Shiga Toxin-Producing *Escherichia coli*: a Public Health Challenge in the Pre-Harvest Stage of the "Farm-to-Table" Continuum

Patrícia Pereira Baltasar

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François C. Elvinger

Monica A. Ponder

William Swecker, Jr.

Korine N. Kolivras

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TECHNICAL ABSTRACT

Escherichia coli is part of the normal gastrointestinal microbiota of many animals, especially cattle. While most strains are commensal, Shiga toxin-producing *E. coli* (STEC) can cause severe human illness.

Pathogenicity of STEC is associated with genes such as those encoding Shiga toxins, enterohemolysin, and intimin. By targeting these genes, highly sensitive molecular-based techniques help detect potentially harmful STEC.

Persistent carriers and environmental contamination may be responsible for maintenance of STEC in cattle farms. Prevalence may be further influenced by diet, distance to contaminated water-sources, wildlife contact, slurry application to pasture, and population density. Relevance in environmental contamination is expected proportional to the amount of STEC shed in feces, but there is no consensus as to which production stage/age is most important.

Distribution and transmission of STEC O157 are widely studied, but risk factors for non-O157 STEC are not as well defined. Understanding what contributes for contamination of animals prior to concentration in high-density feedlots may reveal opportunities for upstream control of shedding and transmission.

Our purpose was to: (a) determine prevalence of STEC in fecal samples from animals in a cow-calf pasture-based production system; (b) describe effects of age class (dam, calf), spatial distribution of cattle, and time-point of sampling on distribution of strains positive for virulence genes *stx*1, *stx*2, *eae*A, and *hly*A; (c) isolate and identify serotypes present in *stx*-positive samples; and (d) assess genetic similarity of isolates.

Understanding factors that influence distribution of STEC strains may help support on-farm management strategies with potential to yield safer beef products.

NON-TECHNICAL ABSTRACT

Escherichia coli are bacteria found in the intestines of cattle and other animals, including humans. Most are harmless, but some produce toxins – Shiga toxins – that can cause severe, even fatal, illness in people who have consumed raw or undercooked contaminated beef or contaminated produce, especially when immunocompromised. These bacteria are variants of *E. coli* known as Shiga toxin-producing *E. coli* (STEC).

We conducted a study to evaluate the effects of cattle bloodlines, age, and physical proximity on the possibility of cattle harboring and shedding closely related STEC.

We collected fecal samples at 3 time points from 90 Angus cows and their respective calves, distributed by 12 paddocks. Small amounts of fecal material were placed in growth media in which STEC can thrive. It is possible to find potentially harmful STEC using a genetic-based method called polymerase chain reaction (PCR). STEC was found in 93.3% (84/90) adults, and 95.6% (86/90) calves. Young animals were more likely to harbor STEC when compared to adults, and populations from different paddocks presented different patterns. *Escherichia coli* colonies were further differentiated. Of 330 STEC colonies 102 were serotype O121, 12 were O103, 4 were O113, and 1 was O157.

There is a high prevalence of animals harboring STEC in the herd. The role of current physical proximity (paddock) seems to be more important than bloodline in the establishment of dominant STEC populations. Animals within bloodline do not share STEC populations.

DEDICATION

To my best friend and "soon-to-be wife", **Leigh Duke**, for reading all the books, and for informing me that the title I had planned to use, "A Heartbreaking Work of Staggering Genius", had already been taken by a more modest soul.

To my "Higher Power", whatever or whoever It might be.

… and …

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… Scientia ac labore …

… Avt viam inveniam avt faciam …

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--- Patrícia Pereira Baltasar ---

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This thesis would not have been possible without the guidance from the members of my graduate committee members, collaborators and colleagues, who contributed to the research and completion of the manuscripts making up this thesis.

Chapter 2:

Monica A. Ponder, PhD (Department of Biomedical Food Science and Technology) is a Professor of Epidemiology of Food and Waterborne Disease and is the corresponding author on this manuscript. She aided in project development, writing, publication, and editing of the manuscript.

François C. Elvinger, Dr. Med. Vet. , PhD, Diplomate, ACVPM & ECVPH is a Professor of Veterinary Epidemiology and Department Head at the Department of Population Health Sciences at the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM). He helped with study design, statistics, writing, and editing of the manuscript.

William S. Swecker Jr., DVM, PhD, Diplomate ACVN is a Director at the VMRCVM Veterinary Teaching Hospital, and a Professor of Production Management Medicine and Clinical Nutrition, at the Department of Large Animal Clinical Sciences. He helped with sample collection, study design, staffing and materials.

Stephen Were, PhD is the Study Design & Statistical Analysis Lab Supervisor at the Blacksburg campus of the Virginia-Maryland Regional College of Veterinary Medicine. He helped with programing design and statistical analysis.

Stewart C. Milton is Laboratory Technician. She helped with collection and processing of samples, laboratory methods, ordering of materials, as well as running repPCR gels.

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CONTENTS

LIST OF FIGURES

CHAPTER 2:

LIST OF TABLES

CHAPTER 2:

Chapter 1: Shiga Toxin-Producing *Escherichia coli* – Literature Review

BACKGROUND

General Description and Early History

Escherichia coli (*E. coli*) is a species of gram-negative, facultative anaerobic, non-spore-forming bacteria. Cells are rod-shaped (*bacilli*), typically about 2.0 micrometers (μm) long and 0.25–1.0 μm in diameter, with an approximate volume of 0.6–0.7 μm^3 (Cronan, 2014). To date, more than 700 serotypes of *E. coli* have been proposed, most of which are considered part of the commensal intestinal microflora of endothermic animals, predominantly cattle (Leimbach et al., 2013).

The body of animals is rich in niches where bacteria can thrive. Of the microorganisms that can use both oxygen and fermentation to produce energy in the form of ATP (facultative anaerobes), *E. coli* is the most abundant (Gyles, 2007). Harmless strains of *E. coli* are beneficial to their hosts by producing vitamin K2, and by balancing colonization of the intestine with non-pathogenic bacteria (Leimbach et al., 2013). Bacterial colonization of the GI tract begins shortly after birth and, by the time of full development, prokaryotic cells in the GI tract outnumber eukaryotic cells: in humans, the adult gastrointestinal (GI) tract contains about of 1kg (~2.2 lbs.) of bacteria, of which between 0.1% and 1% is *E. coli* (Bentley & Meganathan, 1982).

In the late nineteenth century (1885), German bacteriologist and pediatrician Theodor Escherich (1857–1911) introduced then-named "*Bacterium coli commune*" to the Society of Morphology and Physiology (Escherich, 1886). In the lecture "The intestinal bacteria of the neonate and breast-fed infant", Escherich demonstrated the use of groundbreaking pure-culture and bacterial characterization techniques to explore morphology and biochemical properties of this previously unknown bacterial population, which he had recovered from the colon of both healthy and diarrheic children (Escherich, 1886).

In recognition of Escherich's work, the *Bacterium coli commune* was later renamed *Escherichia coli* by Castellani and Chalmers (1919), and it has since become the most extensively studied unicellular life form (Cronan, 2014; Shulman et al., 2007). *E. coli* is now considered the epitome of prokaryote physiology and molecular genetics, as well as one of the best representations of the equilibrium between bacterial commensalism and pathogenicity (Eisenstein & Zaleznik, 2000; Hacker & Blum-Oehler, 2007). Still, until the early 1950s, it was thought that *E. coli*

would only cause gastrointestinal disease in infants. The fact that some pathogenic strains could also cause illness in adults was not definitely proven until adult volunteers were experimentally inoculated with *E. coli* isolated from diarrheic infants, resulting in the development of similar clinical signs (Koya et al., 1954).

The next logical step was to identify the pathogenic strategies of the bacteria. In 1966, researchers demonstrated the ability of chloroform-killed *E. coli* cultures from babies with gastroenteritis to cause dilatation of the ligated gut segment of the small intestine in a rabbit model, while strains from healthy infants did not. This work led to the hypothesis that a heat-liable enterotoxigenic (LT) substance from certain kinds of *E. coli* could produce diarrhea, while *E. coli* from healthy individuals lacked these substances (Taylor & Bettelheim, 1966). A few years later, using porcine-originated strains, it was demonstrated that heat-stable (HT) versions of these substance also seemed to exist (Smith & Gyles, 1970).

While the evolutionary history of STEC is not yet fully understood, it is accepted that the toxin of *Shigella dysenteriae* type I is the likeliest source of the aforementioned enterotoxigenic substances, having made its way into *E. coli* by means of the lambda (λ) bacteriophage. The toxins are now referred to, interchangeably, as Verotoxins (*vtx*, due to specific toxic effects on Vero cells) or Shiga toxins (*stx*); consequently, *E. coli* producing the toxin(s) is known as Verotoxin-producing *E. coli* (VTEC) or Shiga toxin-producing *E. coli* (STEC) (Konowalchuk et al., 1977a; Smith & Linggood, 1971).

Taxonomic Classification and Justification

Escherichia coli strains are included in the domain and kingdom of Bacteria, phylum Proteobacteria, class Gammaproteobacteria, order Enterobacteriales, family Enterobacteriaceae, and genus Escherichia.

Escherichia coli fits into the domain and kingdom of Bacteria, as members of the species all are prokaryotic (anucleated) unicellular microorganisms. The phylum Proteobacteria is composed of bacteria, which are classified as Gram-negative due to composition and structure of their cell walls: presence of an inner phospholipid-made cytoplasmic membrane covered by a thin peptidoglycan layer, and an outer membrane (composed primarily of lipopolysaccharides, the O Antigen, and phospholipids) (Letellier & Santamaria, 2002). Unlike Gram-positive, the peptidoglycan layer of Gram-negative bacteria is too thin to retain the crystal violet dye used in Gram-staining upon washing with an alcoholic solution. Thus, once contrast staining is applied, Gram-negative bacteria display the

contrast stain coloring rather than the violet (Coico, 2005; Letellier & Santamaria, 2002; Neidhardt et al., 1990). Other characteristics of Gram-negatives include: presence of porins (proteins that allow the migration of specific molecules across the outer membrane); surface layers (S-layers) linked to lipopolysaccharides via ionic, protein-protein, carbohydrate-carbohydrate, and/or protein-carbohydrate interactions (rather than directly to the peptidoglycan layer); flagella with four supporting rings (present in *E. coli*); absence of *teichoic* and *lipoteichoic* acids; lipoproteins connected to structural polysaccharides; majority is non-spore-forming, as is the case of *E. coli* (Letellier & Santamaria, 2002; Neidhardt et al., 1990).

Within class Gamma Proteobacteria, *Escherichia coli* are Enterobacteriales due to being facultative anaerobic *bacilli*. Family Enterobacteriaceae further groups those that are motile via peritrichous flagella, grow well at 37ºC, and are Oxidase-negative and Catalase-positive. Enterobacteriaceae are often referred to as “enterobacteria” or "enteric bacteria", as several of its members live in the intestines of animals, including humans (Eisenstein & Zaleznik, 2000). *Escherichia coli* is one of seven species currently recognized in the Genus Escherich. The remainder species are: *Escherichia albertii*; *E. blattae*; *E. fergusonii*; *E. hermannii*; *E. senegalensis*; and *E. vulneris*. Each species displays a set of unique biochemical/metabolic properties, which when combined make it unique (Abbott et al., 2003; Arita, 2004; Wang & Levin, 2009).

Biochemistry and Culture

Escherichia coli can thrive on a diversity of substrates; as a facultative anaerobe, when oxygen is present, the bacterium can produce energy by aerobic respiration; in the absence of oxygen, it can switch to fermentation (anaerobic respiration) to fulfill its energy needs, albeit less efficiently: complete oxidation of 1 mol of glucose leads to a theoretical maximum yield of 26 mol of ATP in aerobic conditions, and of 2 mol of ATP in anaerobic conditions (Kaleta et al., 2013; Madigan & Brock, 2009; Wang & Levin, 2009). Other biochemical characteristics that separate *E. coli* from other bacteria in the same genus are: it ferments lactose; the enzyme lysine decarboxylase is present; it is Vogus-Proskauer negative; produces indole; does not grow on nitrate; and does not produce H_2S (Goodsell, 2009).

E. coli strains best survive and grow at mid-range temperatures of 15 ºC to 45 ºC, with optimum performance at temperatures of 21ºC to 37ºC. Still, survival and growth of some strains has been documented at temperatures as low as 7.5 ºC (Shaw et al., 1971), and/or as high as 49 ºC (Herendeen et al., 1979).

To develop interventions with potential to reduce prevalence of shedding, it is crucial to determine factors mediating STEC prevalence and distribution, which commonly involves isolating STEC from bovine feces. This task, however, is complicated by a number of factors, which make it far from simple or straightforward. Some relevant hurdles that researchers must overcome are: obtaining the correct samples; preserving said samples; avoiding cross-contamination; managing animals to avoid injuries; isolating the wanted bacteria from greater than 10^{12} CFU/g background microflora; and obtaining intact, clean, PCR-inhibitor-free, quality DNA (Baltasar et al., 2014; Bettelheim, 2008; Bettelheim, 2007; Bolton et al., 2011; Boom et al., 1999; Boom et al., 1990). To overcome some of these challenges, researchers have leaned towards different methodologies; this, however, has become a problem in itself, making it hard to compare results between studies.

Variation in methodology starts with sampling; researchers have generally collected fecal material in one of four ways: pen floor fecal pat, rectal fecal grab, rectal fecal swab, or recto-anal mucosal swabs (RAMS). Although sensitivity of these methods has not been methodically measured and compared for non-O157 STEC, differences in isolation of STEC O157 have been observed, albeit not consistently: cultures from rectoanal mucosal swabs were observed to have a greater sensitivity than fecal samples for the detection of STEC O157 (Cernicchiaro et al., 2011; Cobbold et al., 2007; Greenquist et al., 2005; Naylor et al., 2003; Rice et al., 2003); in contrast, others have found fecal-grab samples to be more sensitive compared to RAMS (Khaitsa et al., 2005; Niu et al., 2008); others still found no significant differences between the methods (Cernicchiaro et al., 2011; Davis et al., 2006).

Upon collection, fecal samples are often suspended in an enrichment broth. This to allows multiplication of bacteria prior to streaking onto isolation agar, or as a step prior to DNA extraction. A variety of enrichment procedures have been tried with different broths, concentrations, and incubation conditions. While the enrichment step is often omitted, when properly used, the detection limit can be enhanced to approximately 10^2 CFU/g of sample pre-enrichment STEC (Foster et al., 2003; Hussein et al., 2008; Paddock et al., 2011). Enrichment broths are have been suggested and used for STEC, prior to more specific detection and identification steps (Donnenberg, 2013). Some researchers incorporate selective agents (such as various antibiotics and bile salts) in their enrichment broths (Karama et al., 2008; Kerr et al., 2001; Leung et al., 2001), whereas others prefer to use nonselective broths (Jeon et

al., 2013; LeJeune et al., 2006). Buffered peptone water (BPW) and trypticase soy broth (TSB) are the most commonly used non-selective broths (Cookson et al., 2006; Fukushima et al., 2000; Fukushima & Seki, 2004; Pearce et al., 2004; Shaw et al., 2004); while broths such as these are likely to increase the viability of any STEC present, they may also increase the viability of background microflora. Some authors have used long incubation periods of 18 – 24 h (Ayding et al., 2010; Inat & Siriken, 2010), while others have selected shorter (6 h) ones (Ayding et al., 2010; Fegan et al., 2004; Ibekwe & Grieve, 2003). Some have incubated the broths at temperatures as high as 41-42 °C (Fegan et al., 2004; Inat & Siriken, 2010; Khandaghi et al., 2011), while others preferred lower temperatures of 37°C, closer to optimal growth conditions of generic *E. coli* (Baltasar et al., 2014; Kang et al., 2001; Karama et al., 2008). It has also been suggested that the use of 37°C comes from a publication utilizing the original outbreak strain of STEC O157 (Doyle & Schoeni, 1984; Gonthier et al., 2001; Vimont et al., 2007). Other studies including a more varied selection of strains, the optimal growth temperature for STEC was reported to be 40 °C; however, very few studies have examined optimal growth temperatures in non-O157 STEC (Gonthier et al., 2001; Nauta & Dufrenne, 1999).

