RISK FACTORS FOR SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* IN CATTLE

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ABSTRACT

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Shiga toxin-producing *Escherichia coli* (STEC) is one of the most important food borne pathogens of humans globally, having caused numerous outbreaks in North America and worldwide. Severe clinical disease occurs primarily in children and immunocompromised adults and signs range from mild diarrhea to hemorrhagic colitis to Hemolytic Uremic Syndrome, which can result in kidney failure and mortality.

Cattle are considered the main reservoir of STEC and food or water contaminated with cattle feces is considered to be a major source of human exposure. Common foods implicated in STEC outbreaks include ground beef, unpasteurized milk, leafy vegetables and apple cider. Other domesticated animals and wildlife can also shed STEC, but their importance as a source of human exposure is considered less significant. Human infections have also been reported following direct and indirect contact with animals at zoos, livestock exhibitions and petting farms.

Identifying factors that influence STEC shedding and dynamics in cattle is important for the design and implementation of strategies to prevent STEC transmission. The studies describe in this dissertation are the results obtained from an epidemiological study performed in 11 herds in Mid-Michigan during 2011 and 2012. The primary aims of this project were to identify risk factors for STEC shedding and describe STEC dynamics in cattle. Of specific interest was the potential effect on STEC shedding of pre-existing chronic disease, specifically infection with

Bovine Leukemia Virus (BLV) and *Mycobacterium avium* subsp. *paratuberculosis* (MAP), the causative agent of Johnes disease.

We identified several variables including days in milk and number of lactations, to be important individual factors that influence the risk of STEC shedding in dairy cattle. Intervention strategies could be targeted towards these high risk cattle groups. We also confirmed the importance of seasonality, more specifically warm temperatures, on STEC shedding by cattle. No association was observed between STEC shedding and infection with BLV and MAP.

We found a significant association between the independent variable herd and rate of new infections with STEC; also we found a significant association between herd and persistent STEC negative status, both in dairy herds. However, we were not able to identify specific management factors that influence the risk of STEC shedding over time. These finding highlight the complex and multifactorial nature of STEC epidemiology in cattle.

Based on the results obtained in this dissertation, we conclude that first lactation cows and cows in their first 30 days of lactation have the highest risk of STEC shedding. As a consequence, these specific groups of cattle can be targeted for the design and implementation of intervention strategies at pre-harvest with the aim of reducing STEC infection in humans.

Dedicated to my family

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Accomplishments have not value if you have no one to share them with.

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KEY TO ABBREVIATIONS

AA: Any STEC acquisition

AEE: Attaching and effacing *E. coli*

AL: Any STEC loss

BLV: Bovine Leukemia Virus

cfu: colony-forming unit

eae: gene encoding the *E. coli* attaching and effacing protein, intimin

EAEC: Enteroaggregative *E. coli*

ED: Edema disease

EHEC: Enterohemorrhagic *E. coli*

EIEC: Enteroinvasive *E. coli*

EPEC: Enteropathogenic *E. coli*

ETEC: Enterotoxigenic *E. coli*

ExPEC: Extraintestinal *E. coli*

DAEC: Diffusely adherent *E. coli*

DEC: diarrheagenic *E. coli*

DIM: Days in milk

Gb_3: Globotriaosylceramide

Gb_4: Globotetraosylceramide

HUS: Hemolytic Uremic Syndrome

IMS: Immunomagnetic separation

LEE: Locus of Enterocyte Effacement

L:M: Lymphocyte to monocyte radio

MAP: *Mycobacterium avium* subsp. *paratuberculosis*

MNEC: Meningitis-associated *E. coli*

PP: Persistent STEC positive

RA: Rate of STEC acquisition

RL: Rate of STEC loss

RNI: Rate of new STEC infections

SRP: Siderophore receptor and porin proteins

STEC: Shiga toxin-producing *Escherichia coli*

Tir: Translocated intimin receptor

UPEC: Uropathogenic *E. coli*

VTEC: Vero toxin-producing *E. coli*

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) are one of the most important foodborne pathogens in the U.S. and other developed countries. STEC can cause hemorrhagic diarrhea and hemolytic uremic syndrome (HUS) that can lead to kidney failure and death, particularly in young children (Vanaja, et al 2013).

STEC, also known as Vero toxin producing *E. coli*, is defined by the presence of genes encoding the Shiga toxin (Stx). Two main types of Stx can be produced by STEC: Stx1 (almost identical to Stx from *Shigella dysenteria* type 1) and Stx2 (Gyles 2007; Scheutz, et al 2012). Shiga toxins can be further broken down into subtypes based on differences in biological properties of the toxins (Scheutz, et al 2012). Shiga toxins are bifunctional bacterial toxins, composed by two units A and B, such as cholera toxin (O'Brien and Holmes 1987). The best known receptor for Stx is globotriaosylceramide (Gb_3), a membrane cell surface receptor (Jacewicz, et al 1986).

STEC isolates can be further classified into those that contain the Locus of Enterocyte Effacement (LEE) pathogenicity island and those that do not (Sahl, et al 2013). LEE encodes a type III secretion system that injects infectors into the host cell that produce the formation of attaching and effacing (AE) lesions (Gyles 2007; Sahl, et al 2013). Isolates positive for the LEE island are considered enterohemorrhagic *E. coli* (EHEC), a subset of STEC.

STEC O157:H7 were identified for the first time in the U.S. in 1982 (Riley, et al 1983). Based on the molecular analysis of these outbreak isolates, it was demonstrated that a lambdoid prophage transferred into *E. coli* the genes required to produce Stx, resulting in a newly emergent pathogen (O'Brien, et al 1984). Since its emergence, *E. coli* O157:H7 has been the

most commonly isolated serotype among over 100 STEC serotypes (Vanaja, et al 2013), which has been partially due to enhanced detection protocols from human and animal feces. The burden of illness from serotypes other than O157 (non-O157) STEC has previously been difficult to estimate as culture systems were incapable of differentiating these strains, leading to diagnostic limitations and inadequate surveillance (Farrokh, et al 2013; Vanaja, et al 2013). During the last 10 years, non-O157 serotypes have increased in frequency (Bettelheim 2007) and have contributed to several large-scale outbreaks. Currently, six non-O157 STEC serogroups (O26, O45, O103, O111, O121, and O145) and O157:H7 have been classified by the USDA-FSIS as adulterants of all raw non-intact beef and raw intact beef intended for use in raw-intact products in the U.S. under the Federal Meat Inspection Act (21 U.S.C. 601 (m) (1)).

Transmission of STEC is a complex process that involves different reservoirs, hosts and environments. Ruminants, especially cattle, are the major STEC reservoir. Humans most commonly get infected through the consumption of contaminate food, among the most common food items have been beef products (especially ground beef) or dairy products contaminated with feces. However there have been outbreaks with other less common sources such as unpasteurized apple juice, spinach and salami (Chase-Topping, et al 2008; Hussein 2007; Jay, et al 2007; Menrath, et al 2010). Produce can also get contaminated with STEC through the inclusion of manure in soil and later uptake by plants (Callaway, et al 2013; Franz and van Bruggen 2008). Rainfall events can wash STEC from cattle feces into drinking, recreation or irrigation water supplies, which have led to infection in humans and other animals (Berry and Wells 2010; Callaway, et al 2013; Franz and van Bruggen 2008; Hussein 2007; Solomon, et al 2002). Direct contact with cattle is another route of transmission to humans (Goode, et al 2009). Other animals species such deer and birds can also be sources of STEC (Asakura, et al 1998; Callaway, et al

2013; Dunn 2003; Ferens and Hovde 2011), while person-to-person transmission has also been documented (Gyles 2007; Pennington 2010; Tarr, et al 2005).

According to data collected by FoodNet USA, there were 561 cases (1.17 per 100, 000 people) of non-*O157* associated illness and 552 cases (1.15 cases per 100,000) of STEC *O157* associated illness in the U.S in 2013. In this same year, there were 78 hospitalizations and 2 deaths for non-*O157* and 210 hospitalizations and 2 deaths for STEC *O157* (Crim, et al 2014).

Several researchers have concluded that control measures at the pre-harvest level will have the greatest impact on the reduction of STEC infections in humans (LeJeune and Wetzel 2007; Soon, et al 2011). A solid understanding of the epidemiology of STEC is critical to implementing control measures at the pre-harvest level. Although numerous studies have sought to determine the prevalence of STEC in animal reservoirs and varying geographic locations, additional studies are still needed to better understand the risk factors associated with STEC shedding at both the herd and animal level. Similarly, more research is needed to identify which groups of cattle have the highest risk of STEC colonization and shedding as these groups represent the best targets for pre-harvest intervention strategies. To date, few consistent risk factors have been identified for STEC *O157* shedding in cattle across studies (Cho, et al 2013; Menrath, et al 2010). Some studies have found similar factors while others have found contradictory risk factors. However, most research has been focused on STEC *O157* rather than non-*O157*. Additional large-scale studies are therefore needed to better understand the transmission dynamics of STEC within and across herds with varying management practices.

The overall purpose of the research conducted and presented in this dissertation was to identify new risk factors in beef and dairy cattle that influence STEC shedding. The ultimate goal

was to identify management factors that could be modified to reduce STEC colonization and shedding levels, thereby minimizing the potential risk of human infections.

In chapter one, a literature review on STEC and information available regarding studies on STEC risk factors was presented. This information is useful in understanding what is known and not known about STEC shedding and associated risk factors and was used to inform the development of our subsequent studies.

In chapter two, findings on potential risk factors for STEC shedding at both the herd and individual animal level were reported. By understanding potential risk factors, intervention strategies can be designed to reduce pre-harvest STEC shedding, thus reducing the risk of human infections.

In chapter three, we tested the hypothesis that cattle infected with Bovine Leukemia Virus (BLV) and/or *Mycobacterium avium* subsp. *paratuberculosis* (MAP) were more likely to shed STEC. BLV and MAP are chronic infectious diseases that have significant long-term effects on the immune system (BLV) and gastrointestinal tract (MAP), thus creating a situation where the dynamics of infection and shedding of organisms such as STEC may be altered. If these chronic diseases have an effect on STEC shedding, then implementing management practices to control these important diseases could indirectly influence STEC shedding as well.

In chapter four, we conducted a longitudinal study to investigate rates of STEC acquisition, persistence and loss in cattle and to identify factors that increase or reduce STEC acquisition or persistence. Combining this information with the findings from chapter two could further add to and refine the development of intervention strategies at the pre-harvest level.

