffinized and processed as previously described (Baehler and Moxley, 2000).

Caco-2 cells. Caco-2 cells were grown in 75 cm^2 flasks in 9.6 g/L of minimal essential medium with non-essential amino acids, 2.2 g/L of sodium bicarbonate, 20% fetal bovine serum, and 1% D-+-mannose (Caco-2 media). Caco-2 cells were seeded onto 12 mm coverslips (Fisher) in 24-well plates at a concentration of 1 x 10^5 cells per ml and incubated for 14 days at 37°C in 5% CO_2.

Quantitative adherence assay. Adherence to Caco-2 cells was tested three times independently, each time in triplicate. Strains were streaked for isolation on TSB agar and incubated at 37°C for 24 h. A single colony was picked, transferred into 5 ml of TSB, and incubated at 37°C for 24 h. A 300-µl aliquot was removed, diluted 1:10 into fresh TSB, and incubated at 37°C for 6 to 8 h. This culture was centrifuged at 16,000 x g for 3 min, and the supernatant was removed. The bacterial pellet was washed with PBS, centrifuged, the supernatant was removed, and bacterial cells were resuspended in Caco-2 medium. Cultures were diluted to 10^7 CFU/ml in Caco-2 cell medium, and each well was inoculated with 10^7 CFU and incubated at 37°C in 5% CO2 for 6 h. The inoculum concentration was confirmed by serial dilution in PBS and spread plating. At 3 h after inoculation, the Caco-2 medium was changed. At 6 h, cells were Giemsa stained and examination by light microscopy. Giemsa staining of cells was done by a protocol adapted from Yin *et al*. (2011). Briefly, cells were washed twice with PBS and fixed for 10 min in a 1:1 mixture of methanol and PBS. The fixative was removed and fresh anhydrous methanol was added for 2 min, and then discarded. The monolayer was then rinsed with anhydrous methanol, discarded, and the cells were allowed to dry. The cells were then stained with 10% Giemsa stain for 20 min, rinsed with hematology rinse

(Volu-Sol, Inc.) for 20 sec, and rinsed with water. A coverslip was then applied to the slide with mounting medium (Cytoseal 60).

I**nvasion assay.** Invasion in Caco-2 cells was tested three times independently, each time in triplicate Invasion assays were conducted by methods previously described (Small *et al*., 1987; Luck *et al*., 2005). Bacterial inocula were prepared by growing strains overnight in TSB at 37°C, diluting them 1:10 into TSB, and incubating further for 3 h. A 1 ml aliquot was centrifuged at 13,000 x g, washed with PBS, resuspended in 1 ml of Caco-2 medium, and diluted to a concentration of 1 x 10^7 CFU/ml. Inoculum concentrations were confirmed by serially dilution in PBS and plating on TSB agar. Caco-2 cells were seeded at a concentration of 1 x 10^5 cells per ml into 6-well plates (Falcon 353046), 24 h prior to the experiment. Cell monolayers were washed twice with PBS, inoculated with 10^7 CFU, and incubated for 2 h in 5% CO_2 at 37°C. After 2 h, cell monolayers were washed 5 times with PBS, and Caco-2 media with 100 μg/ml gentamicin was added and incubated for an additional 2 h in 5% CO_2 at 37°C. Afterward, cell monolayers were washed 5 times with PBS and flooded with 1% Triton X-100 for 10 min. One ml of contents was then removed from each well, serially diluted in PBS, and spread-plated on TSB agar to quantify the number of intracellular bacteria.

Fluorescent-actin staining (FAS). FAS assays were conducted in three independent experiments by a combination of methods previously described (Knutton *et al*., 1989; Abu-Ali *et al*., 2010). Caco-2 cells were cultured and inoculated with bacterial strains as described above for the Caco-2 cell adherence assay with the exception that the 300-μl aliquot that was removed was diluted 1:10 into fresh TSB containing 44 mM sodium bicarbonate and incubated at 37°C for 2 h. At the conclusion of the 2 h-