Broth inoculation is often done using a dilution factor of 10: 1 part of feces to 10 parts of broth (e.g. 1.0 g of feces to 10.0 ml of broth; Baltasar et al., 2014). In some cases, larger amounts fecal material were used in larger volumes of broth, still obtaining approximately the same concentrations (e.g. 25.0 g of feces in 225 ml of broth; Khandaghi et al., 2011). Evaluation of effectiveness of different ratios demonstrated that smaller volumes of broth resulted in a greater numbers of fecal samples positive for targeted isolates (Evans et al., 2011). It has also been observed that higher prevalence of target isolates were recognized when samples were run in duplicate (Evans et al., 2011).

Reports stating that the presence of bile salts might alter characteristics of some STEC strains (e.g. growth inhibition of previously injured cells and antimicrobial resistance) have led some researchers to use a modified version of *E. coli* broth, from which bile salts had been removed (Kobayashi et al., 2001b). Considering that bile salts are the selective component in *E. coli* broth, by removing them, the broth becomes non-selective, similar to TSB (Kobayashi et al., 2001b). MacConkey broth could prevent growth of most Gram-positive enteric bacteria, but not select against non-STEC Gram-negative bacteria (Pradel et al., 2000; Renter et al., 2005). A widely popular broth utilizes a selective media, such as *E. coli* broth or Gram-negative broth, enhancing its selectiveness with the addition of antibiotics (e.g. novobiocin 20 mg/l) (Byrne et al., 2003; Ding et al., 2009; Heuvelink et al., 1996; Kleiss

et al., 1995). This is also a disputed procedure since inhibition of some STEC strains by this antibiotic has been documented (Cobbold et al., 2004; Jeon et al., 2013; Kanki et al., 2011). Another type of broth utilizes a non-selective broth like TSB, modified by the addition of novobiocin (8 mg / l), vancomycin (16 mg / l), rifampicine (2 mg / l), bile salts (1.5 g / l) and potassium tellurite (1.0 mg / l) (Joris et al., 2011; Narvaez-Bravo et al., 2007; Posse et al., 2008; Vimont et al., 2007). Meanwhile, adaptations of this method have also included (in addition or as a replacement) LB or BPW broths, with addition of antibiotics like cefsoludin, cefixime, acrifavline, and streptomycin (Chapman et al., 1997; Cobbold & Desmarchelier, 2000b; Kerr et al., 2001; Ogden et al., 2004; Scott et al., 2009). A limitation of these broths is that they have only been validated for a limited number STEC strains, and it is not known whether other STEC would be excluded, or have its growth favored (Posse et al., 2008). Comparing the modified TSB broth described in Posse et al., (2008) with EC broth, it was shown that, by using EC broth alone, there was a one log increase in detection limit, upon testing samples with multiplex PCR (Paddock et al., 2012).

Enrichment tubes maybe held static or gently agitated during incubation. In general, static incubations are used, but some researchers have preferred to agitate the broth tubes during incubation (Renter et al., 2005). To date, little has been done directly compared recovery between static or agitation incubations.

Enriched fecal samples may then be screened for STEC serogroup-specific genes and/or virulence genes by PCR. Generally, a sub-sample of the enriched sample is removed, boiled and centrifuged to lyse the bacterial cells, releasing any DNA into the supernatant. The DNA must then be extracted and purified from the supernatant by phenol-chloroform extraction, or any number of commercial kits. A single or multiplex PCR may then be run on the purified DNA to determine if any genes of interest are present (Baltasar et al., 2014; Paton & Paton, 1998a). The advantage of screening fecal sample is the number of samples that need to be further process may be reduced. While decreasing the number of samples that need to be further processed can greatly decrease cost and logistical concerns, there are disadvantages and concerns with screening samples by PCR. First, while it is generally accepted that PCR is more sensitive than culture based methods, this may not always be the case. PCR screening must be completed swiftly to know which samples will need further processing, hence enhancing their viability. Also, if more than one gene is examined prior to isolation (i.e. a STEC serogroup and stx), the method cannot determine if those genes were in the same isolate. This method relies on gene specific primers, if base pair mutations occur in the primer region of the target genes the PCR may no longer detect that gene. Immunomagnetic separation (IMS) is commonly performed on enriched fecal broths. The procedure consists of mixing a small aliquot of enriched fecal broth with

metallic beads coated with antigens specific to a serogroup (Olsvik et al., 1994). The fecal broth bead mixture is gently homogenized and incubated. Bacteria with matching surface LPS link to the bead surface antigens, and are then extracted with magnets, washed and plated on agar for colony growth. This method has the advantage of concentrating the bacteria of interest while excluding background bacteria, thus providing high specificity and sensitivity (Olsvik et al., 1994). However, there are also important disadvantages: each bead is specific to a target serogroup, so the procedure and subsequent plating must be repeated for the number of serogroups targeted. Currently, only two commercial companies around the world produce magnetic beads, and shortage is not uncommon. Also, beads are only commercially available for 5 serogroups (O26, O103, O111, O145 and O157), although blank beads are available and the procedure to coat beads in a serogroup of the researchers choosing has been published (Olsvik et al., 1994).

Regardless of the material yielded by the previous step (commonly bacteria/bead complexes or enriched broth), isolates in these materials must be plated for colony growth, selection and isolation. Even after selective enrichment step, there is almost always contamination with extraneous microorganisms. To minimize this, while some researchers have chosen plating on simple non-selective agars (e.g. blood agar) (Beutin et al., 1997; Bollinger et al., 2007), others have preferred semi-selective media, such as MacConkey agar (Alali et al., 2004; Alam & Zurek, 2006; Orskov & Orskov, 1992; Wells et al., 1991). Others still, have preferred more selective plating media, possibly at the risk of sacrificing some *E. coli* isolates as well (Chapman et al., 1997; Costa et al., 2011; Davis, 2010). When random colonies are chosen from these agar plates and tested for STEC, success often depends on the ratio of STEC to non-target bacteria (background flora), as well as the ability to differentiate colonies based on morphologic characteristics. To avoid operator discrepancies and consequent bias, some have added a DNA-based step at this point, to help separation of STEC from mixed colony populations (Jenkins et al., 2003).

Cultural isolation of STEC from foods and feces is time-consuming, labor-intensive, and costly. Thus, rapid immunological detection systems have been developed, which significantly reduce the time spent with analysis. These methods include enzyme-linked immunosorbent assay (ELISA), colony immunoblot assay, direct immunofluorescent filter techniques, and several immunocapture techniques: serotyping for the “O” and “H” antigens. Both polyclonal and monoclonal antibodies specific for the O and H antigens are used for these methods. Many of these test systems are able to detect concentrations of less than one O157 STEC cell/g of raw meat after overnight enrichment. Presumptive results can be available after just 24 h; however, isolation of the organisms is

still necessary for further DNA-based confirmation. The primary use of these procedures is to identify food and fecal samples that might be contaminated with STEC. This can be significant drain on time and resources, depending on how many colonies are to be selected.

To date, little work has been done comparing different STEC agars, possibly due to inconsistency of biochemical properties. For example, Hiramatsu and colleagues tried to develop an agar specific for the serogroup O26; it was based on known rhamnose fermentation deficiency, and resistance to cefixime and potassium tellurite. When researchers examined the growth of 102 pathogenic strains of O26 in their newly created agar, only 89 were able to grow, and seven of these were able to ferment rhamnose (Bielaszewska et al., 2005; Hiramatsu et al., 2002).

Without a reliable selective plating media, problems are exacerbated when fecal samples are analyzed. Fermentation characteristics of the background flora cannot be predicted and colonies may phenotypically resemble a target serogroup. While research has taken advantage of the serogroup-specific plating medium for O157 (CT-SMAC) for many years, the search for equivalents that can be used for other serotypes is a relatively recent one (Eblen, 2007).

Escherichia coli as a Model Microorganism

E. coli is the epitome of microbial life, and the best described organism known to science: laboratory strain K12 was one of the first organisms to have its genome completely sequenced and published by *Science*, in 1997 (Blattner et al., 1997; Stein et al., 2002).

Its simple nutritional requirements, fast reproduction and growth rates, ability to grow on chemically defined media, and an extensive genetic toolbox, all make *E. coli* a perfect model organism, and one particularly useful in studies involving molecular genetics, general prokaryote physiology and morphology, and biotechnology (Cronan, 2014; Wang & Levin, 2009).

In biology, a "model" is a living organism (plant, animal, or microbe) that can be used to simulate a given biologic system and/or process (Cronan, 2014). Model organisms have the advantage of providing a common denominator for comparison, and are also part of the strategy to mitigate the use of potentially unethical and/or unfeasible animal and human experiments (Cronan, 2014; Royal Society, 2015). In order to serve as a "model", the organism should be simple, generally well described and understood, widely accessible, and easily manipulated and

maintained (Goldsmith, 1993; Griffiths et al., 2009; Mortlock, 2012). This concept is based on different species sharing similar genes, which have been inherited from common ancestors (e.g. humans and yeast). In any given organism, the same genetic sequence is conserved in the nucleus of every somatic cell; three-letter sequences of DNA molecules are read by ribosomes and converted to amino acids, which then group into proteins. The essence of this coding system is that correspondence between gene-sequence(s) and resulting protein(s) seems to be universal. As many DNA sequences are shared down the evolutionary tree, it is possible to replace genes in model organisms with those of other species without affecting protein function, allowing observation of how similar genes respond to changing experimental conditions across species (Gallup; Griffiths et al., 2009; Mortlock, 2012).

Some cultivated laboratory strains (e.g. *E. coli* K12) are so well adapted to artificial environments that all ability to colonize the GI tract has been lost. Also, several of these have lost their ability to form biofilms (Lederberg & Tatum, 1946; Vidal et al., 1998). While some such features can be protective against antibodies and other chemicals, they require a large expenditure of energy and material resources seldom available in natural environments.

Serotypes and Diversity

There are many different types of *E. coli*, which are commonly referred to as strains, serogroups, or serotypes. The bacteria are serotyped based on surface antigens, specifically the somatic (O), flagellar (H), and capsular (K) antigens (Orskov et al., 1977). Currently, there are from 174 to 181 recognized O antigens (some of which are still being debated), and 56 H antigens defined in the serotyping scheme; however, non-motile (NM) strains have also been identified (Gyles, 2007; Wang et al., 2003). If antigens O, H, and K are be found in nature in as many as the potential combinations, the number of *E. coli* serotypes is could be in excess of 100,000. The number of frequent pathogenic serotypes is, however, limited. Two main groups of such frequent serotypes are (a) serotypes from diarrheal disease and (b) serotypes from extraintestinal disease (Gyles, 2007).

In 1985, the first new species in the genus Escherichia was described, *E. fergusonii* (Farmer et al., 1985). In 2003, a second new species integrated the genus, *E. albertii* (Huys et al., 2003). Hyma et al. (2005) described the evolutionary relationship of *E. albertii* to *E. coli*, and its identity to the diarrheal pathogen *Shigella boydii* serotypes 7 and 13. All of the other named species and serotypes of Shigella are actually members of *E. coli* (Sims and Kim,

2011). There are four other named species of Escherichia: *E. blattae*, *E. senegalensis, E. vulneris*, and *E. hermannii*, but strains of these species are only distantly related to other Escherichia and are not valid members of the genus (Walk et al., 2009).

To date, several BST (bacterial source tracking) methods that attempt to link various animal sources (e.g., geese, humans, cows, deer, gulls, etc.) with types of fecal contamination have been evaluated. Typically, these methods use phenotypic traits, such as antibiotic resistance and other biochemical properties (Hartwood et al., 2000; Parveen et al., 1997), but genotypic profiles generated by molecular methods have greatly expanded scientific understanding of the evolution, ecology, epidemiology, diversity, and population genetics of bacteria, including STEC. These methods include: pulse-field gel electrophoresis (PFGE); multi-locus sequence analysis (MLSA) (Maiden et al., 1998); multiple-locus variable number tandem repeat analysis *(*MLVA*);* repetitive sequence based-PCR (rep-PCR), which rely upon the presence of repetitive DNA sequences (Baltasar et al., 2014; Carson et al., 2003; Dombek et al., 2000); or determination of host-specific 16S rDNA genetic markers to match bacterial fingerprints isolated from a given event (e.g. outbreak or environmental contamination) to bacterial fingerprints isolated from specific host species (Bernhard & Field, 2000); ribotyping (Carson et al., 2001; Hartel et al., 2002; Parveen et al., 1999); denaturing-gradient gel electrophoresis (Buchan et al., 2001); and phage-typing (Chapman et al., 1997). Coupled with the growth fingerprint-based and whole genome sequencing databases, there is an increasing number of studies isolating and characterizing *E. coli* from non-clinical sources (Bernhard & Field, 2000; Elviss et al., 2009; Ezawa et al., 2004). Consequently, understanding of bacterial diversity has increased enormously. These studies have revealed substantially more genetic variation in the genus Escherichia, and five 'cryptic clades' of Escherichia have been identified (Walk et al., 2009).

Sources and Ecology

E. coli is a ubiquitous bacterium; it has been isolated from water, soil, rocks, plants, farm equipment, meat, and feces from a wide variety of animals. Although more frequent in the intestines of homeothermic vertebrates, the bacteria have also been recovered from ectothermic animals. The infection strategy of *E. coli* is to colonize a mucosal site, evade host defenses, and multiply while damaging the host's intestinal lining and competing with other members of the commensal microbiota. The infectious dose for STEC O157:H7 is not known with precision, but it

has been suggested to be relatively low: somewhere between 2-45 organisms, up to 100 organisms (Mellies et al., 2007; Scheffe, 2007; Smith & Linggood, 1971).