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CHAPTER 1

LITERATURE REVIEW: RISK FACTORS FOR SHEDDING OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI (STEC)* IN DAIRY AND BEEF CATTLE

STEC

1. Escherichia coli

Escherichia coli are part of the normal intestinal microbiota and considered the major facultative anaerobic bacterium in the intestinal tract of most mammalian species. *E. coli* are Gram- negative, facultative anaerobe, rod-shaped bacteria belonging to the *Enterobacteriaceae* family (Edwards and Ewing 1972; Escherich 1988; Gyles and Fairbrother 2010). Features used for its identification include a positive indole reaction, negative tests for production of urease and hydrogen sulfide, and failure to utilize citrate as the sole carbon source (Bettelheim 1994). *E. coli*, which has its maximum concentration in the large intestine, is typically present at 10^7-10^9 organisms per gram in feces (Gyles and Fairbrother 2010). *E. coli* are frequently used as indicator organisms for fecal contamination and breaches in hygiene in the areas of food safety and public health (Farrokh, et al 2013).

E. coli are typically nonpathogenic but there is a small proportion of *E. coli* that has acquired genes that enable them to cause intestinal and extraintestinal diseases in humans and animals (Gyles 2007). Among these *E. coli* capable of causing disease are the pathotypes that cause disease outside the intestines called extraintestinal *E. coli* (ExPEC). Examples from this group are Uropathogenic *E. coli* (UPEC) and meningitis-associated *E. coli* (MNEC). Those *E. coli* that

cause enteric diseases are called diarrheagenic *E. coli* (DEC), among these pathotypes are enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC) (traveler's diarrhea), Vero toxin-producing or Shiga toxin-producing *E. coli* (VTEC/STEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) (Gyles 2007; Jafari, et al 2012; Nataro and Kaper 1998). This literature review will focus on the specific pathotype called Vero toxin-producing/Shiga toxin-producing *E. coli* (VTEC/STEC).

2. Shiga toxin-producing *Escherichia coli* (STEC)

STEC is also known as Vero toxin producing *E. coli* because of the cytopathic effect caused when cultured on Vero cells (kidney epithelial cells from African green monkey) (Konowalchuk, et al 1977; O'Brien and LaVeck 1983). STEC are characterized by their ability to produce at least one type of Shiga toxin. The two major types of Stx that STEC can produce are Stx1 (almost identical to Stx from *Shigella dysenteria* type 1, the prototype toxin for this family) and Stx2. These two toxins have 55-60% genetic and amino acid identity homology (Jackson, et al 1987; O'Brien, et al 1982; Stockbine, et al 1985; Strockbine, et al 1986). They are considered genetically related but antigenically distinct, as there is no cross reaction with polyclonal antisera. Biologically, they have similar cytotoxic, enterotoxic and lethal activities (Strockbine, et al 1986). However, Stx1 and Stx2 cross the intestinal epithelial cell barrier by different pathways (Hurley, et al 1999). Shiga toxins can be further broken down into subtypes based on phenotypic differences, biological activity (serologic reactivity, receptor binding, capacity to be activated by elastase in intestinal mucus) and hybridization properties (O'Brien, et al 1994; Scheutz 2014). At present, 107 variants have been identified: 9 variants of Stx1a (including

Shiga toxin from *S. dysenteriae*), 4 of Stx1c, and 1 of Stx1d, and subtypes of Stx2 include 21 variants of Stx2a, 16 of Stx2b, 18 of Stx2c, 18 of Stx2d, 14 of Stx2e, 2 of Stx2f, and 4 of Stx2g (Scheutz 2014; Scheutz, et al 2012) Some Stx2 subtypes are associated with severe symptoms in humans, including bloody diarrhea and HUS (USDA 2012), especially the combination of *eae* gene and *stx2* (Persson, et al 2007). Stx2e is found almost exclusively in strains that cause Edema Disease in pigs (Gyles and Fairbrother 2010; MacLeod and Gyles 1990; Marques, et al 1987).

In essence, Shiga toxins are holotoxins, proteins with an AB_5 quaternary structure, which means that they are composed of two subunits. The first subunit is called Shiga toxin A-subunit (StxA) and it has enzymatic activity. The second is the five B-subunits (StxB) which bind glycolipid cell surface receptors on the host cell surface(O'Brien and LaVeck 1983). The best known receptor for Stx is the globotriaosylceramide (Gb_3) cell surface receptor, a membrane glycolipid of the globo-series (Jacewicz, et al 1986) located in endothelial cells, and found in other cells (Meyers and Kaplan 2000). Once Stx and Gb_3 are bound, the toxin is endocytosed and trafficked retrograde through the Golgi apparatus to the endoplasmic reticulum. During this process the A-subunit is proteolytically cleaved into A1 and A2 fragments. The free A1 fragment is translocated to the cytosol, where its *N*-glycosidase activity cause depurination of the 28S ribosomal RNA, resulting in cessation of protein synthesis, and leading to apoptosis (O'Brien and Holmes 1987; Obrig, et al 1985; Vanaja, et al 2013).

Differentiation of *E. coli* is important for distinguishing pathogenic from nonpathogenic types and for epidemiological investigations (Gyles and Fairbrother 2010). This differentiation can be accomplished using phenotypic and genotypic methods. STEC can be classified by serotyping. The serotype of an *E. coli* is based on the O (Ohne) antigen, which comprises the

polysaccharide portion of the cell wall lipopolysaccharide (LPS), and the H (Hauch) antigen, which is found on the flagella protein (Edwards and Ewing 1972). There are more than 472 STEC/VTEC serotypes (Scheutz 2014). Serogroups are defined by O antigen only; there are actually 174 different O antigens (Gyles and Fairbrother 2010). There are also STEC serotypes that are nonmotile (NM) mutants of strains with an H antigen; that also can produce HUS (Gyles 2007; Gyles and Fairbrother 2010; Karch, et al 1993).

Karmali, et al. (2003) also proposed a seropathotype classification based on their reported frequencies in human illness and their known association with outbreaks and severe outcomes. Five seropathotype classifications have been proposed and include: seropathotype A associated with the "highest" incidence in human disease, it consist of O157:H7 and O157: NM (nonmotile), considered to be the most virulent. Follow by seropathotype B with a "moderate" incidence, considered similar to seropathotype A in causing outbreaks and HUS but with a lower frequency, it includes 13 STEC serotypes. Then seropathotype C includes serotypes infrequently implicated in sporadic HUS but not typically with outbreaks, they are: O5:NM, O91:H21, O104:H21, O113:H21, O121:NM and O165:H25; and seropathotype D is composed of 12 serotypes that have been implicated with sporadic cases of diarrhea but no with outbreaks of HUS. Finally, seropathotype E included at least 14 serotypes not associated with human illness, outbreaks or severe illness (Karmali 2003; Karmali, et al 2003; Scheutz 2014; USDA 2012). This classification is problematic because the majority of STEC isolates are not fully serotyped nor characterized for the presence of virulence factors. As consequence the European Food Safety Authority Panel on Biological Hazards (BIOHAZ) concluded that this classification does not define pathogenic VTEC nor does it provide an exhaustive list of pathogenic serotypes (Scheutz 2014).

STEC isolates can further be classified by the presence of putative virulence factors, for example STEC can be classified into those that contain the Locus of Enterocyte Effacement (LEE) pathogenicity island and those that do not (Sahl, et al 2013). The LEE encodes a type III secretion system that injects effectors into the host cell that result in the formation of attaching and effacing (AE) lesions (Gyles 2007; Sahl, et al 2013). Those STEC strains that are capable of attaching to epithelial cells, effacing microvilli, and eliciting the formation of adhesion pedestals composed of cytoskeletal proteins are called attaching and effacing *E. coli* (AEEC) or enterohemorrhagic *E. coli* (EHEC). EHEC strains of the O157:H7 serotype are the most important EHEC pathogens in North America, however not all O157:H7 are EHEC, some of them lack eae/LEE and are only STEC (Kaper, et al 2004). AEEC strains that lack the bacteriophage genes encoding Shiga toxins are classified as enteropathogenic *E. coli* (EPEC)(Kaper 1996).

The first time STEC was recognized as a threat to public health was in 1982, when two outbreaks of STEC O157:H7 (EHEC) were identified for the first time in the U.S.(Oregon and Michigan) (Riley, et al 1983). STEC O157:H7 was isolated from stool cultures and from ground beef from a suspected lot of meat in Michigan (Riley, et al 1983). Since then, STEC O157:H7 is the most commonly found of the STEC serotypes. Based on the molecular analysis of these isolates , it was demonstrated that a lambdoid prophage, also known as bacteriophage, transferred the genes required to produce Stx into *E. coli,* resulting in a newly emergent pathogen (O'Brien, et al 1984). Phages regulate Stx production through amplification of gene copy number, activity of phage gene promoters, and through release of Stx (Gyles 2007). Through other horizontal gene transfer mechanism, STEC have acquired a variety of virulence factors, such as enterotoxins and fimbriea or pili (Gyles and Fairbrother 2010; O'Brien, et al 1984; Sahl,

et al 2013). One of the most important virulence factors for EHEC is an outer-membrane protein intimin, encoded by *eae* (*E. coli* attaching and effacing protein), which works as an adhesin (Jerse, et al 1990; Moon, et al 1983). The process of attachment and interaction between epithelial cells and *eae*-positive or *eae*-negative STEC is very different (Gyles 2007). Intimin binds to the translocated intimin receptor (Tir), a type III-secreted effector that localizes in the host plasma membrane after translocation into mammalian cells (Kenny, et al 1997). Intimin-binding to Tir induces a downstream signaling cascade that results in the formation of F-actin pedestals, which promote colonization and Stx-mediated disease (Moon, et al 1983). In LEE-negative STEC, binding of STEC to the epithelium occurs in a non-intimate manner (Gyles and Fairbrother 2010), for example, producing autoagglutinating adhesins encoded by the *saa* gene (Bolton 2011; Vidal, et al 2008). LEE- negative STEC also contribute to Shiga toxin mediated disease including HUS, for example, a study reported the cluster of three cases of HUS caused by a STEC O113:H21 strain lacking the *eae* gene (Paton, et al 1999). Strain O113:H21 express a newly identified cytotoxin, Subtilase-like toxin AB (SubAB), also an AB5 toxin (Paton, et al 2004). Other potential adherence factors, such as EibG, have been described in LEE-negative STEC, although their significance for human disease is not as well established as for intimin (Lu, et al 2006).

E. coli O157:H7's lack of β-glucuronidase activity and its inability to ferment sorbitol differentiate it from other *E. coli* strains (Vanaja, et al 2013). Specific biochemical characteristics of *E. coli* O157:H7 allowed the development of several selective media (e.g. CHROMagar O157 and Rainbow agar) to identify and characterize this STEC strain (Bettelheim 2003). Early screening for *E. coli* O157:H7 in human and animal feces was simplified by the development of protocols specific for this STEC strain whereas the burden of illness from non-O157 STEC

strains was not fully recognized. The major problem in detecting non-O157 STEC is that beside the production of Stx, they do not differ significantly in their biochemical characteristics from typical commensal *E. coli*, leading to diagnostic limitations and inadequate surveillance (Farrokh, et al 2013; Vanaja, et al 2013). There has been a steady increase in the number of cases caused by STEC of serotypes other than O157 (Crim, et al 2014; Scallan, et al 2011). This increase may at least partially be due to recent changes in laboratory practices by which non-O157 strains are more likely to be identified than they were in previous years (Gould, et al 2013). Currently, six non-O157 STEC serogroups (O26, O45, O103, O111, O121, and O145) and O157:H7 have been classified as adulterants of beef in the U.S. (USDA 2011). Consequently, rapid, accurate and reliable detection methods are necessary to test for non-O157 STEC in high risk food (Wang, et al 2013).