incubation step, the cells were washed with serum-free Caco-2 medium and PBS, and then fixed by adding 400 µl of 4% paraformaldehyde for 15 min. The fixative was removed and cells were washed twice with PBS. Triton-X-100 (100 µl, 0.1% solution) was added, and after 5 min was removed and cells were washed twice with PBS. RNase A (100 µg/ml) was added and the cells were incubated at 37°C for 20 min, removed and cells were again washed twice with PBS. One unit of Alexa Fluor 488 phalloidin conjugate (Life Technologies) in 1% bovine serum albumin was added and incubated for 20 min. The cells were washed with PBS, propidium iodide (5 µg/ml) was added, and cells were incubated for 15 min. The cells were then washed twice with PBS and coverslips were mounted onto glass microscope slides with Fluoro-gel with tris buffer (Electron Microscopy Sciences, Hatfield, PA) and let stand at room temperature for 1 h. The slides were stored at 4°C, and later examined with a Nikon A1 confocal system on a Nikon 90i upright fluorescence microscope.

Statistical analysis. Mean percent invasion and adherence were compared by an ANOVA with Fisher's least significant difference using SAS v 9.2.

Results

Characterization of strains. STEC strains were characterized by PCR for O-antigen (*wbqEF* or *wzx*), intimin (*eae*), flagellin (*fliC*), and Shiga toxin (*stx*); in addition, they were characterized by standard methods for motility and flagella. The results are summarized in Table 1. Of the 6 strains that produced flagella, all were motile. Within the O groups in the following H antigen and/or [*fliC*] types and *eae* types were detected: O26:H11 (*eae*-β1); O45:H2 (*eae*-ε); O103:H2 (*eae*-ε); O104:[H4] (*eae*-negative);

O111:H10 (*eae*-negative); O121:[H19] (*eae*-ε); and O145:[H28] (*eae*-γ1). Non-O157 STEC were characterized for 18 fimbrial loci by PCR as shown in Table 2.

Adherence to bovine colonic mucosal explants. Bacterial strains were tested for adherence to epithelial cells in bovine colonic mucosal explants. Three independent experiments using colonic tissues from different steers on different days were conducted. By light microscopy, H&E- and IHC-stained sections of non-inoculated colonic explants had normal mucosal morphology and no evidence of STEC bacteria. Explants inoculated with the non-pathogenic *E. coli* strain G58-1 had minimal bacterial adherence to intact epithelial cells. In contrast, all non-O157 STEC adhered to the adhered to the apical surfaces of mucosal epithelial cells (Fig. 1). This is the first report that has demonstrated *E. coli* O104:[H4] binding to colonic mucosal explants from cattle. MT#80 was found intracellularly in epithelial cells.

FAS test for A/E adherence. STEC strains that were tested for adherence to bovine mucosal explants were also tested for A/E adherence by the FAS test (Table 1). STEC O157:H7 strain 86-24 which produces *eae*-γ1 was used as a positive control, and DH5α was used as a negative control. A positive FAS result consisted of polymerized actin (intense green staining) immediately beneath bacteria (red-staining) attached to the host cell membrane (Fig. 2). Based on the FAS test, 5 of 5 *eae*-positive non-O157 STEC strains which collectively contained β1, ε, and γ1 variants of *eae* produced A/E lesions. Also, all STEC strains tested that were *eae*-negative, gave negative FAS test results.

SEM and TEM for detection of A/E lesions and other patterns of adherence. SEM and TEM were conducted on sub-specimens obtained from the above-described explant experiments to test for ultrastructural evidence of A/E lesions and other patterns

of adherence. Since the surfaces of explant tissues were not washed at the conclusion of experiments, most had a heavy mucus layer that covered the epithelial surface and A/E lesions were not visible by SEM. Classical A/E lesions in explants, including pedestals, were detected by TEM as a result of infection with STEC O103:H2 (*eae*-ε) strain MT#80 (Fig. 2).

Invasion of STEC in Caco-2 cells. STEC strains MT#80 and CDC 1994-3024 were tested for their ability to invade Caco-2 cells. MT#80 was significantly ($P < 0.05$) more invasive than DH5α, but not different from CDC 1994-3024. Interestingly, MT#80 was significantly ($P < 0.05$) more invasive compared to the enteroinvasive *E. coli* strain 43893 (Fig. 3).