Ruminants are the prime reservoir of STEC, intestines of whom the organisms commensally inhabit. Once excreted with feces, the bacteria are free to contaminate cattle hides, meat and/or any other products or surfaces contacting the contaminated hides during management, transportation and slaughter processes (Erickson & Doyle, 2007; Shinagawa et al., 2000). Although this might seem straightforward, it is not a simple task for the microorganisms either: before any growth, multiplication and spreading can occur, any microbes trying to colonize the gut of animals will first have to survive the effect of salivary enzymes, as well as the mechanical effects of tongue and teeth. In cattle and other ungulate ruminants, microorganisms are then swallowed and travel down the esophagus into the first compartment of their composed stomachs: the rumen. The rumen contains a complex ecosystem of microbes, whose commensal task is to break down fibrous materials that would, otherwise, remain mostly indigested throughout the gastrointestinal tract. The presence of these microorganisms starting in the rumen (rather than in more distal portions of the gut) improves the efficiency of food breakdown and nutrient absorption. The rumen microflora is also able to use non-protein nitrogen, fixating it into bacterial proteins, which can then be digested and used by animals. Any microorganisms seeking to thrive in this environment will not only have to compete with others, but also resist continuous rumen motility, regurgitation, cud chewing, re-exposure to saliva, and re-swallowing. These mechanical and chemical stresses serve the main purpose of mixing ingesta, and reducing its particle size prior to further digestion. The rumen is a complex ecosystem, where fermentation of dietary components contributes to the growth of bacteria and production of volatile fatty acids (VFAs). Bacterial species compose most of the microflora, but the presence of bacteria-ingesting prokaryotes is indicative of a healthy ruminal ecosystem. Bacterial species can usually be classified according to their preferred substrates as: cellulolytic, pectinolytic, ureolytic, sugar-utilizing, acid-utilizing, proteolytic, lipid-utilizing, hemicellulolitic, and amylolytic species. Thus, type of diet directly affects the balance between gastrointestinal bacteria species; for example, intensive cattle production systems, where animals are fed concentrates containing mostly carbohydrates (sugars and starches), pectins, and some fibers (cellulose and hemicellulose), will have animals producing acetate, propionate, and butyrate, which lower rumen pH. At low pH conditions, acetate production declines, and propionate production increases. Further drop will favor microorganisms that produce lactate, which will then increase and balance the pH (Aharoni et al., 2009; Callaway et al., 2009a; Nutrition, 1996). In sum, rumen microbial growth is affected by such

factors as: the composition of feed (substrate); size of particles (affecting where and in which ruminal layer the particles will end up); feeding pattern, salivation (fluid and mineral flow), intake (affecting passage rate and how long particles stay in the rumen), rate of carbohydrate fermentation; and availability of substrates for microbial growth (Callaway et al., 2009a). Still, microbial activity does not end here, continuing throughout the GI tract. Microorganisms that survive the rumen and continue to more distal portions of the gut will have to endure the presence of more digestive enzymes, peristaltic movement, host immune system attacks, and further competition with pre-established microflora (Pacheco & Sperandio, 2012; Paton & Paton, 1998b; Porter et al., 1997; Zhao et al., 2013).

As environmental conditions in the lower gastrointestinal tract are considerably different from those found on hides, in soil, sediments, or water, enteric bacteria have had to develop mechanisms to cope with both internal host and external environments. Although there is a growing understanding of the molecular and physiologic basis of *E. coli*'s interaction with intestinal cells, there is much yet to learn about the phenotypic responses that allow rapid adaptation to such radically dissimilar conditions, as well as about the activation mechanisms of stress-induced genes (Bolton et al., 1999; Bolton et al., 2011).

In general, non-O157 STEC respond to stresses such as acid, heat, and other stresses induced during food preparation similar to O157 STEC (Buncic et al., 2014; Duffy et al., 2014); however, *Stx*-carrying phages are also widespread in the environment, and seem to be more resistant to chlorination, pasteurization, and composting than their host bacteria (Martinez-Castillo & Muniesa, 2014; Muniesa et al., 1999).

Virulence Factors and Pathogenesis

Although other *Escherichia* bacteria can be pathogenic (e.g. *E. albertii* is associated with diarrheal disease in Bangladeshi children), *E. coli* is the best-known and studied pathogen in the genus *Escherichia* (Abbott et al., 2003). Based on pathogenesis, there are four to six groups (some researchers combine groups) of *E. coli* serotypes, often called pathotypes, which cause illness with different characteristics. The nomenclature is derived from description of the type of lesions and strategies of attack utilized by specific pathogenic strains: EHEC enterohemorrhagic *E. coli* (EHEC); enterotoxigenic *E. coli* (ETEC); enteropathogenic *E. coli* (EPEC); enteroinvasive *E. coli* (EIEC); enteroadherent *E. coli* (EAEC); diffuse-adhering *E. coli* (DAEC); and

enteroaggregative *E. coli* (EAggEC) (Orskov & Orskov, 1992). Despite a multitude of strategies used to distinguish strains of *E. coli*, there is much overlap in the mechanisms of pathogenesis of various pathotypes. Similar virulence pathways may be pursued by more than one type of strain, and microbial evolution means that researchers are always one step, perhaps more, behind bacterial ability to adapt and develop new pathogenic traits (Bollinger et al., 2007; Jenkins et al., 2003; Khan et al., 2002).

Pathogenicity of STEC is thought to be associated with various genes, including those encoding for Shiga toxins (*stx*1 and *stx*2) and Intimin (*eae*A), which are located in the bacterial chromosome and are responsible for inhibition of protein synthesis and attaching-and-effacing enteric lesions, respectively (Bolton, 2011; Farfan & Torres, 2012). Another relevant virulence factor is *hly*A, a plasmid gene encoding for an enterohemolysin (Nguyen & Sperandio, 2012).

Analysis of the toxins coded by *stx*1 and *stx*2 genes revealed that they are AB-type toxins: both toxins are composed of five B subunits and one A subunit, which are encoded on a temperate bacteriophage and inserted into the *E. coli* chromosome. The B subunits bind to globotriaosylceramide (Gb3) receptors, glycolipids of unknown function found in membranes of eukaryotic cells. After endocytosis, the A subunit enzymatically inactivates the 60S ribosomal subunit, thus blocking protein synthesis (Konowalchuk et al., 1977a; Konowalchuk et al., 1977b; Smith & Linggood, 1971). The *stx* genes are either encoded on an inducible prophage that can switch into the lytic phase to produce more virus particles, or the genes may be passed down by being present on prophage remnants in the *E. coli* chromosome (Fogg et al., 2012). The lambda bacteriophage can infect a variety of bacterial strains, promoting exchange of genetic material between them. This form of phage genome inserted and integrated in the bacterial genome or in an extrachromosomal plasmid is called a prophage, i.e. a latent form of the phage, in which the viral genes are present in the genome without causing disruptions to the bacterial cell (Recktenwald & Schmidt, 2002).

The DNA sequence of *stx*1 is highly conserved, with only a few recognized variants: *stx*1a, s*tx*1c, and s*tx*1d. This toxin is also indistinguishable from a toxin produced by *Shigella dysenteriae* type 1, hence the term "Shiga-like toxin" (Burk et al., 2003; Paton et al., 1995). Meanwhile, the sequence of *stx*2 is markedly more divergent, resulting in proteins with only 56% aminoacid homology with Shiga toxin 1. To date, more than 20 variants of *stx*2 have been described: *stx*2, s*tx*2v, s*tx*2va, s*tx*2vh-a, s*tx*2vh-b, s*tx*2vh-c, s*tx*2v-ox392, s*tx*2v-ox393, s*tx*2c, s*tx*2d-ox3a, s*tx*2ox3b, s*tx*2d-ount, s*tx*2O118, s*tx*2O111, s*tx*2O113, *stx*2O48, *stx*2e, *stx*2ev, *stx*2NV206, *stx*2f, *stx*2g, *stx*2h, and *stx*2i (Bertin et al., 2001; de Sablet et al., 2008; Gannon et al., 1990; Ito et al., 1990; Paton &

Paton, 1998a; Paton et al., 1993; Paton & Paton, 1998b). These molecules may be secreted alone or in combination, and variants seem to display different levels of toxicity: for example, *stx*2 variants *stx*2a and *stx*2d are up to 25 times more virulent than *stx*2c and *stx*2b (Fuller et al., 2011). Since there have been different nomenclature schemes applied to variants of *stx*2 without re-testing or using methods similar to the original, it is possible that, at this point, there is some nomenclature overlap, with different names for the same sequence.

Even though the preferred receptor for generic *stx*1 and *stx*2 is globotriaosylceramide (Gb3), variants may show different affinities for other molecules (e.g. the preferred receptor for *stx*2e, a variant of *stx*2 produced by strains of *E. coli* that cause edema disease in swine, is globotetraosylceramide – Gb4). Humans, rabbits, and pigs have vascular receptors for *stx* and develop *stx*-mediated vascular damage. Shiga toxin type 2 has better affinity for the Gb3 receptors than has Shiga toxin type 1, making it many times more powerful than Shiga toxin type 1 (Fuller et al., 2011; Tesh et al., 1993). This is the likely explanation as to why *E. coli* producing Shiga toxin type 2 is more prevalent amongst HUS patients than *E. coli* producing Shiga toxin type 1 alone (Fogg et al., 2012; Fraser et al., 2004; Fuller et al., 2011; Mead & Griffin, 1998; Slutsker et al., 1997).

In cattle, the *Stx* receptor globotriaosylceramide (Gb3) is found in kidney and brain tissues, but not in the gastrointestinal tract or blood vessels (Pruimboom-Brees et al., 2000). This is the most likely explanation as to why cattle have such a close relationship and pacific coexistence with STEC.

The mechanism by which colonization of the lower GI tract occurs is best described for STEC that contain the "locus of enterocyte effacement" pathogenicity island, or "LEE region". Using a type III secretion system, STEC secretes an epithelial receptor for intimin (encoded by *eae*A) called Tir (translocated intimin receptor) into the host cell, along with other secreted proteins (Kenny et al., 1997). This intimate adherence of STEC to the host intestinal epithelium through the Intimin/Tir complex allows the bacteria to become colonized in the host gastrointestinal tract, originating the characteristic "attaching and effacing" (A/E) lesions. These A/E lesions result in loss of enterocyte microvilli, and in formation of pedestals from accumulation of cytoskeletal components (Sherman et al, 1988). At least 33 variants of *eae*A have been identified, encoding proteins with different C-terminals, which bind Tir (Garrido et al., 2006). It is generally recognized that intimin is required for STEC pathogenesis; however, alternative forms of adherence strategies have been proposed (Paton et al., 1998; Vidal et al., 2008). Vidal and colleagues (2008) studied adherence to epithelial cells in *eae*-negative STEC, finding that *saa* and a *psu*-int region

participate in the adhesion process. Putative adhesion genes *efa*1 and *lpf*AO157 have also been described (Vidal et al., 2007).

Multiple copies of *stx*-harboring prophage can be present in a single bacterium, but such configurations have demonstrated instability (Fogg et al., 2012). Loss of the prophage from STEC has been documented both in human infections and cattle-harbored isolates (Bielaszewska et al., 2007; Junqueira et al., 2010).

Detection of Shiga toxins or *stx* genes alone (the common threat among STEC) is not sufficient to elaborate definite statements regarding pathogenicity of *E. coli* isolates; many other virulence factors seem to be implicated in pathogenicity: for example, a virulence plasmid (pO157), and the locus of enterocyte effacement (LEE). The 60MDa plasmid encodes a hemolysin that, associated with specialized transport systems, may allow *E. coli* to use host blood released into the intestine as a source of iron. The LEE contains genes for an adhesion molecule (intimin) and for other factors important to the production of attaching-effacing lesions. As seen before, one of several unique characteristics of gram-negative bacteria is the structure of the bacterial outer membrane: the outer leaflet of this membrane comprises a complex lipopolysaccharide (LPS), whose lipid portion acts as an endotoxin. When gram-negative bacteria enter the circulatory system, the LPS can cause a toxic reaction. The LPS is also what identifies the O-group of the bacteria, while the flagella antigen characterizes the H-group of a given strain.

Risk Factors for Human Infection

Human infection with STEC most frequently develops after ingestion of food contaminated with feces. Those who consume raw or undercooked foods and/or drinks, especially undercooked ground meat, are at greater risk of STEC infection. At lower risk are those consuming treated or pasteurized fluids, as well as appropriately treated or cooked foods, thus, such practices are highly advised. In addition, any food or liquid involved in a recall due to possible *E. coli* contamination should be disposed of immediately. For example, in August of 2010, about 1 million pounds of beef was recalled, in California, due to potential *E. coli* O157:H7 contamination (Bottemiller, 2010). More recent recalls have included, beyond ground beef, components placed into dry pet foods, spinach, and alfalfa sprouts (Marlet, 2016). Recalls of *E. coli*-contaminated foods are usually voluntary, but, if a company refuses to recall its products, the U.S. Food and Drug Administration (FDA) and U.S. Department of Agriculture's Food Safety and Inspection Service (FSIS) have legal authority to detain and seize potentially contaminated food products

from the market (CDC, 2011; FDA, 2011; USDA, 2011)

In the "farm-to-fork" continuum, individuals who, due to the potential for occupational environmental exposure, are at greater risk of infection include: those working directly with livestock; daycare workers and attendees; those living with daycare attendees or livestock handlers; food handlers; recreational water users; and those who travel to locations lacking basic standard food hygiene practices (Lang et al., 2007).

Host physiologic factors also come directly into play: those who are immunocompromised, the very young, and the elderly are at greater risk of infection with lower doses of microorganisms, and are also more likely to suffer from complications and sequelae, some of which can be handicapping and/or life-threatening (Thorpe, 2004).

Clinicopathologic Properties and Public Health Impacts

It is estimated that more than 8.9 million Americans suffer from foodborne illness each year. In 2013, 63,153 cases of infection with *Escherichia coli* O157 were registered, with Non-O157 Shiga toxin-producing *Escherichia coli* accounting for 112,752 (Hoffmann et al., 2012). Foodborne illness caused by the 15 most common foodborne pathogens costs the economy more than $15.6 billion yearly. Of this, *Escherichia coli* O157 is responsible for about $271,418,690, and Non-O157 Shiga toxin-producing *Escherichia coli* for $27,364,561 (Hoffmann et al., 2012).

Escherichia coli O157 was first classified as a human pathogen in 1982, during investigation into an outbreak of hemorrhagic colitis associated with consumption of hamburgers from a fast food chain restaurant (Riley et al., 1983). As the serotype responsible for most human outbreaks of *E. coli*-implicated illness, serotype O157:H7 has been considered a food adulterant since 1994, after yet another mediatic outbreak involving a fast food chain restaurant (Donnenberg, 2013). However, incidence of human illnesses caused by non-O157 STEC serotypes has increased nearly eight-fold between 2000 and 2010 (Bettelheim, 2007; Brooks et al., 2005; Eblen, 2007; USDA, 2011). The top human disease causing STEC serotypes in the United States are O157, O26, O103, O111, O121, O45, O145, O124, O118, O69, and O128 (Scallan et al., 2011). As their public health impact has been progressively recognized, the list of *E. coli* serotypes considered food adulterants expanded, in 2012, to include serotypes O26, O45, O103, O111, O121, and O145 (Bettelheim, 2007; Brooks et al., 2005; Eblen, 2007; USDA, 2011).

Outbreaks of STEC human illness have been linked to consumption of contaminated fresh produce and other commodities (e.g. non-pasteurized dairy products), but 69% of these are attributed to consumption of undercooked contaminated beef, especially ground beef (Painter et al., 2013). Most STEC infections last about a week and tend to resolve spontaneously with little or no treatment; however, supportive treatment becomes an urgent necessity if the patient becomes dehydrated, anemic, or develops further complications. If complications do develop, such as severe dehydration, anemia, hemolytic uremic syndrome (HUS) or thrombotic thrombocytopenic purpura (TTP), the outcomes can decline from good to poor very quickly (Paton & Paton, 1998b).

After an incubation period of 3 to 5 days, the characteristic features of STEC infection include a short period of abdominal cramps and non-bloody diarrhea, with or without significant fever. In about 90% of the cases, watery diarrhea progresses to hemorrhagic colitis, a condition characterized by the presence of blood in the stools. In most infected individuals, these symptoms last about a week, and resolve without any long-term problems or medication. However, the real life impacts of *E. coli* infection can vary widely between individuals, depending on the virulence of the STEC strain and host immune system condition. Up to 61% of cases in outbreaks of STEC illness develop hemolytic uremic syndrome (HUS), a condition characterized by hemolytic anemia and thrombocytopenia, which may culminate in renal failure, with case fatality risks of 5% to 10% (Boyer & Niaudet, 2011; Scallan et al., 2006).

Detection Methods and Diagnosis

Infection with *E. coli* O157:H7 or other Shiga toxin-producing *E. coli* is usually confirmed by detection of the toxin-producing bacteria in stool specimens from infected individuals (CDC, 2011). Furthermore, there are tests designed specifically for the screening of food products, especially beef.