Importance of STEC in Public Health

STEC are one of the most relevant foodborne pathogens in U.S., Canada, and other developed countries. STEC have been also reported in developing countries, however, the proportion of morbidity and mortality caused by STEC in these countries is largely unknown. STEC O157:H7 is the most common serotype isolated in U.S., whereas other non-O157 STEC serotypes are more common in Australia, Germany and Austria (Tarr, et al 2005).

Malaysia, Thailand, Republic of Korea and China are some of the Asian countries where STEC O157 have been reported. In 1996, one of the most largest STEC outbreaks occurred in Japan with 9451 cases reported (Reilly 1998). *E. coli* O104:H4, however, caused an outbreak in Germany that extended to other countries including the U.S. This outbreak caused more

problems in healthy adults than any other outbreak ever reported. This particular strain possessed a combination of virulence genes from both EAEC and STEC (Frank, et al 2011).

The most common cause of acute renal failure in children worldwide is HUS resulted from a gastrointestinal infection with STEC (Tarr, et al 2005). Tarr, et al. (2005) reported a 15% risk of developing HUS in children younger than 10 years diagnosed with an *E. coli* O157:H7 infection. The case fatality rate of patients with HUS is on average 2-7%, but some outbreaks targeting elderly populations has resulted to 50% mortality (Reilly 1998). Unfortunately, the treatment for HUS is supportive and deaths are usually associated with severe extra-renal complications (Pennington 2010). Actually, the administration of antibiotics, antimotility agents or narcotics during diarrheal episodes caused by STEC has been associated with an increased risk of subsequent HUS (Tarr, et al 2005; Vanaja, et al 2013).

Studies have reported that patients with non-O157 infection were less likely to be hospitalized than those with STEC O157 infection (Crim, et al 2014; Gould, et al 2013). In 2012, among 496 serogrouped non-O157 STEC isolates, the most common serogroups were O26 (27%), O103 (23%), and O111 (15%) (Gillis, et al 2013). STEC O157:H7 has been reported to cause the highest frequency of human infections, although there has been a steady increase in the number of cases caused by STEC of serotypes other than O157 (non-O157 strains) (Crim, et al 2014; Gould, et al 2013; Scallan, et al 2011). This increase may partially be due to recent changes in laboratory practices and diagnostics by which non-O157 strains are more likely to be identified than they were in previous years (Gould, et al 2013).

1. Clinical disease in humans

In humans, STEC has a low infection dose of 10 to 100 organism, due largely to its ability to resist highly acidic (pH 1.5-3.0) gastric environments (Menrath, et al 2010; Vanaja, et al 2013). At least three acid resistance mechanisms have been identified in STEC O157:H7 that allow these bacteria to survive in low pH environments. These mechanisms are a glutamate dependent system, an acid-inducible arginine-dependent system, and oxidative systems (Audia, et al 2001; Gyles and Fairbrother 2010).

The incubation period for STEC ranges between two to twelve days. Once established in the intestinal tract, STEC leads to effacement of microvilli, inflammation and active chloride secretion in the large intestine, resulting in watery diarrhea for one to three days followed by hemorrhagic colitis in 90% of the cases. Other common signs are absence of fever and severe abdominal pain (Bolton 2011; Pennington 2010; Tarr, et al 2005). Stx produced by STEC lead to vascular damage and subsequent bloody diarrhea. Toxins are transported in the bloodstream to sites rich in the Stx receptor Gb_3, including the renal glomeruli, the renal proximal tubular epithelium, and the brain (Bolton 2011; Gyles 2007). Stx2 is about 1,000 times more toxic to human renal microvascular endothelial cells than is Stx1 (Gyles 2007).

HUS is characterized by hemolytic anemia, thrombocytopenia, and renal failure (Vanaja, et al 2013). The release of chemokines, including IL-8 and other factors by the host, results in platelet activation and subsequent renal thrombosis which is characteristic of HUS (Gyles 2007). Glomerular capillaries are occluded by these thrombi resulting in ischemic damage to renal endothelium (Vanaja, et al 2013). STEC infection in humans has also been associated with neurological symptoms. There is evidence that Gb_3 is present in neurological tissue making it susceptible to the Stx.

2. STEC pathology in animals

In swine, STEC is the agent responsible for Edema Disease (ED). ED is the only animal disease for which the role of Stx is clearly established (Gyles and Fairbrother 2010; Tseng, et al 2014). STEC with the Stx2e induces a toxemia that causes severe edema in specific sites in post-weaning pigs and young finishing pigs; the most susceptible pigs to ED seems to be those with the fastest growth (Gyles and Fairbrother 2010; Tseng, et al 2014). Pigs lacking intestinal receptors for F18ab fimbriea are resistant to ED. The transportation of pigs and the mixing of pigs from different sources have been mentioned as factors that predispose ED. A body of research exists which led to the identification of the specific receptor for Stx2e called globotetraosylceramide (Gb4), located in epithelial or vascular endothelial cells, due to the impact that ED has had on the swine industry. However, Stx2e can also bind to Gb3. Stx2e binding causes edema and hemorrhage and can present as sudden death without signs of illness. Some affected pigs become inappetent, develop swelling of the eyelids and forehead and show incoordination and respiratory distress (Gyles and Fairbrother 2010).

STEC has also been implicated in calves and lambs with diarrhea and dysentery. STEC colonize the large intestine of calves and cause AE lesions similar to humans. Diarrhea may result from loss of absorptive microvillus surface, activation of secretory activity in epithelial cells, and loosening of tight junctions. Stx1 and/or Stx2 reach the blood system, presumably causing bloody diarrhea, however no systemic signs are observed (Gyles and Fairbrother 2010).

STEC are present in the feces of healthy and diarrheal dogs. HUS occurs in about 5% of the dogs that develop diarrhea caused by STEC. STEC has been implicated as a cause of a syndrome

called cutaneous and renal glomerular vasculopathy (CRGV) in racing greyhounds fed poor-quality ground beef (Gyles and Fairbrother 2010).

STEC reservoirs and transmission

Transmission of STEC is a complex process that involves different reservoirs, different hosts and different environments. To best understand transmission, we can look toward where and how contamination or infection occurs. Ruminants, especially cattle, are the major STEC reservoir. Humans most commonly get infected through the consumption of contaminated beef products (especially ground beef) or unpasteurized milk and dairy products contaminated with feces. However there have been outbreaks with other less common sources such as unpasteurized apple juice, spinach and salami (Chase-Topping, et al 2008; Hussein 2007; Jay, et al 2007; Menrath, et al 2010). Produce can also get contaminated with STEC through the inclusion of manure in soil, which results in STEC uptake directly by plants, leading to human infection (Callaway, et al 2013; Franz and van Bruggen 2008). Direct animal contact is another source of transmission to humans. There have been STEC cases due to direct contact with animals on farms, fairs, and petting zoos (Goode, et al 2009). Rainfall events can wash STEC from cattle feces into drinking, recreation or irrigation water supplies, which have led to infection in humans and other animals (Berry and Wells 2010; Callaway, et al 2013; Franz and van Bruggen 2008; Hussein 2007; Solomon, et al 2002).

Besides cattle, other ruminants that carry STEC are deer (white-tailed deer, red deer), sheep, and goats (Asakura, et al 1998; Callaway, et al 2013; Dunn 2003; Ferens and Hovde 2011). Other species reported to carry STEC at least transiently are pigs, rodents, and birds, such

as starlings, cowbirds, turkeys and egrets (Callaway, et al 2013; Cernicchiaro, et al 2012; Ferens and Hovde 2011). EHEC *O157* can be carried by amphibians, fish and invertebrates, and mollusks, as well as insects such as flies (Berry and Wells 2010; Ferens and Hovde 2011; Heuvelink, et al 1998). Studies have demonstrated that houseflies are not only mechanical vectors, but *E. coli* O157:H7 can likely multiply in their gastrointestinal tract (Soon, et al 2011). STEC can be transmitted from infected humans to uninfected humans through direct contact, which is known as secondary spread. This has been most commonly reported in daycare and elderly facilities (Gyles 2007; Pennington 2010; Tarr, et al 2005)

STEC isolates from cattle have an overall greater genetic diversity, fewer virulence factors, but a greater tolerance for adverse conditions when compared to STEC isolates from humans. STEC isolates associated with severe disease in humans are a minor fraction of the strains found in cattle (Ferens and Hovde 2011). Hence, not all bovine strains are pathogenic to humans and those that are pathogenic have particular characteristics that differentiate them and could be used to develop specific diagnostic tests or control strategies.

Risk factors for STEC shedding by both dairy and beef cattle

The most frequent and consistently reported risk factor for STEC shedding for both beef and dairy cattle production systems has been season of the year. The highest level of STEC shedding occurs during warm months (Callaway, et al 2009; Callaway, et al 2013; Dunn, et al 2004; Hancock, et al 1994; Heuvelink, et al 1998; Kondo, et al 2010; Menrath, et al 2010; Smith, et al 2013). For this reason, almost all studies include season in their study design as a cofounder in their model and purposely programmed their sampling during the warm months (Cernicchiaro, et

al 2012; Cho, et al 2013; Cobbaut, et al 2009; Cobbold, et al 2004; Farrokh, et al 2013; Hussein and Bollinger 2005; Menrath, et al 2010). Seasons not only represent a change in surface temperature, but also season is proxy for other different things such as diet and management practices. Changes in both the host and the environment are possible explanations for this association. One possible reason for the seasonality of STEC shedding is the physiological responses of cattle due to change in day length and heat stress. Another is adverse environmental conditions, such as mud and higher temperatures that favor the growth of STEC outside the animal (Berry and Wells 2010).

Age is also an important risk factor that has been identified for STEC shedding, although there is not an agreement regarding which age group is at the highest risk (Cernicchiaro, et al 2009; Cho, et al 2013; Cobbaut, et al 2009; Farrokh, et al 2013; Hussein and Sakuma 2005; Kuhnert, et al 2005). Cho, et al. (2009) reported that among all cattle, preweaned calves (calves receiving milk or milk replacer) had a higher risk of STEC shedding than adult cows. However, another study reported that calves aged 4 to 12 months had the highest STEC shedding rate compared to all other age groups (Heuvelink, et al 1998). In this same study, cattle older than 3 years were more often found to be shedding STEC than cattle between 1 and 3 years of age (Heuvelink, et al 1998). In adult dairy cows, one study reported a trend of higher shedding of STEC in dairy cows with a parity of ≥ 4 than cows with less than 4 parities. (Cho, et al 2009). In contrast, Menrath, et al. (2010) reported that first calf heifers were at higher risk than those cows with ≥ 2 lactations (older age). Though there is no consensus regarding what age has the highest risk for STEC, the majority of studies reported that younger animals are at higher risk.