Quantitative Adherence to Caco-2 cells. STEC strains were tested for adherence to Caco-2 cells. TY-2482 had significantly higher levels of adherence than all STEC strains except for STEC strain RD8 (Fig. 4). The intimin-positive STEC strains (DEC10B, B8227-C8, MT#80, DA-37, and GS G5578620) were not significantly different from each other or significantly different compared to DH5α.

Discussion

Seven non-O157 STEC strains were characterized for serotype, adherence factors, and virulence factors. Flagella were observed for all strains that were positive for motility in MTM media. Five of seven strains had intimin and only intimin-positive strains tested positive for *efa-1*. Thus, intimin-negative strain must use other adherence factors to colonize the intestine. Non-O157 STEC strains tested positive for 8 to 10 of 18 fimbrial loci. RD8 lacked intimin but tested positive for 8 of 18 fimbrial loci. Previously, RD8

tested positive for aggregative adhesion fimbria type II and was suspected to be as pathogenic as typical intimin-positive STEC in humans (Czeczulin *et al*., 1999). The phenotype of this strain on Caco-2 cells was consistent with an enteroaggregative strain. TY-2482 also lacked intimin and previously tested positive for aggregative adherence fimbria (Bucholz *et al*., 2011).

Non-O157 STEC strains from all six serogroups currently classified as adulterants in raw non-intact beef (O26, O45, O103, O111, O121, and O145) expressing different flagellar types (e.g., H2, H4, H10, H11, H19, and H28) and variants of intimin, viz., β1, ε, and γ1, adhered to bovine colonic epithelium and induced the formation of A/E lesions. Non-O157 STEC strains lacking intimin were also shown to adhere to bovine colonic epithelium; hence, must utilize other mechanisms for attachment. Also, adherence studies were conducted with an enteroaggregative STEC O104:H4 strain that caused a large foodborne epidemic attributable to a non-beef source in Germany in the summer of 2011 (Bucholz *et al*., 2011). STEC O104:H4 adhered to epithelial cells at a significantly higher level compared to intimin-positive STEC. Higher levels of adherence could give a strain an advantage to deliver Shiga toxin to the host. These studies showed this strain has the ability to adhere to bovine colonic epithelium and may do so via fimbria. Hence, if this strain enters the cattle population it may be able to colonize cattle and persist.

STEC are typically thought of as non-invasive pathogens. One STEC strain (MT#80) was consistently observed intracellularly in bovine epithelial mucosal explants. Invasion was confirmed with MT#80 using Caco-2 cells in a gentamicin protection assay. Similarly, Cordeiro *et al*. (2013) found STEC in intracellular compartments of Caco-2 cells with strains persisting up to 96 h, which indicates multiplication. The mechanism by

which STEC enter cells is unknown. Uhlich *et al*. (2002) reported a role for the fimbrial protein curli in invasion of STEC O157 strain ATCC 43894. MT#80 tested positive for curli (loc 7) by PCR. Interestingly, Uhlich *et al*. (2002) also found higher percent invasion in the STEC O157 strain compared to enteroinvasive *E. coli* 43893. Invasion by STEC O157 was also shown in primary bovine rectal epithelial cell cultures (Sheng *et al*., 2011). Previous studies have shown that application of phages was effective in reducing STEC O157 in an *ex vivo* rumen model but not in live cattle (Rivas *et al*., 2010). Intracellular invasion in of STEC in colonic epithelial cells could reduce the effectiveness of antimicrobials and the ability of cattle to clear the pathogen.

In summary, all non-O157 STEC tested adhered to both bovine colonic explants and a human colonic cell line. In colonic explants, STEC O103:H2 strain MT#80 was found to invade epithelial cells. Invasion assays confirmed that MT#80 invaded Caco-2 cells. STEC O104:[H4] strain TY-2482 adhered to Caco-2 cells at a significantly higher level compared to intimin-positive STEC. This study demonstrates that interventions that block the effects of intimin and fimbria in non-O157 STEC may be reduce STEC colonization.

Acknowledgements

This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2012-68003-30155 from the USDA National Institute of Food and Agriculture and by the American Meat Institute Foundation Grant no. 10-516.