Shiga toxin-producing *E. coli* are a heterogeneous group of microorganisms, which have in common the carriage of phage-encoded *stx* genes and, with the exception of the O157 serotype, no biochemical markers are available to facilitate identification (Eblen, 2007; Paton & Paton, 1998b). Efforts to isolate and characterize STEC other that O157 require more sophisticated methods, such as ones targeting portions of the bacterial genome. With new legislation being implemented (adding STEC O-groups O26, O45, O103, O111, O121 and O145 to the list of food adulterants), the establishment of specific, sensitive, fast, reproducible, simple, and accessible methodology for

detection of these serotypes is in demand (Buncic et al., 2014; Paddock et al., 2011; USDA, 2011). Currently, the Food Safety Inspection Services uses PCR to detect presence of *eae* and *stx* in samples. When these virulence genes are simultaneously present, O-groups are identified by immunomagnetic separation. However, until recently, magnetic beads for O-groups O45 and O145 were not yet commercially available (GAO, 2012).

Once colonies are grown on agar plates (even using agars with properties that should allow differentiation of suspect colonies) further testing is required to confirm virulence and serotype. Several methods exist for confirmation, each of which has advantages and disadvantages. One of the most comprehensive methods is known as colony hybridization (Paton and Paton, 1998). During this process, colonies are replicated on a nylon membrane with DNA oligonucleotides matching either *stx* or a specific serotype. Once positive colonies are identified, they can be transferred from the original plate and preserved. With this method, all colonies growing on a plate can be screened, but this can be very time consuming, particularly with large number of samples. All other confirmation methods involve sub-culturing a certain number of colonies for further testing. The number of colonies that are sub-cultured varies widely (ranging from 1 to 50 to "all"), and researchers seldom describe how they determined the number of colonies picked for sub-culturing. Sub-cultured colonies are commonly screened via PCR for *stx* or serotype-specific DNA. These PCR reactions have been combined into a one-step multiplex PCR to detect top virulence (including *stx*) and/or serotype genes (O26, O45, O103, O111, O121, O145 and O157) (Baltasar et al., 2014). While this method is quicker and easier than a hybridization method, it is limited by the fact that a separate reaction must be performed for every colony. Another method to detect *stx* presence is to grow suspect colonies in a broth, centrifuge a sample from the broth, filter the resulting supernatant, and then adding it to Vero cells to observe cytotoxicity (Alexa et al., 2011; de Boer et al., 1994). The gold standard test for serotype determination is agglutination with a standardized set of antisera (Alexa et al., 2011; Blanco et al., 1996); however, this is only practical for serotype reference labs that specialize in this method as a service, and keep the antisera in stock. Individual serotype antisera can be obtained, but this method is more subjective than PCR for serotype determination. A less subjective antisera-based method (latex agglutination) that is faster than PCR is available commercially for the O157 serotype, but it has yet to become commercially available for any other serotypes (Brown-Brandl et al., 2009; Dodson & LeJeune, 2005).

Another proven method to detect STEC contamination in food, especially ground beef and beef trim, is the BAX® (DuPont, Wilmington, DE) Real-Time assay. This is an automated method that uses polymerase chain

reaction (PCR) technology for detection of *E. coli* O157:H7 and other STEC serotypes. Upon enrichment in a broth provided by the manufacturer at 42°C for 10-24 h (or a researcher-selected broth), the system identifies specific DNA fragments, which are unique to STEC. The *BAX®* System uses a lysis buffer that, once added to the enrichment, breaks down cell walls and membranes, releasing chromosomal DNA. Tubes are heated for 20 minutes at 37 °C ± 2 °C and 10 minutes at 95 °C ± 3 °C, and then cooled in a cooling block. PCR tablets are hydrated with lysate and run in a BAX® System Q7 machine. Although this method has been developed to detect *E. coli* O157:H7 in meat products, the manufacturer has expanded the range of serotypes, and some authors have used it successfully to process fecal or RAMS samples (McDonough et al., 2000; Narvaez-Bravo et al., 2013; Stephens et al., 2007b).

As noted, characterization and comparison of bacterial isolates by contrasting restriction enzyme-digested chromosomal DNA fragments (fingerprints) is highly discriminatory, allowing similarities to be readily determined. Utility of a given technique dependents on suitable selection of restriction enzyme(s), and on the method selected to score similarities between fingerprints. Restriction enzymes are selected from amongst those that have suitable frequency of restriction sites for a given enzyme-genome combination. Frequency of sites is calculated from the frequency of di-and tri- nucleotides in sequenced genes from the species of interest, using Markov chain analysis. Fingerprints form when PCR products are run side-by-side on electrophoresis gels, with DNA-size standards. The number, distribution, and intensity of bands are then scored, and scores are compared (numerical profiles) between isolates. Thus, a single electrophoretic gel yields analyzable data, which can be compared with data from other gels (Baltasar et al., 2014; Beutin et al., 1997; Buchan et al., 2001). These approaches have proven efficacy as BST tools, but they can be very time-consuming and labor-intensive. Thus, development of faster, reproducible, and less labor-intensive methods that could serve similar purposes is in demand. Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) has evolved quickly thanks to innovations like new ionization techniques and detection methods (van Baar, 2000). The time from sampling to final characterization is much reduced in comparison to traditional fingerprinting methods. The use of directly visualized restriction enzyme digests of chromosomal DNA (fingerprints) from bacteria offers a rapid and reproducible method for characterization and classification of isolates, applicable to ecological, epidemiological, and population genetic studies (Christner et al., 2014). However, when compared to BOX-A1R primer repPCR, MALDI-TOF seems to have less repeatability, but performs better as a BST tool (Siegrist et al., 2007).

Treatment

The disease induced by STEC can be exacerbated if *Stx* production in the gut is amplified. Antibiotic therapy to treat STEC infections is not recommended because stress induces Stx-prophages into the lytic cycle, resulting in a surge of toxin production. Evidence further suggests that Stx-phages can be transduced to commensal gut flora, escalating the severity of the disease. Thus, apart from supportive care, such as close attention to hydration and nutrition, there is no specific therapy to halt STEC symptoms (Karch et al., 2005; Allison, 2007).

The finding that *E. coli* O157:H7 initially speeds up blood coagulation may lead to future medical therapies that could mitigate complications (Mead & Griffin, 1998).

Prevention Strategies

Prevention of infection requires control measures at all stages of the food chain, from agricultural production on the farm to processing, manufacturing, and preparation of foods, in both commercial establishments and household kitchens. Education in hygienic handling of foods directed at farmhands, abattoirs workers, and those involved in the food preparation at all scales is essential to keep microbiological contamination to a minimum (FAO & WHO, 2009). Once contaminated, the only effective method of eliminating STEC and other potentially harmful bacteria from foods is to introduce a bactericidal treatment, such as heating (e.g. cooking, pasteurization) or irradiation. The Hazard Analysis and Critical Points (HACCP) is a. For STEC in products of cattle origin, HACCP recommends several procedures that can be implemented at the farm, at slaughter, and by the consumer (Havelaar et al., 2010b; Tauxe et al., 2010).

Pre-harvest and Harvest Control in Cattle

Growing international trade, migration and travel have accelerated the dispersal of foodborne and other illnesses, including STEC infections. This has created conditions under which a single source of contamination could spread to people and animals worldwide (Tauxe et al., 2010). The number of cases of disease might be reduced by implementation of various mitigation strategies at the pre-harvest and harvest stages (e.g. screening animals pre-slaughter to reduce introduction of large numbers of pathogens in the slaughtering environment). Good

hygiene prior and during slaughtering practices reduces contamination of carcasses by feces, but does not guarantee the absence of STEC from all cattle-derived products (Buncic et al., 2014).

At the production level, the first critical control point is "reduction of STEC carriage/shedding by animals". Several on-farm procedures have been suggested, which have the potential to (at least in theory) reduce fecal shedding of STEC in cattle. These procedures fit roughly into two categories: reduction of primary exposure through modification of management practices, and interventions to increase animal resistance (Callaway; Callaway et al., 2009b). Reducing animal shedding and consequent hide contamination is likely to increase the effectiveness of well-established generic harvest procedures implemented in abattoirs around the world (Akanbi et al., 2011; Bach et al., 2004; Bonardi et al., 2004; Boqvist et al., 2009).

Simulation research has confirmed that the death rate of microorganisms like *E. coli* O157:H7 is increased with increased cleanliness of chutes, beds, and pens (Vosough Ahmadi et al., 2007). This supports the argument that even basic hygiene practices can, and do, have an impact in cross-contamination of animals (Loneragan & Brashears, 2005). To date, vaccination with attenuated O157:H7 strains has not been effective, likely due to the selection of strains that have lost some of its virulence factors. Such strains do not cause the typical attaching and effacing lesions. Also, since STEC are part of the natural microbiota of cattle, their presence does not stimulate immunity, and vaccination with surface antigens does not result in immunization of animals against subsequent infections (Gyles, 1998). Still, vaccination with type III secreted proteins has been reported to reduce fecal shedding in experimentally infected calves. Thus, research in this field is ongoing (Allen et al., 2011; Ransom et al., 2003; Vogstad et al., 2013). Another major area of focus is the "use of competitive exclusion", in which a microbe that is capable of out-competing the target pathogen is used. The administration of harmless microorganisms (probiotics), such as *Bifidobacteria, Lactobacillus,* or *Butyrivibrio*, with a similar microbiological niche to *E. coli* O157:H7 can reduce the amount of organisms that achieve attachment to intestinal cells, thereby limiting the ability of (at least) *E. coli* O157:H7 to colonize the intestinal tract (Brashears et al., 2003; Shaw et al., 2004; Stephens et al., 2007a; Stephens et al., 2010). The use of antibiotics to control *E. coli* O157:H7 shedding in cattle is controversial. Concerns that widespread use of antimicrobials to reduce STEC populations (particularly *E. coli* O157) may result in selection of antimicrobial-resistant bacteria have halted progress in this area. In the experimental studies that have been undertaken, administration of antibiotics like neomycin was successful in reducing shedding (Ransom et al., 2003). Feeding ionophores (e.g. monensin and lasalocid) has been used to reduce microbial populations and increase

feed/gain ratios; however, these antimicrobials inhibit populations of Gram-positive bacteria in the gut, thereby having the potential to give Gram-negatives, such as STEC, competitive advantage. Researchers that focused on trying different combinations of diet, ionophores, and antimicrobials have demonstrated that, for the most part, inclusion of ionophores either slightly increases shedding of *E. coli* O157, or has no effect (Herriott et al., 1998; Van Baale et al., 2004). The most promising of these combinations included addition of monensin and tylosin (tylan – antimicrobial) to a forage-based diet, resulting in reduction of *E. coli* O157:H7 populations up to 2 log10 CFU/ml in rumen contents (McAllister et al., 2006). Adding crude glycerin and/or distillers' grains with solubles (DDGS), co-products of ethanol production often used as protein and energy supplements in cattle diets, has been shown to increase fecal shedding of *Escherichia coli* O157:H7 (Jacob et al., 2008). Other feed additives that have been tried include ractopamine (β-agonist), organic acids, citrus products, seaweed (tasco), tannins, and phenolics. While some combinations have led to promising decreases in *E. coli* O157 colonization and shedding, most results have been inconsistent across studies (Callaway et al., 2009b). A feed additive that has let to more consistent reduction of STEC carriage, and has sparked some interest amongst cattle producers is sodium chlorate. Sodium chlorate is a relatively inexpensive odorless pale yellow to white crystalline solid. It is highly soluble in water and heavier, sinking and dissolving rapidly. Sodium chlorate seems to be effective because the intracellular bacterial enzyme nitrate reductase does not differentiate between nitrate and chlorate; therefore, the enzyme reduces the chlorate into chlorite in the cytoplasm, and chlorite accumulation is toxic to bacteria (Callaway et al., 2004; Skinner et al., 2005).

Bacteriophages (phages) are highly specific viruses that target bacteria, and are capable of delivering their DNA directly into the cytoplasm of bacterial cells. It has been suggested that modified phages could be utilized as "Trojan horses", to deliver lethal portions of DNA to bacteria of interest (Rozema et al., 2009). A problem with this strategy is the rapid development of bacterial resistance to a single phage; thus, rather than utilizing a single phage type, scientists have been working on "cocktail" mixes, with different phages, which can be used to target multiple serotypes and surface receptors. Also, any selected phage must be exclusively lytic to avoid transference of genetic material between bacteria, which might increase their pathogenicity (Brabban et al., 2005; Law, 2000).

When calves experimentally infected with *E. coli* O157 were fed a high-fiber diet had lower ruminal fatty acid concentration and higher pH than those fed a high-concentrate diet. Cattle fed a high-concentrate diet and switched to a 50% corn silage and 50% alfalfa hay diet prior to assessment, had lower counts of generic *E. coli* (Jordan & McEwen, 1998). In contrast, when cattle were switched from a forage-type diet to a high-grain finishing

ration, fecal and ruminal generic *E. coli* concentrations increased (Berry et al., 2006). This research work has proven that dietary changes during the finishing period have influence in *E. coli* counts. However, cost-benefit analysis is yet to be done, as well as the study of ways to make these dietary shifts more specific (Berry et al., 2006).

Fasting increased *E. coli*, *Enterobacter* and total anaerobic bacterial populations throughout the intestinal tract (Buchko et al., 2000; Gregory et al., 2000), and increased *Salmonella* and *E. coli* populations in the rumen (Brownlie & Grau, 1967). Thus, this approach is not recommended to control STEC populations.

Pre-harvest interventions to reduce cattle shedding of *E. coli* O157:H7 have shown potential to reduce environmental pathogen contamination at the farm level, transportation, slaughterhouse entrance and consecutive steps in the food chain. Still, as these pre-harvest strategies evolve, it must not be forgotten that there is no substitute for good hygiene and procedures in processing plants and food preparation environments. Live-animal interventions to reduce pathogens must be integrated in a logic succession of steps that complement in-plant interventions, and consumer education so that reduction or eliminations of pathogens in the food supply can be maximized.

The following critical control point involves the slaughter of animals. Fecal contamination of hides is believed to be a major source of contamination for slaughter equipment and any other surfaces that may come in contact with said hides. Preconditioning of animals prior to shipment has been proven to reduce shedding (Bach et al., 2004). Also, lower population densities in transport trailers and shorter rides have resulted in diminished hide contamination (Barham et al., 2002; Minihan et al., 2003). Thus, less stressful transportation conditions, increased cleanliness, and more space are essential conditions to maintain animal welfare, reduce stress, and minimizing introduction of potentially pathogenic microorganisms in the slaughter environment (Pfeiffer et al., 2009). Pre-evisceration sanitation of slaughter equipment is also highly recommended (Buchanan & Doyle, 1997; Vold et al., 2000). Additionally, steam vacuums and steam cabinets have been recommended to wash surface contamination off of hides (Arthur et al., 2007). A setback of this approach is that it has been shown that presence of background flora inhibits the growth of *E. coli* O157:H7; thus, application of non-specific elimination of microorganisms may open doors to further contamination by any microorganisms capable of surviving this step, some of which may be pathogens like STEC (Vold et al., 2000).

The method by which foods are processed and prepared for commerce represents another critical control point, which can influence food carriage of STEC and other foodborne pathogens. Traditionally, thermal

inactivation methods have been used to reduce the probability of pathogen survival in a food product (Buchanan & Doyle, 1997). A limitation of this practice is that food, particularly fresh produce, needs to be adequately sanitized while still preserving its raw character. Controlling *E. coli* O157:H7 in fruits, vegetables, dairy products, and juices has been mostly achieved with adequate pasteurization or exposure to ionizing radiation of products (Buchanan & Doyle, 1997). Irradiation, however, has prohibitive costs and is still seen with apprehension by a number of consumers: some have shared concerns that demonstrate a tendency to associate the concept of irradiation with exposure of food products to radioactive materials, which is not the case. Also, some bacterial and fungal toxins are not inactivated by currently used irradiation doses (Havelaar et al., 2010a).