Another risk factor for STEC shedding is contact with other infected species, such as pigs, cats, dogs, rabbits, deer, birds, and pests, like flies and rodents (Berry and Wells 2010;

Cernicchiaro, et al 2009; Cho, et al 2013; Farrokh, et al 2013). Cernicchiaro, et al. (2012) reported an increase STEC risk as the number of birds per milking cow increased. Similarly, the use of mixed animal agriculture was also reported as a risk factor for STEC shedding in cattle (Cernicchiaro, et al 2009).

Additional risk factors include introduction of new animals into groups/pens, animal density, contact between adult cattle and calves (Cernicchiaro, et al 2012), size/number of farm/cattle (Cho, et al 2013; Herbert, et al 2014), transportation and lairage, and stressful situations.

Animal density is especially important for hide contamination, which becomes a significant risk factor for meat contamination at slaughter (Cernicchiaro, et al 2009; Herbert, et al 2014). Cho, et al. (2013), reported that STEC shedding was more common in small dairy herds than in large herds (≥100 cows). Bringing new cattle into the herd or recent animal movements have also been found to increase the risk of STEC shedding at the herd level (Farrokh, et al 2013; Herbert, et al 2014). For instance, the number of times cattle were taken to a livestock exhibition in the previous 12 months was a risk factor for STEC O157 in cow-calf operations (Cernicchiaro, et al 2009). Transportation and lairage are also risk events for transmission or contamination among animals. Direct or indirect transmission among animals can occur during transportation; plus the resulting stressful situation that transportation represent to animals can add to the risk of colonization and shedding (Callaway, et al 2013).

The presence of super-shedders is reported as a STEC risk factor in several studies for both STEC O157 and non-O157 STEC (Berry and Wells 2010; Callaway, et al 2013; Chase-Topping, et al 2008; Chase-Topping, et al 2007; Menrath, et al 2010). A super-shedder is "… an animal that excretes $>10^4$ cfu per gram of feces or the simple identification of outlying counts" (Chase-

Topping, et al 2008). The presence of a super-shedder increases STEC transmission rate, as statistical models have determined (Chase-Topping, et al 2008).

The cleanliness of bedding has been reported as a risk factor for STEC shedding. Herds with clean and dry bedding, as well as with frequent change of bedding, have been associated with a decreased risk of STEC shedding. Also, inorganic bedding, such as sand, has been shown to be less favorable for coliform replication in general, thus decreasing risk of STEC colonization and shedding. Frequent cleaning of the pen surface can also influence the risk of STEC transmission and survival, as it may slow spread within a herd, although it will not completely eliminate STEC (Callaway, et al 2013).

The exact effect of stress on STEC colonization or shedding is still unclear, but increased stress has frequently been reported as a STEC risk factor (Berry and Wells 2010; Chase-Topping, et al 2007; Cho, et al 2013; Farrokh, et al 2013) . Besides transportation, other stressful situations to cattle are weaning, calving, heat stress, handling, loading and unloading, changes in climatic conditions, food and water deprivation (Callaway, et al 2013; Rostagno 2009). One possible explanation for stress contributing to STEC shedding is that the central nervous system and the enteric nervous system have an established communication. Thus stress can lead to the release of hormones into the intestinal tract, that can alter the interactions between the microbiota and the endothelial cells facilitating the infection of the intestinal tract by pathogenic microbiota (Rostagno 2009). Studies have demonstrated that norepinephrine can influence the production of Stx by *E. coli* O157:H7 and its adhesion to the cecal epithelium in cattle (Lyte, et al 1996; Rostagno 2009).

Cattle are often subject to fasting before or during transportation to slaughter. Fasting has been reported to increase the risk of STEC shedding at slaughter facilities while others reported

no effect (Callaway, et al 2009; Callaway, et al 2013; Rostagno 2009). The mechanism linked with STEC and fasting is thought to be by decreasing short chain volatile fatty acids (VFA) and increasing pH in the gastrointestinal tract (Callaway, et al 2013).

Feed, usually called diet and its components, are another important risk factor for STEC shedding. There is a large body of research regarding STEC and diet, which has shown that diet does affect *E. coli* O157 populations, but the magnitude and impact of diet or its components haven't always presented consistent results (Callaway, et al 2009). Example of diet components that affect *E. coli* O157 shedding are percentage of forage and rapidly ruminally fermented grains, among others (Callaway, et al 2009; Cernicchiaro, et al 2009). Some studies reported that barley-based diets increase *E. coli* O157 shedding due to pH increased in feces. Among other feed types linked with an increased risk for STEC shedding are corn silage (Cernicchiaro, et al 2009), distillers grain, brewers grain and wet corn gluten (Callaway, et al 2009; Callaway, et al 2013), while whole cottonseed has been linked with a decreased risk in STEC shedding (Callaway, et al 2013). However a recent study found no association between STEC and distiller grains (Fink, et al 2013). How feeds are processed may also have an effect on STEC colonization and shedding. For example, steam-flaked corn has been associated with an increase risk of STEC when compared to dry-rolled corn (Callaway, et al 2009; Callaway, et al 2013).

Studies have reported that grain-fed cattle shed more *E. coli* O157 than forage-fed cattle (Callaway, et al 2013). In addition, there are conflicting results when looking at whether the switch from a grain base to a pasture grain base diet decreased or not *E. coli* O157 shedding. Some studies report a significant association towards *E. coli* O157 reduction when the switch was made while others reported no association (Callaway, et al 2009; Callaway, et al 2013; Stanford, et al 2005). One possible explanation for the differences in conclusions among studies

is the use of different diets. It is believed that diversity in quality and components of the forage could be the factors responsible for the different results between studies (Callaway, et al 2009; Callaway, et al 2013).

Ionophores (ex. monensin and lasalocid) are included in the diet to inhibit gram-positive bacteria and promote feed efficiency. There are studies that indicated ionophores increase *E. coli* O157 shedding, while another indicated a decrease, and there are even other studies that reported zero effect (Callaway, et al 2009). For example, Cho, et al. (2013) reported that the use of monensin for weaned calves, and the use of decoquinate (a quinolone derivative) for preweaned calves decrease the risk of STEC shedding. The lack of consistent results about the effect of ionophores in STEC makes necessary more research the find the right answer. Also this lack of consistency in results exposed the importance of more consistent methodology and study design among future studies.

In addition to the presence of other species, the type of cattle production system factors into the risk of STEC shedding. When differences in the level of risk for STEC shedding between dairy and beef farms have been reported, dairy farms usually present higher levels of STEC shedding (Cobbaut, et al 2009; Cobbold, et al 2004). Similarly, the type of cattle, more specifically, the raising of female cattle for breeding, increased the risk of STEC shedding compared to raising of cattle for beef (Chase-Topping, et al 2007).

There are risk factors that have been reported exclusively for the dairy or beef production system. Among the risk factors for dairy is stage of lactation. According to several studies, the risk of STEC shedding is higher in lactating cows than in dry cows (Cho, et al 2009; Dunn, et al 2004; Fitzgerald, et al 2003; Mechie, et al 1997). Another group in dairy production at higher risk for STEC shedding is cull cows; cull cows are those cows selected to go to slaughter. In a

study by Cho, et al (2009), cows that were scheduled to be culled were more likely to be shedding STEC than those not scheduled to be culled. Menrath, et al. (2010), reported several risk factors for detection of STEC in dairy cattle feces in Germany. They found that cows with a somatic cell count lower than 100,000 cells/ml in milk, milk protein content higher than 3.0% and a body condition score higher than 3.50 had significantly or tendency towards increased risk of shedding STEC; while cows with blood urea content lower than 150 mg/L milk had a decreased risk. These measures in milk are related to the diet, health and stress of the cow. So they could be taken as a proxy for these other factors that have been reported to influence STEC shedding.

Several studies reported their findings about dairy herd management practices and its association with STEC shedding. The use of total mixed ration (TMR) for lactating dairy cows was reported to increase STEC shedding (Cho, et al 2013). The use of manure piles for manure storage was also reported to increase STEC shedding, and the use of three or more different ventilation systems (ex. doors, fans, curtains) on the farm also increased STEC shedding (Cernicchiaro, et al 2012). Garber (1999) reported a higher risk for STEC shedding in those herds that use flushed water to remove manure compared with other methods of manure removing. Hancock, et al. (1994), reported an increase risk for STEC presence in cattle when owners apply slurry to pasture.

Some risk factors reported specifically for beef cattle (feedlot and/or cow-calf operations) deal with parturition and weaning as events that increase the risk of STEC shedding in cows and calves respectively (Gannon, et al 2002). In addition, Sargeant, et al (2003) described a positive relationship between the water tank's sediment and the water in those water tanks being STEC positive as well as the cattle who drink that water in feedlots, with capacities >1000 heads, being

STEC positive. Also Smith, et al. (2005) reported the recovery of *E. coli* O157:H7 from water tanks as a risk for STEC positive cattle. The use of corn silage supplementation in winter (silage preparation) is another herd management practice reported to increase the risk of STEC shedding (Cernicchiaro, et al 2009).

Intervention strategies at the pre-harvest level

Since establishing cattle as the main reservoir of STEC, control measures have been developed and implemented during the pre and post-harvest periods to reduce the risk of beef contamination and subsequent human infection. These measures have helped to reduce the number of STEC cases in humans and the public health burden (Gillis, et al 2013).

Several studies have concluded that control measures at the pre-harvest level will have the highest impact in the reduction of STEC infections (LeJeune and Wetzel 2007; Soon, et al 2011). Callaway, et al. (2013), summarized the reasons very clearly and concisely "… 1) reducing the amount of pathogens entering processing plants will reduce the burden on the plants and render the in-plant interventions more effective; 2) reducing horizontal pathogen spread from infected animals (especially in "super-shedders") in transport and lairage; 3) will reduce the pathogenic bacteria burden in the environment and wastewater streams; and 4) will reduce the direct risk to those in direct contact with animals via petting zoos, open farms, rodeos and to animal workers". LeJeune and Wetzel (2007) grouped the pre-harvest interventions into 3 categories "1) exposure reduction strategies; 2) exclusion strategies and 3) direct antipathogen strategies".