Table 1. Characterization of non-O157 Shiga toxin-producing *Escherichia coli* strains and tests for the ability to adhere and induce attaching-effacing lesions using bovine colonic mucosal explants and Caco-2 cells

Strain	Source	Serotype	Motile[a]	Flagella[b]	*stx*	*eae*	*efa-1*	*iha*	*ehxA*	Adh[c]/FAS[d]
DEC10B	human	O26:H11	+	+	*stx*$_1$	β1	+	+	+	+/+
B8227-C8	bovine	O45:H2	+	+	*stx*$_1$	ε	+	-	-	+/+
MT#80	human	O103:H2	+	+	*stx*$_1$	ε	+	-	+	+/+
TY-2482	human	O104:[H4]	+	+	*stx*$_2$	-	-	+	-	+/-
RD8	human	O111:H10	+	+	*stx*$_2$	-	-	-	-	+/-
DA-37	human	O121:[H19]	+	+	*stx*$_2$	ε	+	-	+	+/+
GS G5578620	human	O145:[H28]	-	-	*stx*$_1$	γ1	-	-	+	+/+

[a]Motility detected after culture in motility test medium for 1 to 10 passages. [b]Flagella detected in cultured bacteria by Presque-Isle staining method. [c]Detection of bacterial adherence to epithelial cells in bovine colonic mucosal explants by light microscopy of tissue sections immunohistochemically stained with antibodies specific for O antigen of inoculum strain. [d]Fluorescent actin staining for detection of attaching-effacing lesions on Caco-2 cells.

Table 2. Characterization of fimbrial loci in Shiga toxin-producing *Escherichia coli*

		Fimbrial gene (locus)																	
		Z0024	*Z0146*	*Z0686*	*Z0872*	*Z1290*	*Z1538*	*csgD*	*Z2204*	*Z3279*	*Z3597*	*Z4501*	*Z4971*	*Z5221*	*Z5917*	*Z0360*	*Z0118*	*cs1-like*	*yqi-like*
Strain	Serotype	(*loc1*)	(*loc2*)	(*loc3*)	(*loc4*)	(*loc5*)	(*loc6*)	(*loc7*)	(*loc8*)	(*loc9*)	(*loc10*)	(*loc11*)	(*loc12*)	(*loc13*)	(*loc14*)	(*loc15*)	(*loc16*)	(*loc17*)	(*loc18*)
DEC10B	O26:H11	-	-	+	-	+	-	+	+	-	-	+	-	-	+	+	+	+	+
B8227-C8	O45:H2	-	-	+	-	+	-	+	+	-	-	+	-	-	+	+	+	+	+
MT#80	O103:H2	-	-	+	-	+	-	+	+	-	-	+	-	-	+	+	+	+	+
TY-2482	O104:[H4]	-	-	+	-	+	-	+	+	-	-	+	-	-	-	+	+	+	+
RD8	O111:H10	-	-	+	-	-	-	+	+	-	-	+	-	-	-	+	+	+	+
DA-37	O121:[H19]	-	-	+	-	+	-	+	-	-	-	+	-	-	+	+	+	+	+
GS G5578620	O145:[H28]	+	-	+	+	-	-	+	+	-	-	-	+	-	+	+	+	-	-
933	O157:H7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-