The ultimate goal of these prevention strategies, although not without increased costs for the cattle production industry, has the potential to greatly reduce the risk of consumer outbreaks (Buchanan & Doyle, 1997). Unfortunately, most of these prevention strategies focused exclusively on *E. coli* O157, leaving out other serotypes of STEC that have been shown to be at least as concerning from a public health point of view.

Household-based Prevention

Although it is unreasonable to advocate avoidance of raw fresh fruits and vegetables that could be contaminated as a prevention measure, it is practical and highly recommended to educate consumers about food-safety practices that would limit contamination of uncooked foods within the consumer kitchen. These recommendations should in all cases be implemented, especially "cook thoroughly", so that the core of the food reaches at least 70 °C. Fruits and vegetables should always be washed carefully, especially if they are eaten raw. If possible, vegetables and fruits should be peeled. Vulnerable populations (e.g. small children, the elderly, immunocompromised) should avoid the consumption of raw or undercooked meat products, raw milk and products made from raw milk. Regular and proper hand washing (particularly before food preparation and/or consumption, and after toilet contact) is highly recommended (Sanders, 2001). Following these recommendations is particularly relevant for people who take care of small children, food handlers, immunocompromised individuals, and the elderly. The bacterium can be passed directly from person-to-person, as well as through food, water, and direct contact with animals, including those in petting zoos (Davis et al., 2005). A lesser-known fact is that STEC infections have been caused by contact with recreational waters (Soller et al., 2010). Thus, it is

especially important to protect waterways, wetlands, and water reservoirs from contamination with fecal materials, especially those from cattle operations (Bolton et al., 2011; Dorner et al., 2004). Also, drinking water should be treated with antibacterial non-toxic substances or filtered, particularly when sourced from open-air water reservoirs and waterways. Other complimentary home-based strategies include: avoid cross contamination of foods (surfaces, utensils); keep food refrigerated or frozen and properly separated; and make sure food is kept at appropriate temperature at all times (no fluctuations) (Scheffe, 2007).

Surveillance

The Centers for Disease Control and Prevention (CDC) defines epidemiologic surveillance as the "ongoing systematic collection, analysis, and interpretation of health data essential to the planning, implementation, and evaluation of public health practice, closely integrated with the timely dissemination of these data to those who need to know" (CDC, 2012).

Early recognition and resolution of outbreaks are crucial to prevent the spread of infections. The Center for Disease Control and Prevention has occupied a central role in the coordination of efforts leading to the recognition of outbreaks, and identification of their sources. As a result of this epidemiological work, in collaboration with State Public Health Laboratories and Health Departments, the amount of people affected by outbreaks can be limited (Cary et al., 2000).

Knowing where to look for different types of data can save much time and resources. Information may be gathered during outbreak investigations, in specialized research studies, and/or from public health network surveillance databases (CDC, 2011). Answering questions and improving prevention is the pinnacle of a continuous team learning effort towards improvement. When formal and/or informal surveillance detects a problem (e.g. STEC infections), a well-planned epidemiological investigation is the best available tool to clarify relevant clues about its etiology, source and impact. In some cases, particularly if the pathogen and its transmission routes are understood, control and prevention measures can be directly applied. In other cases, further research may be needed to answer new questions raised by the emergence of infection, and to interpret any epidemiological findings. Results of said research can then be used to develop and implement new or improved control and prevention strategies. In either case, surveillance further helps document the effectiveness of control and prevention efforts. With succession of

events, one hopes to see the incidence of infection decrease, as prevention becomes increasingly successful. Assembling sufficient evidence can be extremely time and resource consuming: information is often scattered, people are not always cooperative, and different individual interests may come into play. With greater attention and support, the wheel of public health control and prevention will eventually spin faster.

In the United States, the FoodNet system has been extremely useful order to monitor the burden and to conduct swift assessments of new threats, to generate consistent and comparable information despite variation in local notification requirements. Since outbreaks can be either local or widespread, with advent of subtype-based surveillance networks such as PulseNet, in the United States and other locations around the world, and EnterNet in Europe, more geographically dispersed outbreaks can be detected.

Prevalence and Distribution in Domestic Ruminants

Shiga toxin-producing *E. coli* strains are present worldwide, at prevalences that range from anywhere from 0 to 100% (de Boer et al., 1994; Geue et al., 2002). Differences in prevalence of cattle shedding O157 STEC, in survey studies, have been linked to factors such as diet, production stage, type of production, sex, distance to contaminated water-sources, contact with wildlife, seasonality, cattle breed, slurry application to pasture, weight, and animal population density. In randomized controlled trials, the following factors have been shown to affect shedding: administration of antibiotics; administration of probiotics; lack of pre-conditioning prior to transportation;

As primary reservoirs, ruminant animals are asymptomatic carriers of STEC. Prevalence in cattle seems to vary widely across studies, due to factors such as differences in target populations, sampling strategies, potential seasonal changes in prevalence, intermittent shedding, and differences in methodology of screening and isolation. Furthermore, access to farms/sampling sites is often off limits to researchers and representatives of State and Federal agencies (Fernandez et al., 2009; LeJeune et al., 2001).

In one of the most comprehensive reviews to date, Hussein and Bollinger described worldwide prevalences of *E. coli* O157 in cattle ranging from 0.7% to 27.3% in animals on irrigated pasture, 0.9% to 6.9% in those on rangeland forages, and 0.3% to 19.7% in feedlots (Hussein & Bollinger, 2005). Presence of non-O157 *E. coli* ranged from 4.7% to 44.8% in grazing cattle, and from 4.6% to 55.9% in feedlots (Hussein & Bollinger, 2005). The Animal and Plant Health Inspection Service (USDA) tested *E. coli* isolates from 1,305 fecal samples collected between 1991

and 1992 from dairy heifers. At the time, 5.9% of samples tested positive for *stx* genes through DNA hybridization, in what was one of the first surveys to expand knowledge beyond *E. coli* O157 in North American cattle (Cray et al., 1996). More recently, Cobbold et al. found animal prevalence of STEC in Washington State herds to be 8% in dairy cattle, 11% in range beef cattle, and 3% in feedlots (Cobbold et al., 2004). Barkocy-Gallagher et al. found *stx* genes to be harbored by 34.3% of beef cattle presented for slaughter in three large meat-processing plants in the Midwest, and non-O157 STEC were present in 19.3% of all samples (Barkocy-Gallagher et al., 2003).

In Australia, a longitudinal study in dairy herds found STEC in 14.6% of animals (Cobbold & Desmarchelier, 2000a). In Ireland, 40% (480/1,200) of fecal samples from dairy and beef cattle were positive for *stx* virulence genes through PCR. In Iran, a cattle survey found that 12.1% (51/420) of animals shed STEC, and 146 strains were isolated from these animals: 10.3% of these were O157, and the remaining were non-O157 (Tahamtan et al., 2010). In Japan, a total of 358 rectal swabs from dairy cattle in 78 farms were tested for presence of *stx* by PCR. Animal prevalence of 46% in calves, 66% in heifers, and 69% in cows was found. However, when nested PCR was used, all samples were turned out positive (Kobayashi et al., 2001a). In France, a study of beef cattle, dairy cattle, and young bulls found *stx* genes in 18.1% of fecal samples (Rogerie et al., 2001). In Brazil, prevalence of STEC in Minas Gerais 39.2%, (40/102) in beef cattle, and 17.5% (18/103) in dairy cattle (Oliveira et al., 2008). Clearly, prevalence of STEC in the SVAREC herd is at the high end of these values, but comparing data across studies is of limited value due to differences in target populations, sampling strategies, potential seasonal changes in prevalence, intermittent shedding, and differences in methodology of screening and isolation. For example, *E. coli* O157 tends to be more prevalent during spring and summer, while non-O157 *E. coli* is found more frequently in the fall (Barkocy-Gallagher et al., 2003; Cho et al., 2009; Cobbold et al., 2004; Ferens & Hovde, 2011; Sargeant et al., 2007). It has been suggested that colonization of the rectoanal junction may result in longer periods of shedding: cattle shedding O157 *E. coli* for less than one week were positive by fecal culture, but not by culture of rectal swabs, while animals with positive cultures for an average of 26 days had positive rectal swabs early in the study. Re-infection from environmental sources, particularly water troughs and fecal pads, may also keep *E. coli* strains circulating in the farm (Bolton et al., 2011; Ferens & Hovde, 2011; LeJeune et al., 2001). In animals kept exclusively on pasture, it has been suggested that STEC shedding may last for longer periods of time when compared to animals fed high-grain diets, although results across studies are inconsistent (Callaway et al., 2009b).

A clearer picture of STEC presence in herds is achieved in longitudinal studies, by addressing potential fluctuations in prevalence through sampling in multiple time-points.

In reports from the early 90s, it was suggested that calves always shed more STEC than adult cows. For example, prevalence of 8% in dams and 19% in calves was described in the United States, and of 9% in cows and 25% in calves in Canada (Wells et al., 1991; Wilson et al., 1992). Although more recent studies also describe similar scenarios, as methods become more sensitive, differences are not present in all surveys: Kobayashi et al. reported a higher prevalence in cows than in calves, while Cerqueira et al. found no differences (Cerqueira et al., 1999; Cho et al., 2009; Kobayashi et al., 2001a). In studies where distinction was made between pre- and postweaning calves, preweaning animals harbored STEC less frequently than those of any other age class, while postweaning animals had the highest prevalence. Laegreid et al. noted similarly high prevalence of *E. coli* O157 in Midwestern beef calves, suggesting that presence of STEC in preweaning calves may be more common than what has been previously described (Laegreid et al., 1999). In addition, it has been suggested that calves kept in groups prior to weaning may be more likely to shed the bacteria than calves in separate pens prior to weaning (Heinrichs et al., 1994).

In Brazil, Oliveira and his research partners found *stx*1 in 15.6% (28/180) of beef, and in 26.7% (16/60) of dairy cattle; *stx*2 was found in 30% (54/180) of beef and 53.3% (32/60) of dairy cattle. Simultaneous presence of *stx*1 and *stx*2 sequences was found in 54.4% (98/180) of beef, and 20% (12/60) of dairy cattle. Virulence gene *eae* was found in 0.6% (1/180) of beef and 15% (9/60) of dairy cattle (Oliveira et al., 2008). Once again, following the logic previously presented for differences in prevalence between and within herds, a common trend is hard to identify across studies. Regardless, when looking at fecal samples as a whole, it is impossible to know the distribution of virulence genes by isolate, which would be more relevant from a public health standpoint. In Ireland, Monagah et al. (2011) found a prevalence of *stx*2 in isolates from beef and dairy cattle, and soil samples (Monaghan et al., 2011). Virulence genes *stx*1, *stx*2, or a combination of both was present in 22%, 42%, and 36% of isolates, respectively. The *eae*A sequence was found in 18% of samples (Monaghan et al., 2011). Gould et al. summarized FoodNet data collected from 2007 to 2010 from patients with STEC infections, and concluded that most non-O157 isolates (74%) were positive for *stx*1 alone, while 17% were positive for *stx*2 only, and 9% had both virulence genes. Among patients with O157 *E. coli* infections, *stx*1 alone was present in 51% of isolates, *stx*2 alone was found in

47% of isolates, and the remaining isolates had both genes. All isolates from HUS patients had *stx*2 (Gould et al., 2013).

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Chapter 2: Shiga Toxin–Producing *Escherichia coli* Distribution and Characterization in a Pasture-Based Cow-Calf Production System

Patrícia Baltasar[1], Stewart Milton[2], William Swecker, Jr.[2], François Elvinger[1], and Monica Ponder[3]*

[1]Department of Population Health Sciences and [2]Department of Large Animal Clinical Sciences, Virginia-Maryland Regional College of Veterinary Medicine, and [3]Department of Food Science and Technology, College of Agriculture and Life Sciences, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061, USA

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ABSTRACT

Shiga toxin producing *Escherichia coli* (STEC) are commonly found in the gastrointestinal (GT) of cattle tracts. In this study, prevalence and distribution of *E. coli* virulence genes (*stx*1, *stx*2, *hly*A, and *eae*A) were assessed in a cow-calf pasture-based production system. Angus cows (n=90) and their calves (n=90) were kept in three on-farm locations, and fecal samples were collected at three consecutive time-points (July, August and September, 2011). After enrichment of samples, detection of *stx1*, *stx2*, *eaeA*, and *hlyA* was done by multiplex PCR (mPCR). Fecal samples positive for *stx* genes were obtained from 93.3% (84/90) of dams and 95.6% (86/90) of calves at one or more sampling time-points. Age class (dam, calf), spatial distribution of cattle (on-farm locations B, H, K) and sampling time-point influenced prevalence and distribution of virulence genes in the herd. Of 293 *stx*-positive fecal samples, 744 *E. coli* colonies were isolated. Virulence patterns of isolates were determined through mPCR: *stx*1 was present in 41.9% (312/744) of isolates, *stx*2 in 6.5% (48/744), *eae*A in 4.2% (31/744), and *hly*A in 2.4% (18/744). Prevalence of non-O157 STEC was high among the isolates: 33.8% (112/331) were O121, 3.6% (12/331) were O103, 1.8% (6/331) were O113. One isolate (0.3%) was identified as serotype O157. Repetitive element sequence based-PCR (rep-PCR) fingerprinting was used to study genetic diversity of *stx*-positive *E. coli* isolates. Overall, rep-PCR fingerprints were highly similar supporting the hypothesis that strains are transmitted between animals but not necessarily from a dam to its calf. Highly similar STEC isolates were obtained at each sampling time-point, but isolates obtained from dams were more diverse than isolates from calves, suggesting that strain-to-strain differences in transfer may exist. Understanding transfer of *E. coli* from environmental and animal sources to calves may aid in developing intervention strategies to reduce early life stage colonization of cattle.

INTRODUCTION

Escherichia coli is part of the normal gastrointestinal microbiota of many endothermic animals. While most strains are commensal, Shiga toxin-producing *E. coli* (STEC) is capable of causing severe human illness (Frank et al., 2011; Prevention, 2011). In the United States, it is estimated that STEC cause approximately 265,000 human illnesses every year. Serogroup O157:H7 is the STEC serogroup most frequently linked to outbreaks, and has been considered a food adulterant since 1994. The incidence of human illnesses due to non-O157 STEC serogroups has increased substantially in recent years (Bettelheim, 2007; Brooks et al., 2005; Eblen, 2007; USDA, 2011). Thus, as their public health impact has been progressively recognized, serotypes O26, O45, O103, O111, O121, and O145 were added to the list of food adulterants (Bettelheim, 2007; Brooks et al., 2005; Eblen, 2007; USDA, 2011).

Up to 61% of cases in outbreaks of STEC illness develop hemolytic uremic syndrome (HUS), a condition characterized by hemolytic anemia and thrombocytopenia, which may culminate in renal failure with case fatality risks of 5 to 10% (Scallan et al., 2006). Outbreaks have been linked to fresh produce and other commodities, but 69% of these are attributed to consumption of contaminated beef (Painter et al., 2013). Pathogenicity of STEC is thought to be associated with various genes, including those encoding for Shiga toxins (*stx*1 and *stx*2) and Intimin (*eae*A), which are located in the bacterial chromosome and are responsible for inhibition of protein synthesis and attaching-and-effacing enteric lesions, respectively (Bolton, 2011; Farfan & Torres, 2012). Another relevant virulence marker is *hly*A, a plasmid gene encoding for an enterohemolysin (Nguyen & Sperandio, 2012). By targeting these virulence genes, highly sensitive molecular-based techniques enable the detection of potentially harmful STEC strains, even when these are present in small numbers (Bolton, 2011; Fagan et al., 1999).