1. Exposure reduction strategies

1.1. Environmental exposure

Avoiding muddy feedlot pens and providing dry bedding helps with the reduction of STEC as well as preventing fecal-oral infection or re-infection (Soon, et al 2011). The treatment of manure with carbonate and alkali has also been demonstrated to inactivate *E. coli* in cattle manure (Berry and Wells 2010; LeJeune and Wetzel 2007). Some plant essential oils added to cattle waste such as carvacrol, eugenol and thymol have been reported to reduce or eliminate *E. coli* (Berry and Wells 2010; Doyle and Erickson 2012; Varel and Miller 2004). It is also important to apply hygienic practices during transportation (Doyle and Erickson 2012).

1.2. Wildlife exclusion

Although cattle are the main reservoir for STEC, contamination of feed and water with fecal material from wildlife could introduce into cattle new STEC strains, through the fecal-oral route, so avoiding access of wildlife from the farm should be attempted as much as possible (Soon, et al 2011).

2. Exclusion strategies

2.1 Probiotics

Probiotics are defined as "a preparation of a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora in a compartment of the host and that exert beneficial health effects in this host" (Schrezenmeir and de Vrese 2001). The probiotics are also called direct fed microbials (LeJeune and Wetzel 2007). An important probiotic that has been frequently reported capable to reduce the shedding of STEC is *Lactobacillus acidophilus.* The specific strain that appears to be most efficacious and already

available on the market is NP51. Some point out that the selection of the right strain is very important for successful reduction of STEC (Cull, et al 2012; LeJeune and Wetzel 2007; Loneragan and Brashears 2005; Sargeant, et al 2007; Soon, et al 2011; Stephens, et al 2007). Some other effective probiotics reviewed include, either individually or in combinations, *Enterococcus (Streptococcus) faecium, L. casei, L. fermentum, L.gallinarum, L. platarum, Propionibacterium freudenreichii,* and *Streptococcus bovis* (Berry and Wells 2010).

2.2 Prebiotics

Prebiotics are defined as "organic compounds such as fructo-oligosaccharides, inulin and galacto-oligosaccharides that are unavailable to, or indigestible by, the host animal, but are digestible by specific bacterial species" (LeJeune and Wetzel 2007; Soon, et al 2011). When probiotics and prebiotics are administered together it is called Synbiotics (Doyle and Erickson 2012).

2.3 Other diet supplements

There are studies that reported an inhibitory effect of a additive product from brown seaweed (*Ascophyllum nodosum*) on *E. coli* O157:H7 (Bach, et al 2008; Braden, et al 2004) but more information is required to confirm this event, as some mentioned brown seaweed is not an efficacious intervention (Loneragan and Brashears 2005).

3. Direct antipathogen strategies

3.1 Antimicrobial Compounds

Studies have demonstrated a reduction in STEC shedding after cattle received oral neomycin sulfate, an aminoglyoside antibiotic. However, there are concerns regarding this practice due to the fear of developing antibacterial resistance in humans (Berry and Wells 2010; LeJeune and Wetzel 2007; Loneragan and Brashears 2005). Another negative side is that supplementation of milk replacer with Neomycin may increase *E. coli* O157:H7 shedding in very young calves (Berry and Wells 2010). Reports also claim STEC shedding reduction with the use of sodium chlorate, whether via feed or water in cattle. The application of chlorate for this use is pending U.S. Food and Drug Administration review and approval (Anderson, et al 2005; Berry and Wells 2010; LeJeune and Wetzel 2007; Loneragan and Brashears 2005; Sargeant, et al 2007).

3.2 Bacteriophage Therapy

Studies have reported the use of bacteriophage (virus of bacteria) as an effective therapy to decrease *E. coli O157:H7* in cattle and or other ruminants (Sheng, et al 2006). One advantage of phages is their narrow target spectra, specifically in this case STEC (Soon, et al 2011). Bacteriophages have been administered orally through water or feed and directly to the recto-anal junction (RAJ) (Berry and Wells 2010; Sheng, et al 2006). Some concerns regarding the use of bacteriophages are the development of phage resistance and the possibility of genetic materials being transferred to bacterial hosts (Soon, et al 2011).

3.3 Vaccination

Several studies have showed that cattle vaccination decreases shedding of *E. coli* O157 and there are even results that indicate that cattle vaccination is considered the most effective measure to reduce human exposure to *E. coli* O157 (Smith, et al 2013). Even with prices

between $2.29 and $ 9.14 USD, vaccination can be a cost effective intervention measure (Smith, et al 2013). Loneragan, et al. (2005), discussed the potential advantages for the use of vaccine that include: “1) cattle producers are familiar with administration of vaccines; 2) incorporation into existing management of cattle would be fairly simple; 3) vaccines could be used in all sectors of the industry”.

One vaccine has been developed against *E. coli* O157:H7 type III secreted proteins (Bioniche Life Sciences, Inc., Belleville, Ontario, Canada) (Berry and Wells 2010). Type III secreted proteins are critical for *E. coli* O157:H7 intestinal colonization in cattle. This vaccine is fully licensed for use in Canada (Berry and Wells 2010). The vaccine license is conditional for the U.S. market (Vande Walle, et al 2013). Another vaccine targeting siderophore receptor and porin proteins (SRP) (Epitopix, LLC, Wilmar, MN) currently has a conditional license for use in cattle in U.S. (Berry and Wells 2010). The SRP is a cell membrane receptor that is essential for iron transport into cells. This vaccine produces antibodies which bind the *E. coli* O157:H7 SRP thus essentially starving the cells of iron, leading to their eventual death (Cull, et al 2012). There are studies recommending a three-dose regimen (Berry and Wells 2010; Vande Walle, et al 2013) while others used a two-dose regimen (Cull, et al 2012). A new technical approach for *E. coli* O157:H7 vaccine design is the use of bacterial ghosts (BGs), as inactivated whole-cell envelope vaccines. BGs are empty bacterial cell envelopes, which display all surface components, including colonization factors in a non-denatured form and are able to induce a strong mucosal immune response (Vande Walle, et al 2013). There is still the necessity to develop more studies to explore the efficacy of this BGs vaccine.

Conclusions

Historically *E. coli* O157 has been the "star" strain in the group of serotypes belonging to STEC because of the frequency of reported outbreaks caused by *E. coli* O157. But this does not mean that the other non-O157 STEC bacteria are a less important threat to public health. This may be just a reflection of the lack of laboratory techniques to detect these other bacteria. As a result, scientists have been working on the development and improvement of isolation and detection methods for non-O157 STEC. The effort to improve non-O157 STEC detection has led to more frequent detection of both STEC O157 and non-O157 STEC. For example, the immunomagnetic separation (IMS) assay is a sensitive method that was design to detect *E. coli* O157. This same methodology is now being adapted to non-O157 serotypes thus leading to improved and more reliable detection.

Over the years, many different risk factors have been identified in association with STEC shedding. Some studies have found similar factors while others have found contradictory risk factors. It is also important to discuss the difficulty to compare results between studies, due to the differences among laboratory methods and study design (Cobbaut, et al 2009; Sargeant, et al 2003). In careful evaluation of the literature, it is evident there is a diversity in the methodology applied by the different researches to detect STEC. This makes it harder and sometimes impossible to compare results between studies. For example, the association between several risk factors, such as bedding type or house type and STEC shedding in cattle hasn't been determined yet, because the results among the available studies cannot be compared due to the differences among laboratory methods. Regarding study design, the lack of uniformity in study design, sampling strategies and animal premises analyzed (Cho, et al 2013; Cobbaut, et al 2009; Menrath, et al 2010; Sargeant, et al 2003) makes it difficult to derive definite, well supported and

consistent conclusions (Sargeant, et al 2003). Therefore the different STEC research groups should be more consistent with the methodology so results can be compared.

Risk factors that affect different production systems have been identified including season and age. This is advantageous for the development and application of intervention strategies aimed to control or prevent the transmission among animals and also to humans. Although the identification of common risk factors is relevant, it will be equally beneficial and probably easier to target risk factors specific for each production system. In this way, intervention strategies designed for each production type could be implemented.

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CHAPTER 2

RISK FACTORS FOR SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* (STEC) SHEDDING IN CATTLE

Abstract

Shiga toxin-producing *Escherichia coli* (STEC) are one of the most important foodborne pathogens in the U.S. and other developed countries. STEC can cause hemorrhagic diarrhea, and sometimes hemolytic uremic syndrome (HUS). STEC is defined by the presence of genes encoding the Shiga toxin (Stx), of which Stx1 and Stx2 are the major types, but additional subtypes have also been described. Cattle are the primary reservoir for STEC, and food or water contaminated with cattle feces is the most common source of infections in humans.

The purpose of our study was to identify risk factors for STEC shedding in cattle. During the summers of 2011 and 2012, a cross-sectional study was performed on 1,096 cattle in 5 dairy herds and 6 beef herds. A fecal sample from each animal was enriched in *E. coli* broth (EC) and plated on selective media. In addition, a portion of the broth was subjected to immunomagnetic separation targeting *E. coli O157*, and multiplex PCR was used to detect the presence of *stx1*, *stx2* and the gene encoding intimin (*eaeA*). STEC prevalence was 21% (80/378) in beef cattle, which was significantly higher than the 13% (95/718) in dairy cattle (OR: 1.76; 95% CI: 1.25-2.47). A multivariable model, with herd included as a random effect, was used to evaluate both herd-level and cow-level risk factors for dairy cattle. Dairy cattle were more likely to shed STEC when the average temperature was > 84°F 1-5 days before sampling (OR: 2.5; 95% CI: 1.25-4.91). Dairy cows were more likely to shed STEC in their first lactation (OR: 1.8; 95% CI: 1.1-

2.8) and when they were < 31 days in milk (OR: 3.9; 95% CI: 2.1-7.2). Descriptive epidemiologic studies such as this one will hopefully foster hypothesis-testing and intervention strategies aimed at mitigating STEC shedding in cattle, thereby reducing the risk of human infections.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) is one of the most virulent and pathogenic foodborne pathogens in both developed and developing countries (Reilly 1998). STEC can cause hemorrhagic diarrhea and sometimes hemolytic uremic syndrome (HUS) that can lead to kidney failure and death, particularly in young children (Vanaja, et al 2013). STEC belonging to serotype O157:H7 has been reported to cause the highest frequency of human infections, although there has been a steady increase in the number of cases caused by STEC of serotypes other than O157 (non-O157 STEC) (Crim, et al 2014; Scallan, et al 2011). This increase may at least partially be due to recent changes in laboratory diagnostic practices by which non-O157 strains are more likely to be identified than they were in previous years (Gould, et al 2013). The incidence of U.S. reported non-O157 STEC cases increased from 0.12 per 100,000 population in 2000 to 0.95 per 100,000 in 2010, while the incidence of STEC *O157* decreased from 2.17 per 100,000 in 2000 to 0.95 per 100,000 in 2010 (Gould, et al 2013). In year 2013, there were 561 cases (1.17 per 100, 000 people) of non-O157 and 552 (1.15 cases per 100,000) for STEC O157. In this same year there were 78 hospitalizations and 2 deaths associated with non-O157 and 210 hospitalizations and 2 deaths associated with STEC O157 (Crim, et al 2014). These findings support the Gould et al, (2013) report that patients with non-O157 STEC infection were less likely to be hospitalized than those with O157.