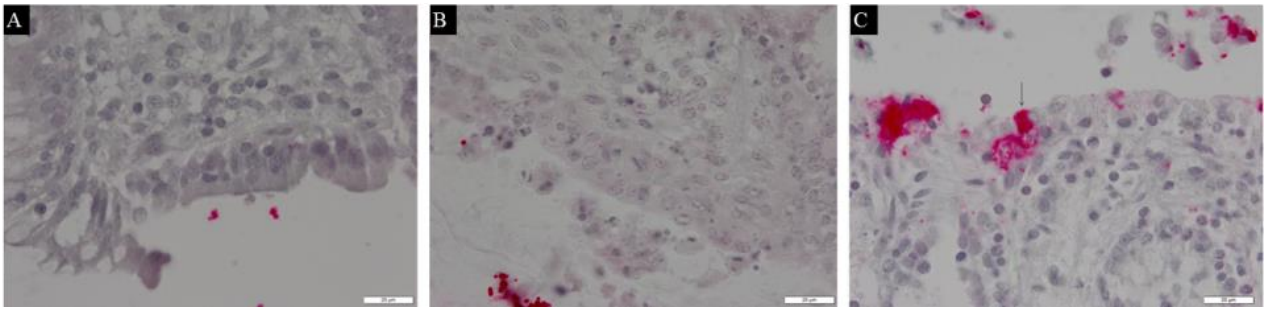

Figure 1. Light photomicrographs of bovine colonic mucosal explants uninoculated, inoculated with non-pathogenic G58-1, and inoculated with STEC O103:H2 strain MT#80. Light photomicrograph of 4-µm thick paraffin sections of bovine mucosal explants stained immunohistochemically using control rabbit antiserum, or anti-O101 serum, or anti-O103 antibodies for corresponding bacterial O-antigen, alkaline phosphatase-labeled goat anti-rabbit serum, Fast Red and hematoxylin counterstain. (A) Non-inoculated control tissue has surface-layer epithelial cells with normal columnar morphology. (B) Inoculated with non-pathogenic *E.coli* strain G58-1, G58-1 bacteria (stained red) are seen overlying the mucosal surface among sloughed epithelial cells and mucus, but none are adherent to intact, cuboidal epithelium. Intact cuboidal epithelial cells are immature replacement enterocytes that have emerged from the intestinal crypts. (C) Inoculated with STEC O103: [H11] strain 15612-1. Mucosal epithelial cells are cuboidal in shape, indicating they are immature replacement enterocytes that have emerged from the intestinal crypts.

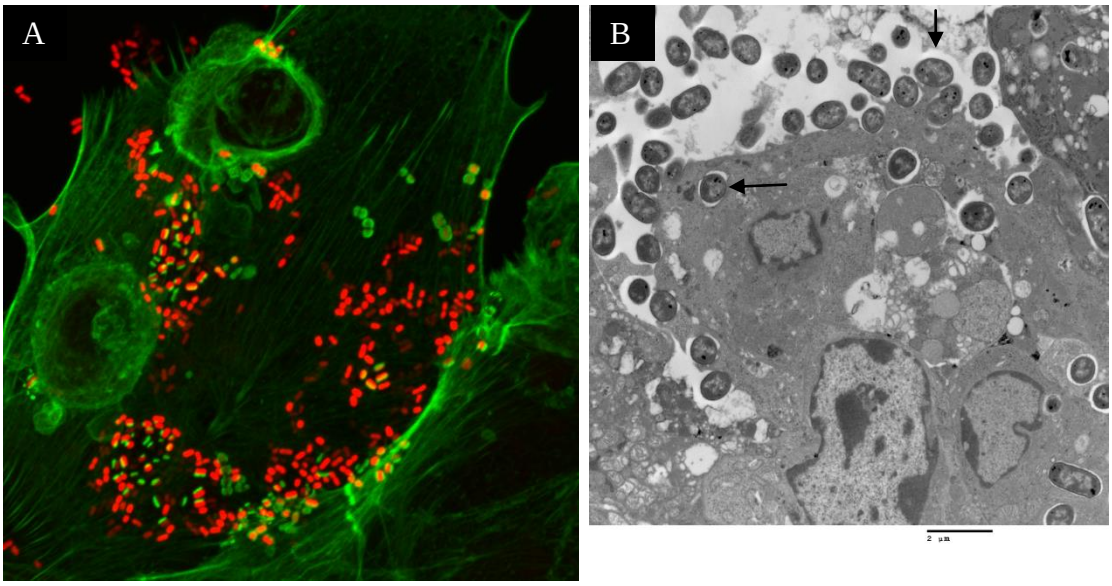

Figure 2. (A) Confocal laser scanning photomicrograph of fluorescent-actin staining (FAS) of Caco-2 cells and (B) transmission electron photomicrograph of bovine mucosal explant infected with STEC O103:H2 strain MT#80. The FAS test was performed to detect attaching-effacing (A/E) lesions in which the actin cytoskeleton is aggregated immediately beneath the bacteria and anchors it to the host cell. Nucleic acids (hence Caco-2 cell nuclei and bacteria) are stained red by propidium iodide, and actin is stained green by Alexa Fluor 488 phalloidin. (B) Transmission electron photomicrograph of MT#80 colonizing the epithelial surface of a bovine mucosal explant. Numerous bacteria colonize the epithelial surface and several in this field have induced attaching-effacing lesions (vertical arrows). Bacteria also have been taken up into phagosomes (horizontal arrows).