Cattle are considered important reservoirs of STEC, and a combination of factors such as presence of animal carriers and environmental contamination may be responsible for maintenance of specific isolates in the farm for extensive periods (Callaway et al., 2009; Ferens & Hovde, 2011; Polifroni et al., 2012). Prevalence of STEC among cattle seems to be further influenced by diet, distance to contaminated water-sources, contact with wildlife, slurry application to pasture, and animal population density (Fernandez et al., 2009; LeJeune et al., 2001). The role played by dams and calves in environmental contamination is expected to be proportional to the amount of STEC shed in feces, but there is no consensus as to which age class is the most relevant (Cerqueira et al., 1999; Cho et al., 2009; Kobayashi et al., 2001; Renter et al., 2005; Renter et al., 2004).

Distribution, transmission, and maintenance of STEC, especially of serogroup O157, have been systematically studied in feedlots and dairies, but risk factors for non-O157 STEC in cow-calf pasture-based systems are not well characterized (Renter et al., 2005; Renter et al., 2004). Understanding factors that, at the farm level, contribute for primary contamination of animals that will be concentrated in high-density feedlots may reveal opportunities for upstream control of shedding and transmission (GAO, 2012; Sargeant et al., 2007; Smith et al., 2010). As non-O157 STEC have been increasingly associated with human illness, filling this gap in knowledge needs to be prioritized.

The purpose of this study was: (a) to determine the prevalence of STEC in fecal samples from animals in a cow-calf pasture-based production system; (b) to describe the effects of age class (dam, calf), spatial distribution of cattle (on-farm locations), and time-point of sampling (July, August, September) on distribution of *E. coli* positive for virulence genes *stx*1, *stx*2, *eae*A, and *hly*A; (c) to isolate and identify *E. coli* serotypes present in *stx*-positive fecal samples; and (d) to assess genetic differentiation of STEC isolates.

Understanding factors that influence distribution of potentially pathogenic *E. coli* strains will help support on-farm management strategies with the potential to contribute to safer beef products.

MATERIALS AND METHODS

The Virginia Tech Institutional Animal Care and Use Committee (09-147) approved all cattle care and management procedures here described.

Cattle and Farm Characteristics

Angus cows (n=90) and their calves (n=90) were housed at the Shenandoah Valley Agricultural Research and Extension Center (SVAREC) of Virginia Tech. Climate at the site is temperate, with mean air temperature of 21.9 ºC in July, 24.9 ºC in August, and 19.8 ºC in September (NOAA, 2011). Pasture was composed of (in order of abundance): tall fescue (*Festuca arundinacea Schreb*), orchard grass (*Dactylis glomerata L.*), bluegrass (*Poa pratensis L.*), white clover (*Trifolium repens L.*), and red clover (*T. pratense L.*). All animals were maintained in an exclusive pasture-based cow-calf system, within the same premises in three locations (B, H, K) with 30 cow-calf

pairs each (stocking rate of 1.54 animal units/ha). On-farm location replicates of a pasture trial. Locations were fenced and separated by a hard surface road, and a minimum of 250 meters. Calves were born in a 60-day calving season (March-April) and were weaned at the end of September. Water was provided via automatic, ball-type waterers (MiraFount, Grinnel, IA). Samples included in this study were collected prior to weaning.

Sample Collection and Processing

Fecal samples were obtained *per rectum* on July 12, August 7, and September 15 of 2011, during routine procedures. Feces were collected using nitrile disposable gloves, and immediately transferred into sterile polypropylene centrifuge tubes. A total of 503 fecal samples, 258 from dams and 245 from calves, were collected at the three sampling time-points. Samples were then transported to the laboratory on ice (for approximately 2 h, at an average temperature of 5ºC) and stored at -80˚C until processing. Enrichment for coliforms was performed by incubating 1 g of feces in 9 ml of Lauryl-Tryptose Broth (LTB; Difco, BD, Sparks, MD) for 72 h at 37˚C. Feces in LTB (1 mL) were transferred into Brilliant Green Bile Broth 2% (BG; Difco, BD), and incubated at 37˚C for 72 h to enrich for coli-aerogenes bacteria, including *E. coli*.

Detection of *E. coli* Virulence Genes in Fecal Samples

After enrichment, fecal samples were screened for presence of *E. coli* virulence genes *stx*1, *stx*2, *eae*A, and *hly*A using a previously described multiplex PCR (mPCR) protocol (Fagan et al., 1999). A primer designed to amplify a fragment of the 16S rDNA gene (*E. coli* O157:H7 str. Sakai positions 5' 188 – 3' 1334) was added to the primer mix. The fecal enrichment in BG was washed for DNA extraction by centrifugation at 4000 x g for 10 min and re-suspension of the pellet in 1 ml of 1% buffered peptone water (Sigma-Aldrich, St. Louis, MO). Fecal DNA was extracted from 600 µl of washed enrichment using the ZR-96 Fecal DNA Kit (Zymo Research, Irvine, CA) following manufacturer instructions. Multiplex PCR reactions were undertaken in volumes of 25 µl containing 10 mM pentaplex primer mix, 10 mM dNTPs (USB, Cleveland, OH), 10% DMSO, 25 mM $MgCl_2$ (USB, Cleveland, OH), Fisher Buffer A (Fisher Scientific, Pittsburgh, PA), 1.5 U of *Taq* DNA polymerase (Fisher Scientific), and 20 ng of template DNA. An *iCycler iQ5* PCR (Bio-Rad, Hercules, CA) was programmed with the following settings: 95˚C for 5 min; 30 cycles of 90˚C for 15 sec, 53˚C for 30 sec, and 72˚C for 30 sec; and 72˚C for 10 min. An

outbreak-linked strain (ATCC 43894) from the American Type Culture Collection (Manassas, VA) served as a positive control for each set of mPCR reactions, and double-distilled water (ddH_2O) served as negative control. Amplified DNA fragments were resolved through electrophoresis, and success of individual reactions was indicated by amplification of the *E. coli* 16S rDNA fragment.

Selection of Fecal Samples for Further Characterization

To undergo further characterization, fecal samples had to meet the following criteria: (a) positive for *stx* genes by mPCR and (b) be collected from animals with more than two generations present in the herd. The latter criterion was included in order to achieve a more stable genetic background among animals whose samples were used for isolation of *E. coli* colonies.

Rep-PCR fingerprinting of *E. coli* isolates

Repetitive element sequence based-PCR (rep-PCR) using the BOX-A1R primer was selected to generate genomic fingerprints of colonies with identified O-groups and of colonies with more than two virulence genes. DNA was obtained from 500 µl of *E. coli* cultures in TSB using the Gentra Puregene Yeast/Bacteria Kit (Qiagen, Valencia, CA) following manufacturer instructions. PCR reactions were undertaken as described (Pesapane et al., 2013).

Rep-PCR gel images were converted to "tagged image file format" (TIFF), and imported into Bionumerics software version 7.1 (Applied Mathematics, Austin, TX, USA) for analysis. Gels were normalized against an external reference standard consisting of bands ranging from 300 bp to 2000 bp. Both top and bottom 10% of each gel were excluded from the analysis. Bands were manually selected in each lane, and lanes with few or no bands, representing PCR failure, were omitted. Normalization with the same set of external standards allowed comparisons to be made across multiple gels. Similarity between fingerprints was calculated with the Cosine correlation coefficient, a method that considers band position and band intensity. This coefficient was selected as that delivering the highest average similarity for the positive control strain (*E. coli* O157:H7; ATCC 43894) included in all gels. Optimization and position tolerance settings were set at 0.9% and 1.0%, respectively. The initial analysis included 170 isolates. To avoid bias due to overrepresentation of strains, duplicate DNA fingerprints corresponding to *E. coli*

isolates from the same animal and sampling time-point with fingerprints >99.8% similar were represented by a single isolate in the final analysis (Johnson et al., 2004). Isolates with fingerprints >90% similar were considered as members of the same cluster and isolates with fingerprints >98.5% similar were considered "highly similar" (Byappanahalli et al., 2007; Johnson et al., 2004).

Similarity scores calculated using Cosine's coefficient were used to construct Unweighted Pair Group Method using Arithmetic mean (UPGMA) dendrograms. A comprehensive dendrogram was built encompassing all retained cattle isolates (n=74) in addition to 10 control strains from human origin (Table 1; Figure 1; (Orskov et al., 1977; Valadez et al., 2011; Wells et al., 1983)). Partial dendrograms (not shown) were constructed to facilitate calculation of diversity indices for isolates from dams (n=39), calves (n=35); location B (n=26), location H (n=24), and location K (n=24). The Shannon diversity index assesses species diversity in a population and was calculated from the UPGMA dendrograms as follows:

- $$H' = -\sum_{i=1}^{s} p_i \ln p_i ,$$

where S is the total number of clusters, and p_i is calculated as $[n_i]/ [N]$, where n_i is the number of isolates in a cluster and $[N]$ is the total number of isolates (Anderson et al., 2006; Byappanahalli et al., 2007).

Maximum similarity Jackknife analysis was performed in Bionumerics 7.1 (Applied Mathematics) to determine how accurately similarity coefficients were able to predict origin of each isolate. Jackknife analysis consists of removing one isolate at a time from the database, calculating new similarity coefficients for the remaining isolates, and resubmitting the isolate back to the database while determining to which group or category it is most similar. The percentage of isolates correctly assigned to its original category is then calculated. The overall cophenetic correlation coefficient was calculated using the comprehensive UPGMA dendrogram. Genetic differentiation of *E. coli* isolates was assessed in Arlequin V3.5 by analysis of molecular variance (AMOVA) and it is translated by the fixation index (F_{ST}). Binary band-matching characters were generated from the rep-PCR fingerprint data (Excoffier et al., 1992).

Statistical Analysis

Descriptive analysis was performed using JMP Pro V10.0 (SAS Institute Inc., Cary, NC). The "GLIMMIX" procedure in SAS V9.3 (SAS Institute Inc., Cary, NC) was used to create a multivariate logistic regression model. Individual animals were considered experimental units, and a variable was created to pair dams with their respective calves in 90 dam-calf pairs. Both animal identification and dam-calf pairing were included in the "RANDOM" statement. Data was analyzed by time-point of sample collection (July, August, September). Response variables were the presence or absence of virulence genes *stx*1, *stx*2, *eae*A, or *hly*A. Independent variables were animal age class (dam, calf), on-farm location (B, H, K), and interaction between the two variables. The CLASS statement included animal identification, dam-calf pair, animal age class, and on-farm location. Orthogonal contrasts were included in the ESTIMATE statement to calculate odds ratios with 95% confidence limits. Odds ratios with associated *P*-values of $P \leq 0.05$ were considered significant.

RESULTS

Virulence Genes Detected in Fecal Samples

The prevalence of cattle shedding *stx*-positive *E. coli* at one or more sampling time-points was 93.3% (84/90) in dams and 95.6% (86/90) in calves. Seventy percent (352/503) of fecal samples had one or more virulence genes, with 58.3% (293/503) being positive for either *stx*1 or *stx*2. Virulence gene *stx*1 was present in 38.8% (195/503) of fecal samples, *stx*2 in 47.3% (238/503), *eae*A in 36.4% (183/503), and *hly*A in 18.5% (93/503) (Table 2). Two combinations of virulence genes dominated: 75 specimens were positive for *stx*1, *stx*2, and *eae*A, and 59 were positive for *stx*2 only. Odds ratios associated with the presence of individual virulence genes *stx1*, *stx*2, *eae*A, and *hly*A in fecal samples were influenced by both animal age class and on-farm location, and varied across sampling time-points (Table 3). While 4 dams were negative at each sampling, 16 were always positive. Only 2 calves were negative at each sampling, while 6 were always positive.

Virulence Typing and O-group Identification of *E. coli* Isolates

Seven hundred and forty-four colonies were isolated from 80 animals with *stx*-positive fecal samples. Of these isolates, 44.5% (331/744) were *stx*-positive, from 68 animals. Among isolates with at least one virulence gene, 38.3% (285/744) of isolates had one virulence gene, 3.9% (29/744) had two, 2.8% (21/744) had three, and one isolate carried all four. The virulence gene most frequently detected was *stx*1, carried by 41.9% (312/744) of isolates. Virulence gene *stx*2 was present in 6.5% (48/744) of isolates, *eae*A in 4.2% (31/744), and *hly*A in 2.4% (18/744). Thirteen different combinations of virulence genes were observed, but most isolates (n=270) were exclusively positive for *stx*1 (Table 4). Different combinations of virulence genes were carried by *E. coli* isolates of the same O-group. Serotype O121 was identified in 33.8% (112/331) of isolates, O103 in 3.6% (12/331), O113 in 1.8% (6/331), and O157 in 0.3% (1/331) (Table 4).

Rep-PCR Fingerprinting Analysis

The number of *amplicons* within each fingerprint ranged from 10 to 30, with sizes from approximately 200 bp to 4000 bp. Minimum similarity between isolates, as expressed at the root of the comprehensive dendrogram, was 69.3%, with 12 clusters of isolates with fingerprints >90% similar. Clusters III, IV, V, and VIII contained 86.5% (64/74) of isolates, and clusters IV, V, and VIII contained 9 "highly similar" fingerprints (>98.5% similar). All control strains, with exception of that representing serotype O121, were part of cluster VIII. The Shannon diversity index calculated for the comprehensive dendrogram was 1.42, excluding control strain fingerprints (Figure 1).

Similarity scores of isolates from dams were between 76.1 and 99.5%, and scores of isolates from calves were between 82.6 and 99.7%. The Shannon diversity index was 1.90 for dam isolates and 0.91 for calf isolates. A lower percentage of isolates from dams (35.9%) was attributed to their correct age class when compared to isolates from calves (71.4%) when Jackknife analysis was performed.

Similarity scores of isolates from animals in farm location B were between 81.8 and 99.6%, scores of isolates from animals in farm location H were between 80.8 and 99.7%, and scores of isolates from animals in farm location K were between 74.5 and 98.2%. The Shannon diversity indices were: 1.23 for B; 1.20 for H; and 1.56 for K. Through Jackknife analysis, isolates from animals in location B and location H were correctly attributed to their

original location 46.2% and 54.2% of times, respectively. Isolates from animals in location K were correctly attributed to that location 8.3% of times.

E. coli isolates presented little genetic differentiation as demonstrated by the low F_{ST} values, and variation "within" rather than "between" age classes and farm location accounted for nearly all variation among isolates (Table 5). Isolates from dams were more diverse than isolates from calves (F_{ST} = 0.01; p = 0.02), suggesting differences in transmission between strains. On-farm farm location was not associated with differences in genetic differentiation among isolates (F_{ST} = 0.004; p = 0.22; Table 5), suggesting that environmental factors may keep strains circulating in the farm.

DISCUSSION

Prevalence and characterization of *E. coli* serotype O157:H7 in cattle herds has been extensively described, and risk factors for colonization and shedding have been identified (Chase-Topping et al., 2007; Cho et al., 2009; Cobbaut et al., 2009). However, prevalence and ecology of non-O157 STEC in cattle, in particular O-groups O26, O45, O103, O111, O126, and O145, is poorly understood (Brooks et al., 2005; Cho et al., 2009; Frank et al., 2011). Knowledge of factors mediating STEC distribution must be expanded beyond a single serotype to design on-farm interventions with potential to improve the safety of beef products. Shiga toxin-producing *E. coli* have in common the carriage of phage-encoded *stx* genes and, with exception of the O157 serogroup, no biochemical markers are available to facilitate their identification (Paton & Paton, 1998). Isolation and characterization of STEC other than O157 require sophisticated molecular-based methodologies (Fagan et al., 1999). Multiplex PCR was used to detect *E. coli* positive for virulence genes *stx*1, *stx*2, *eae*A, and *hly*A in fecal samples.