STEC is defined by the presence of genes encoding Shiga toxins (Stx), which are carried on a bacteriophage (O'Brien, et al 1984). The two major Stx types are Stx1 and Stx2, but additional subtypes (e.g., Stx2c-2g) have also been described (Scheutz, et al 2012). The *eaeA* gene, which is present on the LEE island and encodes for the intimin protein, allows STEC to intimately adhere to the intestinal mucosa (Fagan, et al 1999; McDaniel, et al 1995). All STEC

strains have at least one *stx* subtype, though the locus of enterocyte effacement (LEE) pathogenicity island may be variably present. STEC strains with the LEE island and *stx* are referred to as enterohemorrhagic *E. coli* (EHEC), while STEC refers to *stx*-positive strains that lack the LEE island. EHEC typically causes more severe clinical symptoms in humans relative to STEC (Beutin, et al 2007; Reilly 1998), though the 2011 STEC *O104:H4* outbreak in Germany that contributed to over 50 deaths (Frank, et al 2011) is an exception.

Cattle are the primary reservoir for STEC, and food or water contaminated with cattle feces is the most common source of infection for humans (Kuhnert, et al 2005).Other sources of STEC infection include direct contact with domestic animals, such as swine, dogs and cats, and wildlife including wild-white-tailed deer (Asakura, et al 1998; Beutin, et al 1993; Rounds, et al 2012).

STEC prevalence has been shown to vary across food animal production systems in the U.S and other countries. For example, the prevalence of STEC O157 infections was 45%, 19% and 8% in cow-calf operations in Ontario, feedlots in Scotland, and dairy cattle in Washington respectively (Cernicchiaro, et al 2009; Chase-Topping, et al 2007; Hancock, et al 1994). Additionally, worldwide the prevalence of non-O157 STEC reported in feedlots and beef cattle on pasture ranged between 4.6% to 55.9% and 4.7% to 44.8%, respectively (Hussein 2007). Factors associated with low or high herd prevalence estimates, however, are not fully understood. It is therefore important to determine which production systems represent the greatest risk of STEC infection for the efficient implementation of pre-harvest and post-harvest intervention strategies.

Several prior studies have reported a higher prevalence of STEC shedding in pasture cattle and dairy farms than in feedlots (Cobbold, et al 2004), while others have found differences

attributable to geographic location. Studies in Sweden and Korea, for instance, have reported significant regional differences. Positive cattle samples appeared to be concentrated in the southern and central parts of Sweden (Kistemann, et al 2004), while in Korea, the region of Gyeonggi and Gangwon, had higher prevalence rates than other parts of the country (Kang, et al 2014). Other studies have not observed differences across region (Sargeant, et al 2003). Although numerous studies have sought to determine the prevalence of STEC in animal reservoirs and varying geographic locations, additional studies are still needed to better understand the risk factors associated with STEC shedding at both the herd and animal level. Similarly, more research is needed to identify which groups of cattle have the highest risk of STEC colonization and shedding as these groups represent the best targets for pre-harvest intervention strategies.

To date, few consistent risk factors have been identified for STEC O157 shedding in cattle across studies (Cho, et al 2013; Menrath, et al 2010). This lack of consistent risk factors is even more dramatic for non-O157 STEC, due to the scarcity of research studies (Menrath, et al 2010). For STEC O157, several risk factors including season, herd management practices (manure removing), age, level of animal-to-animal contact, stress and diet have been suggested to be important (Cernicchiaro, et al 2009; Cho, et al 2013; Dunn, et al 2004; Garber 1999). However, most research has been focused on STEC O157 rather than non-O157.

To guide STEC shedding prevention strategies, additional large-scale studies are needed to better understand the transmission dynamics of STEC within and across herds with varying management practices. Here, we conducted a cross-sectional study of 1,096 animals from five dairy and six beef herds during the summers of 2011 and 2012. Our goal was to identify factors important for STEC shedding throughout Mid-Michigan. The identification of risk factors for

STEC shedding in cattle could aid in the improvement of intervention practices aimed at reducing the level of STEC entering the human food supply.

Materials and methods

1. Study design and herd selection

A convenience sample of dairy farms and beef feedlots were contacted and selected for inclusion in the study based on the availability of good records, proximity to East Lansing Michigan, adequate animal handling facilities and willingness to participate in all phases of the study. Eleven of twelve herds contacted agreed to participate. One herd chose not to participate because of concerns regarding animal welfare. The farm owners provided written informed consent to participate in the study, and each received a monetary incentive following study completion. This study was approved by the Michigan State University Institutional Animal Care and Use Committee (AN12/10-223-00) and this study was supported by the USDA NIFA Grant #2011-67005-30004.

Phase I involved completing a questionnaire designed to collect demographic information and data related to potential STEC risk factors. Phase II focused on sampling a representative number of animals within each herd and culturing for STEC. Herds were visited and sampled between May 11th and August 16th of 2011 (n=5) or between May 21st and August 27th of 2012 (n=6). Season was gauged based on the day of the equinoxes and solstices indicated on the Gregorian calendar.

1.1 Questionnaire

Two questionnaires were designed; one for dairy farms and another for beef feedlots. Both were pre-tested on the managers of representative farms. Questionnaires were administered to the farm owners or managers during a face-to-face interview at the first visit. The same person administered the questionnaires for all 11 farms. The questionnaires consisted of both closed and open-ended questions addressing farm demographics, animal movements, farm management practices, and herd health management strategies (**Table 2.1** and **Figure 2.4**).

1.2 Sampling

The number of cattle sampled per herd was based on the type of herd and number of cattle. In dairy herds with fewer than 175 animals, all adult cattle were sampled. In dairy herds with greater than 175 animals, a convenience sample of 175 cattle was selected with representation from each management group. In the beef feedlot herds, we selected cohorts of cattle within the feedlot that were managed as one unit and then sampled all cattle within that cohort. A summary of herd demographics and animals sampled can be found in **Table 2.2**.

Fresh fecal samples collected by rectal palpation using individual obstetrical sleeves, were placed in plastic bags (Whirl pak). Samples from the first four herds (496 animals) were transported to the laboratory on ice where they were stored at 4°C and then processed within 48 hours. The remaining seven herd's samples were transported to the laboratory in a cooler without ice and processed within 8 hours. This change in protocol was made because a prior study found that ice storage decreased the likelihood of STEC recovery from feces [Mindy Brashears, personal communication].

The date, time, latitude and longitude were recorded for each farm sampled. In addition,

the maximum, minimum and average temperatures from the day of sampling and the preceding five days were recorded using data from the closest weather station (Quality Controlled Local Climatological Data (NOAA)).

2. Laboratory protocol for STEC detection and isolation

Five grams of feces were inoculated in 2X EC broth (Oxoid Ltd.; Waltham, MA) supplemented with novobiocin (8mg/l), rifampin (2mg/1) and potassium tellurite (1mg/1) for 24 hours at 42°C (Jason, et al 2009) followed by subculture on STEC CHROMagar™ (CHROMagar, Paris, France) and sorbitol MacConkey (SMAC) agar. A portion of the EC culture was also processed by immunomagnetic separation using Dynabeads® (Invitrogen Corporation, California, USA) specific for *E .coli O157* followed by subculture to O157 CHROMagar (CHROMagar, Paris, France) and sorbitol MacConkey (SMAC) agar. Up to 20 presumptive STEC single colonies were selected from each plate, inoculated into Luria-Bertani (LB) broth for growth overnight at 37°C, and confirmed by PCR using a previously described protocol (Tarr, et al 2002) with either the Taq 2x MeanGreen Master Mix or Kappa2G Multiplex Master Mix (Kapa Biosystems, Massachusetts). The multiplex PCR used to confirm STEC single colonies detects the presence of *stx1*, *stx2* and *eaeA* (intimin). Individual colonies with at least one *stx* gene were considered to be STEC, and fecal samples from individual cattle were considered positive if at least one STEC isolate was recovered.

3. Data analyses

The data was analyzed using SAS 9.3 (SAS Institute Inc., Cary, NC, USA). The dependent variable used in the analysis was the positive or negative STEC status of the animal. An animal was considered positive when STEC could be recovered by culture. The analysis performed in this study was based on the STEC results at the animal level and not at the isolate level. There was an average of four isolates per STEC positive animal, with a range of one to 19 isolates.

The distribution of the independent variables was analyzed. Those independent variables with non-normal distributions were transformed into binary or categorical variables based on their average or quartiles. All the categorical variables are based on answers provided by the farmers. The average on the temperatures variables were calculated based on the data collected from the weather stations. For the univariate analysis the independent variables were analyzed in groups, those groups were herd characteristics, housing, cleaning, herd treatment, diet, contact with other animals, and environmental conditions. In **Tables 2.4 and 2.6** the independent variables analyzed were displayed. See **Table 2.7** for key of the variables names.

Variables with potential confounding were identified. The univariate analysis was used to identify variables to be included in a multivariable model, using a backward manual selection procedure. The point of significance was $P < 0.15$ for inclusion into the multivariable model, however, the point of significance for the final multivariable model was $P < 0.05$ (Dohoo, et al 2010). Herd was always included as a random effect in the univariate analyses. Additional models were constructed within the groups allowing a correlation between the variables up to 0.9 (Dohoo, et al 2010). Odds ratios (ORs) and their 95% confidence intervals (95% CI) were estimated for each variable in both the univariable and multivariable analyses. Year and season

variables were unique to each herd and were therefore removed from the analysis because each herd was only sampled once.

Separate univariable models were constructed to analyze the dairy and beef data using logistic regression and generalized linear mixed models (GLMM). The data structure was different for beef and dairy herds and beef cattle had no cattle-specific independent variables. Because three of the five beef herds were raised at different times at the same location, their herd-level risk factors were mostly identical or correlated, thereby preventing construction of a valid multivariable model.

For the dairy data, a base "full" model was created to include all variables that were significantly associated with STEC-positivity in the univariate analyses, and then the final multivariable model was created through a backward manual selection process. Additional variables were evaluated with the base model, depending on their relationships to other variables, as well as, biological plausibility. For example, variables that examined factors associated with housing such as access to pasture or use of free stalls, were evaluated in this way.