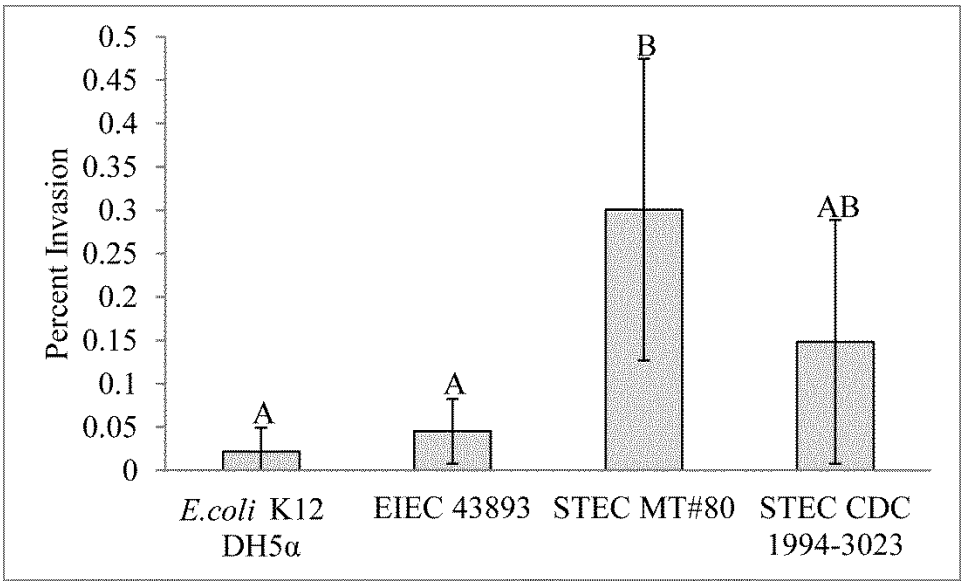

Figure 3. Percentage of Caco-2 cell invasion by various *E. coli* strains. Means with the same letter are not statistically significant. Error bars represent standard deviations. EIEC, enteroinvasive *E. coli*; STEC, Shiga toxin-producing *E. coli*.

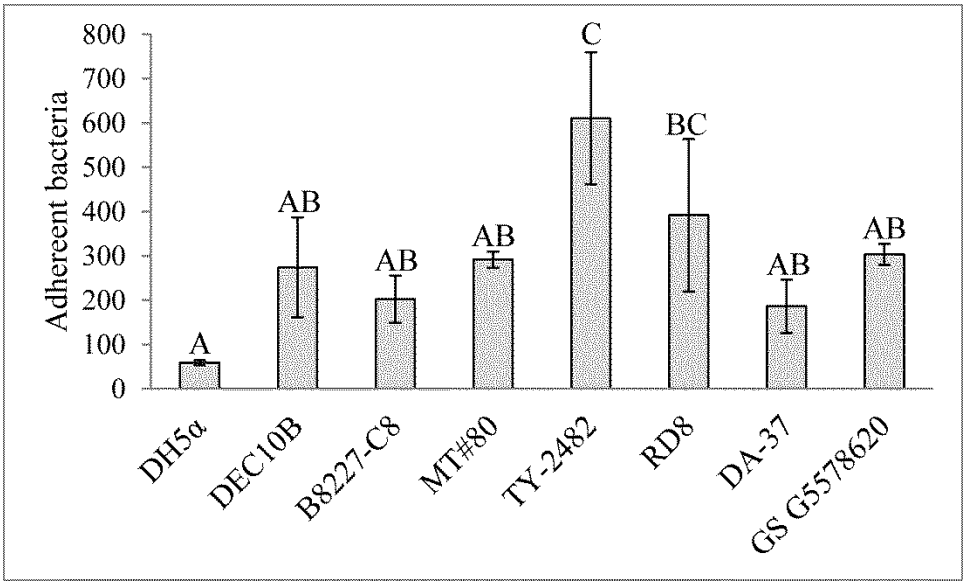

Figure 4. Adherence of Shiga toxin-producing *E. coli* strains to Caco-2 cells. Adherence was quantified by recording the total number of adherent bacterial cells per 100 cells. Error bars represent standard deviations. Means with the same letter are not significantly different.

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