Point prevalence of STEC shedders in this herd ranged from 50.3% to 71.7%, surpassing previous prevalences reported in grazing cattle of 0.7% to 27.3% for STEC O157 and 4.7% to 44.8% for STEC non-O157 (Hussein, 2007). Nevertheless, comparing STEC prevalence between studies may be of limited value – due to, among other factors, differences in sampling strategies and enrichment protocols (Ferens & Hovde, 2011; Hussein & Bollinger, 2005). Likelihood of finding STEC increases when samples are enriched, showing that method sensitivity may play an important role in testing outcomes (Ferens & Hovde, 2011; Hussein et al., 2008). To

improve chances of growing *E. coli*, fecal samples were placed on ice and transported as quickly as possible to the laboratory in order to: (1) minimize growth of competitive microbiota in the fecal matter; and (2) to improve the survival of any STEC present in the samples. Upon arrival, samples were immediately stored at -80˚C to restrict further biological and chemical reactions, such as breakdown of nucleic acids. Cryopreservation of biological cells, at -80˚C results in a high freezing rate, with formation of small ice crystals that are less likely to damage cell membranes (Souzu et al., 1989). Thus, we anticipate that the majority of STEC survived the freeze/thaw process, and that the extended enrichment steps improved the detection and isolation of surviving coliforms. Generic enrichment was followed by enrichment for coli-aerogenes bacteria.

Sampling was performed at time-points within summer and early fall to increase the likelihood of assessing the prevalence of STEC in the herd at its peak – serogroup O157 is most prevalent in cattle feces during summer, while non-O157 strains are most prevalent in the fall (Barkocy-Gallagher et al., 2003; Cho et al., 2009; Cobbold et al., 2004; Ferens & Hovde, 2011). Once exposed, individual animals seem to shed STEC for relatively short periods of time (Ezawa et al., 2004); however, colonization of the recto-anal junction and re-introduction from environmental sources, particularly water troughs and feces, may keep *E. coli* strains circulating in farms for weeks or even months (Ferens & Hovde, 2011; LeJeune et al., 2001). Cattle in our study were kept exclusively on pasture – conditions under which cattle may shed STEC for longer periods when compared to animals fed high-grain diets, although results across studies are inconsistent (Callaway et al., 2009).

Within each sampling time-point, the prevalence of virulence genes varied between dams and calves: while in July and September calves were more likely to shed STEC, dams were more likely to shed in August (Table 3). This supports previous findings that *E. coli* populations of young animals do not mirror those of dams (Kobayashi et al., 2001; Polifroni et al., 2012; Wilson et al., 1992). In early prevalence studies, it had been suggested that calves might always shed STEC more frequently than their dams, although this trend has not been evident in recent studies (Cerqueira et al., 1999; Cho et al., 2009; Kobayashi et al., 2001). In studies where distinction was made between pre- and post-weaning calves, pre-weaning animals harbored STEC less frequently than any other age class, while post-weaning animals had the highest prevalence. These differences may be a consequence of weaning stress, more intensive housing conditions during the post-weaning period, and changes in diet and physiology associated with shift from a pre-ruminant to a ruminant gastrointestinal tract (Chase-Topping et al., 2007; Ferens & Hovde, 2011; Fernandez et al., 2009; Rogerie et al., 2001; Wells et al., 1991). Nonetheless, relatively high prevalence of STEC in

pre-weaning calves was recorded in this study, as had also been noted for *E. coli* O157 in Midwestern beef calves, suggesting that the presence of STEC in pre-weaning calves may often be underestimated (Laegreid et al., 1999). Calves kept in groups prior to weaning may also be more likely to shed the bacteria than calves kept in separate pens prior to weaning (Heinrichs et al., 1994). Co-housing of calves included in this study may have contributed to the high prevalence of STEC observed in the pre-weaning calves.

Despite differences in prevalence of shedding, no consistent differences in distribution of *E. coli* virulence genes were detected among animals in different on-farm locations. Only the virulence gene *stx*1 was more likely to be present in fecal samples from location H in July and from location B in September (Table 3).

Isolates of STEC serogroups O121, O113, O103, and O157 were present in the herd, and similar virulence patterns were confirmed in isolates of the same O-group, as previously described (Beutin et al., 1997). Serogroup O121 isolates dominated, suggesting that they may either outcompete other serogroups in the gastrointestinal tract of cattle, or outgrow them during enrichment, culture and isolation procedures. Enrichment and culture bias may also be responsible for differences in presence of virulence genes both in fecal samples and in isolates, by potentially inhibiting the growth of specific isolates below the threshold of PCR detection. Selection of additional colonies from fecal samples, refinement of culture conditions, and research on increasingly sensitive and affordable tests are important considerations to further expand knowledge of STEC transmission dynamics among animals within cattle herds.

Repetitive element sequence based-PCR fingerprints of STEC isolates were closely related, as demonstrated by the high percentage of similarity translated by Cosine's correlation coefficient (Figure 1). This supports the existence of vehicles for transmission other than direct transfer. Only isolates from location K diverged slightly from those originated in locations B and H, which may be a consequence of its relative geographic seclusion and less frequent exposure to personnel and vehicles. Members of the herd have likely shared isolates among each other, within location or between adjacent locations B and H, potentially through personnel and vehicles, wildlife or contaminated run-off, as previously reported (Fernandez et al., 2009; Hancock et al., 2001; LeJeune et al., 2001; Wilkes et al., 2011).

Fixation indices (F_{ST}) obtained from MANOVA analysis also confirm the close genetic relationship between isolates (Table 5). Significant differences in genetic diversity were observed between isolates from animals

in different age classes. Isolates from calves presented less genetic variability among themselves when compared to those from dams: the Shannon diversity index was lower in calves, and a higher percentage of isolates from calves was correctly attributed to their group of origin when Jackknife analysis was performed. This suggests that particular STEC strains have a distinct ability to colonize immature gastrointestinal systems. Variability of isolates between different groups (on-farm locations and age classes) was always substantially lower than variability within groups. This may be the first study to identify differences in genetic variability between STEC isolates from calves and dams utilizing rep-PCR fingerprinting. *E. coli* strains used as positive controls in this study had been isolated from human patients. With exception of the O121 strain, all control strains were in the same cluster, together with a few cattle isolates. High similarity between rep-PCR fingerprints of pathogenic isolates of human origin and those from cattle re-emphasizes the potential public health impact of these animals as sources of human infection with STEC. Furthermore, the close relationship between potentially pathogenic STEC isolates of different O-groups stresses the utility of whole-genome techniques to characterize public health relevant isolates beyond their O-group and presence of a limited number of virulence genes.

Virulence gene distribution may provide clues for determining the potential of isolates to impact human health. In 2013, FoodNet reported characterization of a total of 941 non-O157 STEC isolates of human origin; of these, 74% was positive for *stx*1 alone, 17% was positive for *stx*2 only, and 9% had both virulence genes (Gould et al., 2013). In the same report, of 1213 O157 STEC isolates, 2% carried *stx*1 alone, 47% carried *stx*2, and 51% of isolates carried both genes. All patients with HUS had STEC isolates carrying *stx*2 (Gould et al., 2013). In this cattle study, 38.0% (283/744) of isolates were positive for *stx*1 and negative for *stx*2, 2.6% (19/744) were positive for *stx*2 and negative *stx*1, and both *stx*1 and *stx*2 were present in 3.9% (29/744) of isolates. Although presence of virulence genes does not always equate with pathogenicity, the United States Department of Agriculture uses preliminary screening for simultaneous presence *stx* genes and *eae* as criterion to decide whether samples should undergo further testing (USDA, 2012). Of 744 isolates, 27 were positive for *stx*1 or *stx*2 and *eae*A. Of these, four were *E. coli* O121 positive for *stx*2 and *eae*A simultaneously, which supports the role of cattle as potential sources of STEC illness in humans. The least frequent virulence gene was *hly*A, which was found in 18 isolates. Colonization of young animals with STEC strains of public health concern emphasizes the need for interventions at the cow-calf level of production, prior to relocation of calves to feedlots.

Prevention of on-farm contamination of cattle has the potential to improve safety of beef products, and an essential part of this process relies on understanding the biology and ecology of host-pathogen interactions. We have demonstrated the widespread presence of STEC in a pasture-based cow-calf herd, and identified O-groups that have been responsible for outbreaks of human illness in cattle. Prevalence of STEC in cattle may often be underrepresented due to animal factors, such as shifts in bacterial shedding, laboratory methodology, and sampling strategies. Differences in STEC colonization and shedding across animals in different age classes stress the need to tailor on-farm contamination prevention and control strategies. Animals of different ages do not share the same behavior and physiology, which may justify some of the differences observed in this study. A cow-calf system designed to reduce carriage of STEC by animals should focus on preventing transmission of STEC from dams to calves. Future studies should address the relevance of individual animals versus environmental contamination in maintenance and spread of STEC strains within and between herds, while addressing potential differences between strains.

FIGURES

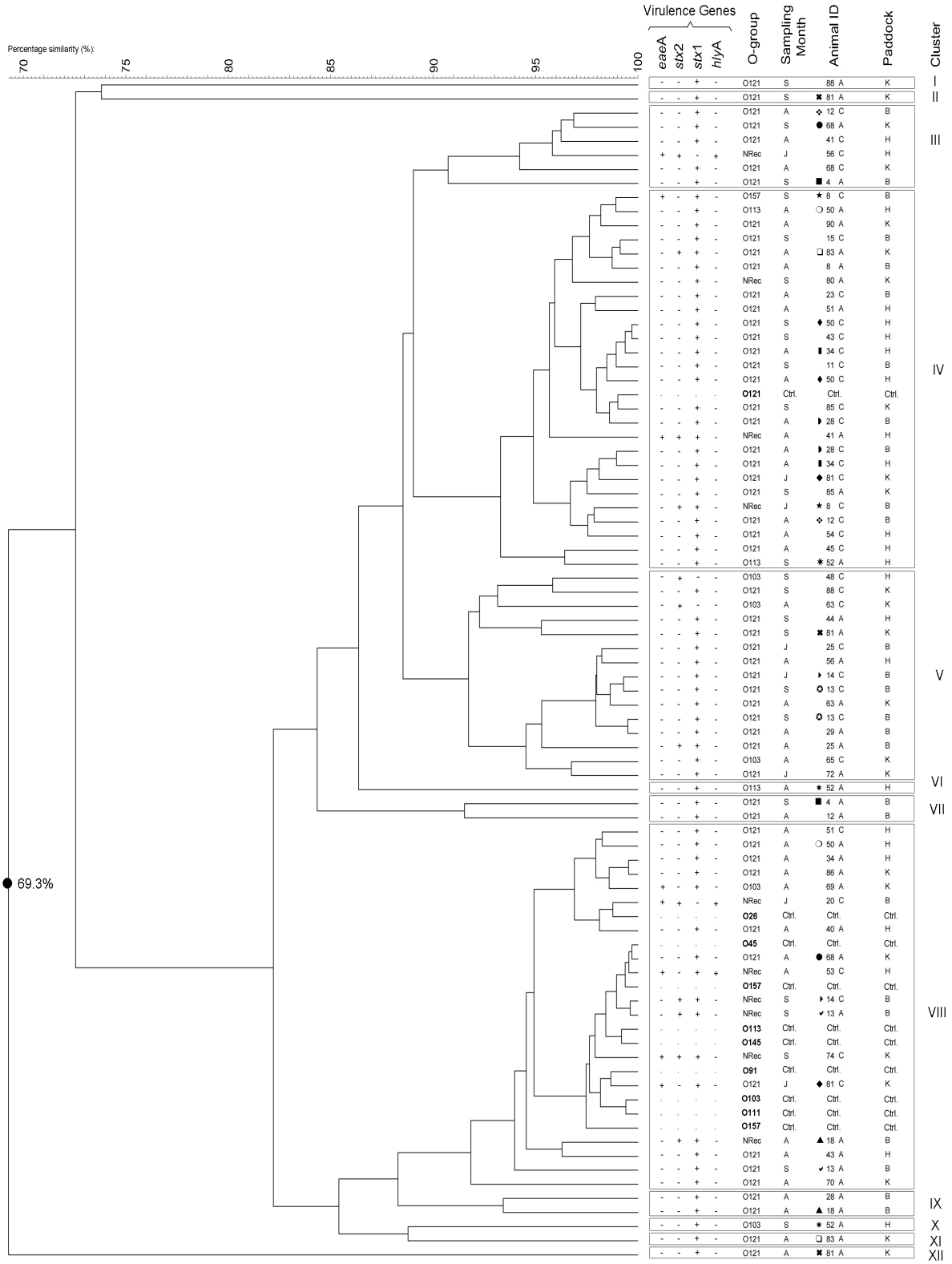
Virulence Genes
eaeA
stx2
stx1
hlyA
O-group
Sampling Month
Animal ID
Paddock
Cluster
Percentage similarity (%):
70
75
80
85
90
95
100
69.3%
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII

Figure 1: Dendrogram obtained from rep-PCR fingerprint analysis of *E. coli* isolates from cows and calves in the herd. Identical isolates from the same animal and sampling period were not included. Comparisons were constructed using Cosine's correlation coefficient. The top ruler represents percentages of similarity between isolates, and the numeric value at the root is the cophenetic correlation calculated for the dendrogram as a whole. Characterization of isolates includes virulence profile, serotype (N.D. = 'not determined') and sampling month. Correspondence of isolates to the animal from which they were obtained is also shown: "Animal ID" is composed by a number, which identifies each pair composed by a dam and its calf, and a letter informing about the animal age class (A= 'Adult'; C = 'Calf'). Isolates originating in the same sample – same animal and period – are marked with a unique symbol adjacent to the "Animal ID" (n=16). On-farm locations, in which the animals were kept, are represented the letters "B", "H", and "K". Rectangles were drawn around "clusters" formed by isolates more than 90% similar. Roman numerals (I to XII) identify each cluster.

TABLES

Table 1: Reference strains used as positive controls for mPCR reactions (O-group determination) and rep-PCR comprehensive dendrogram construction.

Bacterial Strain	Culture ID	Isolation Source	Reference
E. coli O26^{-}:H^{-}	H311b	Human Feces, Denmark	(Orskov et al., 1977; Valadez et al., 2011)
E. coli O45:H2	05-6545	Human Feces, Canada	USDA-ARS
E. coli O91:K^{-}:H^{-}	ATCC 23980	Human Feces, Denmark	(Orskov et al., 1977; Valadez et al., 2011)
E. coli O103:K^{+}:H8	ATCC 23982	Human Feces, Denmark	(Orskov et al., 1977; Valadez et al., 2011)
E. coli O111^{-}:H^{-}	ATCC 33780	Human Feces, Scotland	(Orskov et al., 1977; Valadez et al., 2011)
E. coli O113:H21	6182-50	Human Feces, Australia	(Orskov et al., 1977; Valadez et al., 2011)
E. coli O121:H19	03-2832	Human Feces, Canada	USDA-ARS

E. coli O145 K^-:H^-	E1385 (3)	Human Feces	(Orskov et al., 1977; Valadez et al., 2011)
E. coli O157:H7	ATCC 43894	Human Feces, MI	(Orskov et al., 1977; Valadez et al., 2011)
E. coli O157:H7	ATCC 43895	Raw hamburger, OR	(Wells et al., 1983)

Table 2: Determination of pattern of virulence genes *stx*1, *stx*2, *eae*A, and *hly*A by mPCR in 503 fecal samples (Panel A) and total number of samples positive for individual virulence genes (Panel B), in dams and calves in July, August, and September.