Results

1. Descriptive statistics

A total of 1,108 animals were sampled during the course of this study; 724 (65%) were dairy cattle and 384 (35%) were beef cattle. Six beef and six dairy cattle were excluded from the analysis due to missing STEC laboratory results, leaving a total of 1,096 individual cattle in the final analysis. Notably, STEC was detected in cattle from all 11 herds. The animal level prevalence ranged between 6.4% and 53.7% (**Figure 2.1**) with an average of 16%. Among the 378 beef cattle sampled, 80 (21.2%) were positive for STEC as were 95 of the 718 (13.2%) dairy cattle tested. STEC prevalence was significantly different between dairy and beef cattle ($P < 0.0007$) with beef cattle being 1.8 times more likely to be STEC positive than dairy cattle (95% CI: 1.25- 2.47). The overall STEC prevalence was 10% for 2011 and 23% for 2012 ($P < 0.0001$) and the overall STEC prevalence was 18% for spring and 15% for summer ($P < 0.1888$).

Among the 522 STEC isolates recovered from all 175 STEC-positives animals, *stx1* and *stx2* genes were detected in 52 (29.7%) and 73 (41.7%) of animals, respectively; while 33 (18.9%) animals were positive for STEC strains with both *stx1* and *stx2* (*stx1/2*) genes present. There were 17 (9.7%) animals that had multiple STEC strains isolates with distinct stx profiles between them (**Table 2.3**). In addition, 20 (25%) beef cattle and 47 (49%) dairy cattle had *stx*-positive isolates without the *eaeA* gene, while 36 cattle (12 dairy and 24 beef) had both *stx* genes as well as *eaeA* gene and thus, could be classified as EHEC. Differences in the *stx* distribution were also observed across herds. One beef herd was positive only for *stx1* (**Figure 2.2**), while the other herds were all mixed with multiple *stx* profiles. Additionally, beef cattle had a higher likelihood of having *stx2* than did dairy cattle (OR: 2.2; 95%CI: 1.05- 4.08; p-value: 0.04). There

were 288 EHEC (any *stx* gene with *eaeA* gene) strains isolated, which came from a total of 108 (62%) cattle (**Figure 2.3**). There were 17 animals that had both an EHEC strain and also a STEC strain. All the herds had at least one EHEC isolate, although there was a great variety in the number of EHEC isolates among herds, some having up to 22 EHEC isolates while others had only one or two EHEC isolates.

2. Univariate analyses of STEC shedding in dairy herds

The univariate analyses of all the variables analyzed in the dairy herds are present in **Table 2.4.** In the following paragraphs we described the most relevant findings from the univariate analysis in dairy.

2.1. Individual host factors:

Multiple host factors including number of lactations, days in milk, antibiotic treatment, etc were evaluated to identify associations with STEC shedding. Only two variables, however, were important in the univariate analysis. First was number of lactations. Cows in their first lactation were at highest risk for shedding STEC (OR: 1.6; 95%CI: 1.04- 2.58, p-value: 0.04) relative to cows with more lactations. The number of cows in their first lactation was 279 (40%); of those 56% were sampled during 2011. Also of those 279 first lactation cows 84% was sampled in the summer. Cows with more lactations numbered 426 (60%). Of those 59% were sampled during 2011 and 87% were sampled in the summer.

STEC shedding was more common in the first 30 days of lactation (OR: 3.8; 95% CI: 2.07- 6.90; p-value: <0.0001) relative to cows who had been lactating more than 30 days. A total

of 579 (82%) cows had been lactating more than 30 days, of these 53% were sampled during 2011 and 86% were sampled in summer. Cows in the first 30 days of lactation numbered 70 (10%). Of these 70 animals 71% were sampled during 2011 and 89% were sampled in the summer. The other 8% were dry cows. These 54 dry cows belonged to 4 of the 6 dairy herds. These variables were further evaluated in the final model (**Table 2.4**).

2.2. Environmental factors:

2.2.1 Herd characteristics

Significant herd-specific variables associated with STEC shedding included the "culling rate" (OR: 0.5; 95% CI: 0.22- 1.25; p-value: 0.15). Herds with low culling rates had a decreased risk of STEC shedding. Also included in this category were "proportion of the herd that is lactating" (OR: 0.4; 95% CI: 0.12- 1.35; p-value: 0.14) and "proportion of the herd that is dry". These last two variables were correlated, as a consequence, only the first was chosen for the analysis. Those herds with "percentage of dry cows" between 1.7- 4.0% were more likely to shed STEC relative to herds with either a higher or lower percentage of dry cows.

2.2.2 Housing characteristic

Cattle with access to pasture or a dry lot did not present a higher risk of shedding STEC compared to cattle that did not have access (OR: 0.7; 95%CI: 0.29- 1.64; p-value: 0.40), although there was a tendency for cattle with access to pasture to have an increased risk of STEC shedding. Similarly those first lactation cows that were housed separate from cows with more lactations had a increased tendency for STEC shedding (OR: 1.1; 95%CI: 0.44- 2.78; p-value:

0.84), which is in agreement with the higher rates of STEC shedding among cows that belong to herds that housed separated transition cows from the other cows (OR: 2.0; 95%CI: 0.96- 4.11; p-value: 0.06). Neither variable, however, was significant in the univariate analysis.

2.2.3 Cleaning characteristics

Dairy farmers that cleaned feeders every day had a trend for a lower risk of STEC shedding when compared to those that cleaned less frequently (OR: 2.0; 95%CI: 0.96- 4.11; p-value: 0.06).This could be due to the elimination of an environment that can favor STEC contamination or even multiplication. Nonetheless, cow environmental cleanliness scores, which represents a visual subjective evaluation of the farm's cleanliness by the interviewer, were not significantly associated with STEC shedding (OR: 1.9; 95%CI: 0.80-4.53; p-value: 0.15). The animals and bedding cleanliness scores were high, medium and low thirds. There was a low variability among the cleanliness scores which possibly accounts for the lack of significance.

2.2.4 Treatment characteristics

In five herds that had a history of using antimicrobials for treatment of respiratory disease the odds of STEC shedding was significantly lower (OR: 0.3; 95%CI: 0.19- 0.52; p-value: <0.0001) than herds that did not use antimicrobials; only one herd did not use antimicrobials. Products reported to be used for treating respiratory disease included ceftiofur, florefenicol and tulathromycin. In contrast, herds with a history of using antimicrobials for treatment of foot infections (OR: 2.5; 95%CI: 0.74- 8.23; p-value: 0.14) and metritis (OR: 2.5; 95%CI; 0.74- 8.23; p-value: 0.14) had a non-significant higher risk of STEC shedding; only one dairy herd did not use antimicrobials. The most common product for foot infections was copper-sulfate, whereas

ceftiofur and oxytetracycline was used for Metritis. The prophylactic use of anthelmintics, a measure applied by four of the six dairy herds, was significant associated with STEC shedding (OR: 0.4; 95%CI: 0.23- 0.84; p-value: 0.01); those herds that use anthelmintics had a lower likelihood of STEC shedding.

2.2.5 Diet

Cows fed a diet that included a "direct-fed microbial product" had less risk of STEC shedding (OR: 0.4; 95%CI: 0.23- 0.83; p-value: 0.0111). No other diet variables such as percentage of corn silage, distiller's grains, and cottonseed were significant. Neither was significantly associated the use of Rumensin on the diet with STEC shedding. We also examined the association of STEC shedding with the use of TMR, but the association was not significant. Neither was significant the association between level of NEL in the diet and STEC shedding. All farms had different diets for dry and lactating cows. Some of the farms had different diets according with the level of milk production.

2.2.6 Contact with other animals

Two herds that had continuous exposure (OR: 2.7; 95%CI: 1.09- 6.52; p-value: 0.03), and three herds with frequent exposure to "rodents" and "raccoons" (OR: 1.3; 95%CI: 0.53- 3.00; p-value: 0.03) were at higher risk for STEC shedding than the one herd with rare exposure to "rodents" and "raccoons". On the other hand, cows that did not have contact with "dogs" and "deer" were at less risk for STEC shedding. This is in agreement with the literature as deer have been reported to be a source of STEC, so the lack of contact with deer should reduce the risk of STEC shedding in cattle. All the herds had frequent or constant contact with birds; as a

consequence, contact with birds was not significant associated with STEC shedding. Only one dairy herd was reported to have contact with other species, more specifically horses.

2.2.7 Environmental conditions

Regarding the temperature, the "average maximum temperature 1-5 days before sampling" was the most predictive of the correlated environmental temperature variables and was therefore selected for analysis in the multivariable model. For example, "temperature average" and "minimum temperature average" on the day of sampling were highly correlated. Overall, there was higher risk of STEC shedding when the temperature was high. At an average maximum temperature 1-5 days before the sampling less than 28.9°F, for instance, there was a lower probability of STEC shedding relative to cattle sampled at a higher temperature (OR: 2.0; 95%CI: 0.99- 4.03; p-value: 0.05).

2.3. Multivariable analysis for dairy:

Independent variables were evaluated individually. Twenty-eight variables yielded no significant associations with STEC-positivity at p-value cut off of ≥ 0.15 and sixteen more at p-value ≥ 0.05 (**Table 2.4**). For example, "contact with cats" and "contact with raccoons" were not significant predictors of STEC shedding in this study. Therefore, these variables were not incorporated in the subsequent steps of the model building process.

The variables included in the final model were "average maximum temperature five days before sampling", "lactation status" and "days in milk (DIM)" as fixed effects with herd as a random effect (**Table 2.5**). The variance of the herd random effect was 0.2012, which yielded an

Intra class correlation (ICC) of 0.06. In all, a total of 692 animals were included in the final model; 26 animals had missing values for one or more of the variables examined.

Cattle in their first lactation were 1.76 times more likely to be shedding STEC than cattle in their second or higher lactation (OR 1.8; 95% CI 1.09- 2.83; p-value: 0.0204). Also, cows in their first 31 days of producing milk were 3.9 times more likely to be shedding STEC than cows with 31 or more days producing milk (OR 3.9; 95% CI 2.12- 7.18). Furthermore, dry cows were less likely to shed STEC, although the association was not significant (OR 0.7; 95% CI 0.20- 2.41; p-value: 0.5590). Higher average temperatures (>28.9 F) in 1-5 days before sampling increased the likelihood of STEC shedding 2.5 times compared with lower temperatures (OR: 2.5; 95%CI: 1.25- 4.91; p-value: 0.0092).

3. Univariate analyses of STEC shedding in beef herds

All the variables analyzed were displayed in **Table 2.6**. Eleven variables yield no significant associations with STEC-positivity at a p-value of 0.15. Therefore, these variables were not incorporated in the subsequent steps of the building process for the multivariable model, that was described in the data analysis section was not possible to built. The intraclass correlation for the herd random effect for beef herds was low (0.076) which means that there was more variability within herds than between herds. As a consequence there was not enough variability to use herd as a random effect.