A. Virulence genes				**JULY**		**AUGUST**		**SEPTEMBER**		**Total**
*stx*1	*stx*2	*eae*A	*hly*A	Dams (n=78)	Calves (n=85)	Dams (n=90)	Calves (n=82)	Dams (n=90)	Calves (n=78)	**503**
+	+	+	+	0	4	4	3	0	5	16
+	+	-	+	0	0	7	3	0	1	11
+	-	+	+	0	4	1	4	0	4	13
-	+	+	+	0	4	2	1	0	1	8
+	+	+	-	1	9	24	0	20	21	75
+	-	-	+	2	1	1	0	2	1	7
+	+	-	-	3	4	15	1	5	10	38
-	+	+	-	2	4	8	4	5	3	26
+	-	+	-	0	1	1	3	0	1	6
-	+	-	+	1	2	1	1	0	0	5
-	-	+	+	0	6	0	6	0	1	13
+	-	-	-	11	4	5	3	4	2	29
-	+	-	-	8	15	7	23	2	4	59
-	-	+	-	9	0	7	3	6	1	26

-	-	-	+	6	1	1	4	3	5	20
-	-	-	-	35	26	6	23	43	18	151

B. Virulence genes (total)							
*stx*1	17	27	58	17	31	45	195
*stx*2	15	42	68	36	32	45	238
*eae*A	12	32	47	24	31	37	183
*hly*A	9	22	17	22	5	18	93

Table 3: Association of animal age class and on-farm location with presence or absence of virulence genes in fecal samples. Only odds ratios with associated P value ≤ 0.05 are presented.

Sample Collection	Dependent Variable	Explanatory Variable		OR (95% CI)	*P*-value
JULY	***stx*1**	Location:	H	2.82 (1.13 – 7.05)	0.03
			K	1.00	
	***stx*2**	Age Class:	Dam	0.24 (0.11 – 0 50)	0.0002
			Calf	1.00	
	***eae*A**	Age Class:	Dam	0.30 (0.14 – 0.65)	0.003
			Calf	1.00	
	***hly*A**	Age Class:	Dam	0.36 (0.15 – 0.87)	0.02
			Calf	1.00	
AUGUST	***stx*1**	Age Class:	Dam	11.07 (4.42 – 27.71)	<0.0001
			Calf	1.00	
		Location*Age Class:	H*Dam	9.70 (2.54 – 37.02)	0.001
			H*Calf	1.00	
			K*Dam	70.74 (7.72 – 648.18)	0.0003
			K*Calf	1.00	

	***stx*2**	Age Class:	Dam	4.09 (2.06 – 8.12)	<0.0001
			Calf	1.00	
	***eae*A**	Age Class:	Dam	3.40 (1.60 – 7.18)	0.002
			Calf	1.00	
		Location:	H	3.70 (1.40 – 10.24)	0.009
			K	1.00	
			B	3.47 (1.31 – 9.16)	0.013
			K	1.00	
	***hly*A**	Location*Age Class:	H*Dam	0.25 (0.06 – 0.97)	0.04
			H*Calf	1.00	
SEPTEMBER	***stx*1**	Age Class:	Dam	0.34 (0.17 – 0.67)	0.004
			Calf	1.00	
		Location:	B	3.53 (1.34 – 9.08)	0.01
			K	1.00	
	***stx*2**	Age Class:	Dam	0.38 (0.20 – 0.73)	0.002
			Calf	1.00	
	***hly*A**	Age Class:	Dam	0.19 (0.06 – 0.59)	0.004
			Calf	1.00	

Table 4: Virulence patterns and O-groups as determined by mPCR in 744 *E. coli* isolates and number of animals represented.

Virulence genes				**Number of colonies**		**Total animals represented**		**O-groups detected**	
*stx*1	*stx*2	*eae*A	*hly*A	Dams	Calves	Dams	Calves	Dams	Calves
+	+	+	+	1	0	1	0	O121	-
+	+	-	+	1	0	1	0	N.D.[1]	-

+	-	+	+	0	5	0	1	-	N.D.[1]
-	+	+	+	0	8	0	2	-	N.D.[1]
+	+	+	-	6	1	3	1	O121, N.D.[1]	N.D.[1]
+	-	-	+	2	0	1	0	N.D.[1]	-
+	+	-	-	12	8	6	3	O121, N.D.[1]	N.D.[1]
+	-	+	-	3	3	1	3	O103, O121	O121, O157, N.D.[1]
+	-	-	-	159	111	18	15	O103, O121, N.D.[1]	O113, O121, N.D.[1]
-	+	-	-	0	11	0	6	-	O103, N.D.[1]
-	-	+	-	0	4	0	1	-	N.A.[2]
-	-	-	+	1	0	1	0	N.A.[2]	N.A.[2]
-	-	-	-	159	251	9	15	N.A.[2]	N.A.[2]

1. None determined: colonies negative for O26, O45, O91, O103, O111, O113, O121, O145, or O157.

2. Not applicable: O-groups were not determined for colonies that were not *stx*-positive.

Table 5: Analyses of Molecular Variance (AMOVA) for *E. coli* isolates collected from cattle in the SVAREC herd.

Source of Variation	**Observed Partition**					
	df	Sum of Squares	Variance	% Total	F_{ST}	*P*-value
Age Classes						
Between Age Classes:	1	15.7	0.15	1.48	0.01	0.02
Within Age Classes:	72	729.2	10.13	98.52		
Spatial Distribution						
Between Paddocks:	2	22.2	0.04	0.37	0.004	0.22
Within Paddocks:	71	722.7	10.18	99.63		

Isolates were grouped following characteristics of the animals from which they were isolated. Sources of variation, i.e. age class and spatial distribution, were considered separately to best assess genetic variability of the *E. coli*

isolates. Isolates were first considered in 2 age class categories: dams (n=39) and calves (n=35), and then in 3 spatial distribution categories: paddock B (n=26), paddock H (n=24), and paddock K (n=24). Analysis of Molecular Variance was performed in Arlequin V3.5 using the binary band pattern generated in Bionumerics software version 7.1 (Applied Math, Austin, TX, USA).

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Chapter 3: General Conclusion and Future Directions

The combined efforts of the food industry, and federal, state and local regulatory and public health agencies are credited with making the standing of U.S. food supply amongst the safest in the world. Americans spend more than $1 trillion on food each year, nearly half of it in restaurants, schools, and other places in and outside the home. Still, a number of well-publicized outbreaks and sporadic cases of foodborne illness have occurred, and still occur every year. The costs are measured not only in dollars, but also, and more importantly, in human lives, decreased quality of life, and production losses. Most of the products responsible for these illnesses have animal origin, or were contaminated by animal and/or human waste. Since 1982, when *E. coli* O157 was recognized as a major pathogen, research has focused on understanding its sources, ecology, diversity, virulence factors, prevalence, and risk factors for human illness. Much has been learned in the 34 years since the outbreak that brought this serotype of *E. coli* to the attention of public health agencies; however, only recently have other potentially pathogenic *E. coli* serotypes been investigated, and it is not obvious whether they obey by the same principles. Furthermore, research has focused mostly on samples from feedlots and dairy productions, and a better understanding of STEC-cattle interactions requires expansion to other systems of production.

To help fill in the aforementioned gap in research, the purposes of our work were to determine prevalence of all *stx*-carrying fecal samples from animals in a cow-calf pasture-based production system; describe effects of age class (dam, calf), spatial distribution of cattle, and time-point of sampling on distribution of strains positive for virulence genes *stx*1, *stx*2, *eae*A, and *hly*A; isolate and identify serotypes present in *stx*-positive samples; and to assess genetic similarity of said isolates utilizing repPCR.

In this study, we have demonstrated the widespread presence of STEC serotypes in a cow-calf grass fed production system. Of the 744 colonies isolated in the three time-periods investigated, only one was STEC O157. This emphasizes the fact that, in order to minimize contamination of animals and their surrounding environment, knowledge of factors mediating STEC presence and distribution must be expanded beyond a single serotype. Still, any improvements in detection and quantification of *E. coli* O157 in feces, and further understanding of its ecology in cattle could potentially lead to strategies to reduce overall pathogen prevalence and individual loads, while protecting the environment. Meanwhile, it is important to acknowledge and understand the differences between

serotypes, in particular so that reduction or removal of STEC O157 does not end up giving other pathogens a competitive advantage. Although impossible to know with certainty from a single study, we may have witnessed this event at play, for the first time, in this herd. Although preservation and isolation methods could have contributed (or been solely responsible) for the observed dominance of serotype O121 in *stx*+ isolates, which amounted to a total of 33.8% (112/331) of *stx*-positive isolates, the contribution of cattle management operations cannot be fully excluded. Given that serotype O121 was significantly linked to the development of bloody diarrhea in human-based studies undertaken between 1983 and 2002 (Brooks et al., 2005), it is of particular interest to understand the characteristics of this serotype, particularly when it comes to carriage of virulence genes. We found four *E. coli* O121 colonies carrying *stx*2 and *eae*A simultaneously, one of which carried all four virulence genes searched: *stx*1, *stx*2, *eae*A, and *hly*A.

Whether serotype O121 is more frequent in cow-calf grass-fed production systems when compared to other production models is a question to be answered by future research, which must be expanded beyond a single herd, while preserving methodology similar to that of this study.

Carriage of these isolates by calves may also reflect upon contamination of animals throughout the remaining stages of production, all the way to the consumer's table. Although we did not assess actual production of toxins by our isolates, they do seem to carry enough virulence genes to raise some concern. This, coupled with point prevalence of STEC shedders in this herd ranging from 50.3% to 71.7%, leaves a lot of work to be done by post-harvest decontamination procedures. To reduce this pressure, it is imperative to prevent on-farm contamination and cross-contamination of cattle, which can only be done by determining factors mediating STEC prevalence and distribution, as well as understanding the biology and ecology of host-pathogen interactions. Since cattle are major reservoirs and rarely demonstrate clinical signs consistent with STEC infection, the best available way to assess these factors involves isolating STEC from bovine feces.

Prevalence of *stx*+ animals in the herd is one of the highest reported in a cow-calf pasture-based production system. Differences between paddocks in distribution of virulence genes suggest that physical proximity of animals contributes to the establishment of dominant *E. coli* populations. Contradicting several claims, calves may not consistently shed more *stx*+ *E. coli* than adult cattle and the fact that isolates from calves presented less genetic variability among them than those from dams suggests that, once installed, particular STEC strains have a distinct ability to colonize immature gastrointestinal systems. Further research to answer this question would be highly

desirable. Differences in STEC colonization and shedding across animals in different age classes stress the need to tailor on-farm contamination prevention and control strategies. Animals of different ages do not share the same behavior and physiology, which may justify some of the differences observed in this study. A cow-calf system designed to reduce carriage of STEC by animals should focus on preventing transmission of STEC from dams to calves and so on, all the way to harvest. Future studies should address the relevance of individual animal shedders versus environmental contamination in maintenance and spread of STEC strains within and between herds, while addressing potential differences between strains.

Besides demonstrating the widespread presence of STEC in a pasture-based cow-calf herd, we also identified O-groups that have been responsible for outbreaks of human illness in cattle (O103, O113, O121, and O157) further confirmed that isolates included in the same O-group may harbor different virulence gene combinations, emphasizing their diversity. This further raises the question on whether the legislation and resulting procedures should focus on removal of products contaminated with given *E. coli* serotypes, or rather focus on the presence of virulence genes and/or the proteins they originate. If focusing on virulence genes, it is urgent to establish, with as much certainty and consensus as possible, which exact genes must be present for illness to occur.

Prevalence of STEC in cattle may be underrepresented due to animal factors, such as shifts in bacterial shedding, laboratory methodology, and sampling strategies. Although longitudinal study designs may minimize some of these issues, it is by no means guaranteed that researchers will ever develop a method 100% sensitive, particularly without sacrificing much needed specificity. Furthermore, collection of large fecal samples (> 5.0 g) is sometimes difficult, as animals tend to evacuate more often when stressed, resulting in empty rectums upon immobilization in the chute. When swabs are used, the weight of the sample cannot be determined (the swab is generally placed in 10.0 ml of broth) (Cobbold & Desmarchelier, 2000; Fukushima & Seki, 2004). Also, presence of reaction inhibitors, such as humic acids, may be responsible for decreased PCR efficiency. To avoid this, DNA-extraction methods with steps to exclude PCR inhibitors should be preferred, and conserved DNA regions should be used in PCR procedures to confirm success of individual-sample reactions. If success of reactions is not confirmed, artificially low ratios of adult animals shedding STEC may be obtained when compared to calves, since, as we observed, successful PCR reactions are more likely to be obtained with samples collected from calves.

To overcome some of these and other challenges, researchers have leaned towards different methodologies, which has, in itself, become a problem. Comparison of results across studies can be a very laborious (if possible)

endeavor. In order to compare shedding prevalence from animals naturally exposed to different environmental factors and management practices, it is important to unify methodology. Efforts to isolate and characterize STEC beyond the O157 serogroup require methods more sophisticated (targeting surface antigens or bacterial genome) than a simple culture medium. Still, it would be a major time and resource saver to have a selective and differential culture media (similar to CT-SMAC for the O157 STEC serogroup) available for other major STEC serotypes. The closest available tool comes in the form of agars such as CHROMagar STEC (CHROMagar, Paris, France) and Rainbow STEC (Biolog Inc., Hayward, CA). Although their exact composition is proprietary information that has not been disclosed, these media have both selective and chromogenic properties, and are available commercially to target major STEC serogroups involved in outbreaks of human illness (e.g. O26, O48 O111, O157). However, they too come with familiar issues: relying on principles such as antibiotic resistance and fermentation characteristics of targeted STEC serogroups (e.g. novobiocin and potassium tellurite); they have only been tested on a limited number of serotypes; chromogenic differences between colonies of different serotypes are often very subtle; and they forget that the same STEC serotype, it is known that resistance and fermentation characteristics may vary widely.

Although not without its merit and placement, it is likely that progress in the development of an ideal culture or enrichment media for each and every strain of interest may take precious time and resources that could, otherwise, be invested in already proven methods such as whole genome sequencing, which may (particularly in some instances, geographic settings, and economic resources) still have prohibitive costs, but are likely to become cheaper once optimized to be more efficient (improve repeatability, consume less resources, occupy smaller less-specialized spaces, be less expensive, simple to use and to interpret, can be used on field research, etc.).

Furthermore, likelihood of finding STEC increases when samples are enriched, showing that method sensitivity can play an important role (Ferens & Hovde, 2011; Hussein et al., 2008). We used a generic enrichment, followed by enrichment for coli-aerogenes bacteria to improve chances of growing any *E. coli* present in fecal samples. This has the disadvantage of encouraging the growth of some background flora that might be able to survive in the selective broth and compete with STEC for the same niche.

Several studies have explored methods to reduce pathogenic bacteria in cattle. Some of these methods have been described above, under “Pre-harvest and Harvest Control in Cattle”. In order to convince the cattle production industry of the importance of such expenditures, it is essential to collaborate with individuals and associations to understand how much producers know and care about STEC, and which information sources or vehicles could be

effectively and efficiently used to inform producers about the best cost-benefit interventions that could be applied to reduce STEC contamination in their farms. In the context and continuation of these efforts, perhaps the development of a special certification could serve as an extra encouragement to install certain on-farm pathogen mitigation strategies, demonstrating that, at the source of a certified product, exceptional measures were taken to protect the consumer.

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