3.1 Environmental factors:

3.1.1 Herd characteristics

Herds with crossbreds were less likely to shed STEC than Holstein or Angus feedlots. Crossbreds represent 64% of the beef animals sampled, followed by 22% Holstein and 14% Angus. The other variables feedlot animal capacity, number of cattle fed annually, weight at arrival, weight at sale, and cattle purchased off site were significantly associated with STEC shedding. However, there should be caution in interpreting these results as once the variables were categorized only one herd was different than the rest. Cow environmental cleanliness scores, which represent a visual subjective evaluation of the farm's cleanliness was not significant associated with STEC shedding. None of the herds was classified in the low third of cleanliness.

3.1.2 Housing characteristics

Only one of the herds did have exposure to pasture, and this was the herd with the highest STEC prevalence. The other variables, times the waterers were washed, times the animal's holding areas were clean, and the use of disinfectant in these areas were not significantly associated with STEC shedding. Bed and animals cleanliness were a subjective evaluation of the farm cleanliness. Only one herd was evaluated in the middle third; all others were in the cleanest third.

3.1.3 Treatment characteristics

The use of "anthelmintics" was significant as those herds that used an anthelmintic had a decreased risk of STEC shedding (OR: 0.2; 95% CI: 0.04- 0.57; p-value: 0.01). Treatment for respiratory diseases (OR: 5.9; 95%CI: 2.79- 12.70, p-value: <0.0001) and treatment for foot infection (OR: 5.9; 95%CI: 2.79- 12.70, p-value: <0.0001) were correlated, so only one of them was chosen for the next model building step. Those farms that used only one type of antibiotic for respiratory diseases had a lower risk of STEC shedding compared with farms that used several types of antibiotics. Those herds that used only oxytetracycline for foot infections had a higher risk of STEC shedding compared to those herds that use others antibiotics.

3.1.4 Diet

Herd managers that fed a total mixed ration (TMR) to their cattle (OR: 6.6; 95%CI: 1.77- 24.31; p-value: 0.01), and used ionophores (OR: 0.2; 95% CI: 0.04- 0.57; p-value: 0.01) had a lesser risk of STEC shedding. Only one beef herd did not use either TMR or ionophores. Because these two variables were correlated, only one was selected for the next step in the model construction. Beef herds had very similar diets; the only herd with different diet was the herd that raised its animal on pasture. Contrary to dairy cattle, direct feed microbials was not significant.

3.1.5 Contact with other animals

Those herds with constant contact with opossums, deer, dogs, and skunks had a higher risk of STEC shedding. All beef herds had contact with cats. Contact with other species presented a lower risk of STEC shedding. Voles and weasels were the other species that some of these herds had contact with.

3.1.6 Environmental conditions

The herds sampled during 2011 had less risk of STEC shedding (OR: 0.19; 95%CI: 0.06-0.65; p-value: 0.0077). In the case of the variables that denoted temperature, all of the variables were significant associated with STEC except the average maximum temperature at day of sampling. Overall, the risk of STEC shedding was higher when the temperature was high.

Discussion

The objective of this study was to identify risk factors in dairy and beef cattle for STEC shedding, which could ultimately lead to STEC intervention strategies.

We found that all the dairy and beef feedlot herds we sampled were positive for STEC. These herds had at least 6% of their cattle positive to STEC shedding. In agreement with our study, a Swiss study reported a 100% STEC farm prevalence in the dairy farms they sampled (Kuhnert, et al 2005). Other studies had reported lower STEC prevalence; however these studies were testing only for STEC O157 (Dunn, et al 2004; Hancock, et al 1994; Heuvelink, et al 1998; Sargeant, et al 2003); (Cobbaut, et al 2009) or only non-O157 STEC (Renter, et al 2007), while we were testing for all STEC. The improvement of laboratory techniques and diagnostic tools may also be responsible for our result that all examined herds were found to contain STEC. In addition, it could be that Michigan has a higher STEC prevalence than other parts of USA, as this is the first study performed in Michigan. There are studies that reported differences in STEC prevalence by region (USDA 2003) while others report no differences (Sargeant, et al 2003).

The finding of *stx2* as the most frequent gene detected in our cattle sampled is in agreement with previous studies (Kuhnert, et al 2005; Mechie, et al 1997; Polifroni, et al 2012). Shiga toxin 2 is the more dangerous of the two Shiga toxins to humans, as a consequence finding *stx2* as the most frequent gene has important implications from the public health perspective. It is also important to report that 68% of the animals shed EHEC isolates, which are more virulent and likely to result in HUS (Karmali, et al 1983). Indeed, more than half of STEC isolates from animals had the *eaeA* gene. It is important to identify the most common *stx* genes in the STEC bacteria isolated due to the implication in case of human infection.

We found that beef herds had a higher risk of STEC shedding than dairy herds. This finding is opposed to a previous reported study (Cobbold, et al 2004). A potential explanation for this difference could be that beef cattle were younger than dairy, so dairy animals had been already exposure to STEC, and as consequence had a good immune response already working. Studies have reported that younger cattle have higher risk of STEC shedding than older cattle (Cho, et al 2009; Heuvelink, et al 1998). Other possible explanation for the incongruent results could be that our study was in the Midwest region, during summer/spring seasons whereas Cobbold' s study was done in the Pacific Northwest, during fall and winter so there are environmental and management practices differences.

Dairy cattle in their first lactation were found to be at a higher risk for shedding STEC, this is in agreement with earlier studies. Fitzgerald, et al. (2003), for instance, found that primiparous cows shed more STEC than multiparous cows, although this difference was not significant (p-value >0.10). Similarly, a German study found that first calf heifers were at a higher risk of STEC shedding than older cows (Menrath, et al 2010). Also a longitudinal study in the UK reported the prevalence of *E. coli O157:H7* to be highest in cows two years of age, which is the typical age for the first calving (Mechie, et al 1997). Other studies have found the opposite results. Cho et al. (2009) reported that cows with parities of ≥ 4 were 1.7 times more likely to shed STEC compared to cows with <4 parities, although this association was non-significant. A study in Switzerland also found that as the number of lactations increased, the risk of STEC positivity increased (Kuhnert, et al 2005). A possible explanation for the opposite results between studies could be differences in methodology.

The reasons for our observed association between first lactations animals and STEC are not clear but could be related to the fact that first lactation dairy cows have different energy

requirements and are often in more severe negative energy balance than older cows (Edrington, et al 2004). This negative energy balance could alter the digestive microbiota composition (Edrington, et al 2004), which could favorite STEC colonization and shedding more during the first lactation. This negative energy balance is one of many differences between older and younger dairy cattle.

In our study, the risk of STEC shedding was found to be highest in the first 31 days of lactation. Other studies have reported variable findings with regard to STEC shedding and stage of lactation. A longitudinal study in the UK found that *E. coli O157:H7* shedding peaked in the first month of lactation, followed by low levels of shedding and then a less intense increase at seven months postpartum (Mechie, et al 1997). These investigators speculated that modifications in diet may explain the change in STEC shedding reasoning that diet change could potentially modify the digestive tract microbiota, favoring STEC growth. A negative energy balance could also explain the increased risk for STEC shedding in the first 31 days of lactation. During early lactation, there are significant physiological and metabolic changes that occur and investigators have suggested that the high metabolic demand associated with early lactation could potentially favor intestinal STEC colonization and shedding (Dunn 2003; Edrington, et al 2004). In contrast, Edrington, et al. (2004) did not find any difference in STEC *O157:H7* shedding between cows in early lactation (<60 DIM) versus late lactation (>150 DIM). Similarly, Fitzgerald, et al. (2003) reported no effect of DIM on STEC *O157:H7* shedding. In complete contrast, a study performed in Germany found that cows with more than 50 DIM have higher risk of shedding STEC with the highest risk being in cows with more than 350 DIM (Menrath, et al 2010). There were also differences in methodology and the way of classified the DIM among these studies.

Seasonal variation in STEC shedding has been widely reported, with shedding highest in the summer months (Berry and Wells 2010; Cobbold, et al 2004; Dunn, et al 2004; Gautam, et al 2011; Kondo, et al 2010; Smith, et al 2005). Our findings support these earlier findings. Potential reasons for these findings include increased growth and survival in the environment at higher ambient temperature (Smith, et al 2005). Similarly, higher temperatures could lead to changes in normal microbiota and immunological functions that may favor STEC colonization and shedding in cattle (Smith, et al 2005).

There are two important limitations to our study with respect to the beef herds' sample. We found that one herd was very different than the other herds. Several independent variables for this herd were different from the rest of the beef cattle herds. Another limitation as previously mentioned was the correlation between the beef herds that were raised at different times at the same location. For these reasons we were unable to build a multivariable model for the beef herds and interpretation of the univariate analysis should be done with caution.

In summary, the implementation of control strategies in dairy herds should focus on those groups of animals that are at higher risk for STEC shedding. Thus first lactation cows and cows within their 31st DIM are a group with a high risk for STEC shedding. Therefore control strategies could be specifically targeted to this group. Although these two specific groups of cows do not usually get into the food chain, their milk can impact food safety. Also these animals serve as potential source of contamination for other animals on the farm and the environment. Elucidation of STEC determinants should lead to intervention strategies to control STEC infection in cattle, and indirectly, to reduce transmission to people.

APPENDIX

Table 2.1. Areas explored in the questionnaire for dairy and beef herds.

Area	Dairy and Beef	Only Dairy	Only Beef
Farm demographics	• Herd Size	• Closed or open herd • Overall culling rate	• Cattle breed • Percentage cattle gender • Average arrival and sale weight • Length of time on feed
Farm and animal management	• Source and number of cattle added during past 12 months • Contact with other domestic or wild species • Cleaning process and frequency for feedbunks, waterers and areas where animals are housed • Percentage cattle receiving a total mixed ration • Composition of diet • Fly control • Type of bedding • Water source	• Access to pasture • Grouping of lactating, transition and sick cows • Number of milkings per day • Type of lactating cow housing	• Housing if animals post-arrival
Herd health management	• Anthelmintic used • Antimicrobial used • Morbidity and mortality • Used of feed additives • Growth promoters		

Table 2.2. Herd identification, type of production system, total number of animals in each herd, number of animals sampled in each herd, number of animals tested for STEC and year of sampling.

Herd	Type of herd	Total number of animals	Number of animals sampled (% of total)	Number of animals STEC tested	Year of sampling
B1	Beef	136	136 (100)	134	2011
D2	Dairy	320	154 (48)	149	2011
B3	Beef	36	36 (100)	32	2011
D4	Dairy	3000	175 (9)	174	2011
D6	Dairy	98	94 (96)	94	2011
D7	Dairy	12000	100 (1)	100	2012
B8	Beef	54	54 (100)	54	2012
D9	Dairy	243	100 (41)	100	2012
D10	Dairy	530	101 (19)	101	2012
B11	Beef	83	83 (100)	83	2012
B12	Beef	75	75 (100)	75	2012

Figure 2.1. Prevalence of STEC by herd. D= dairy, B= beef

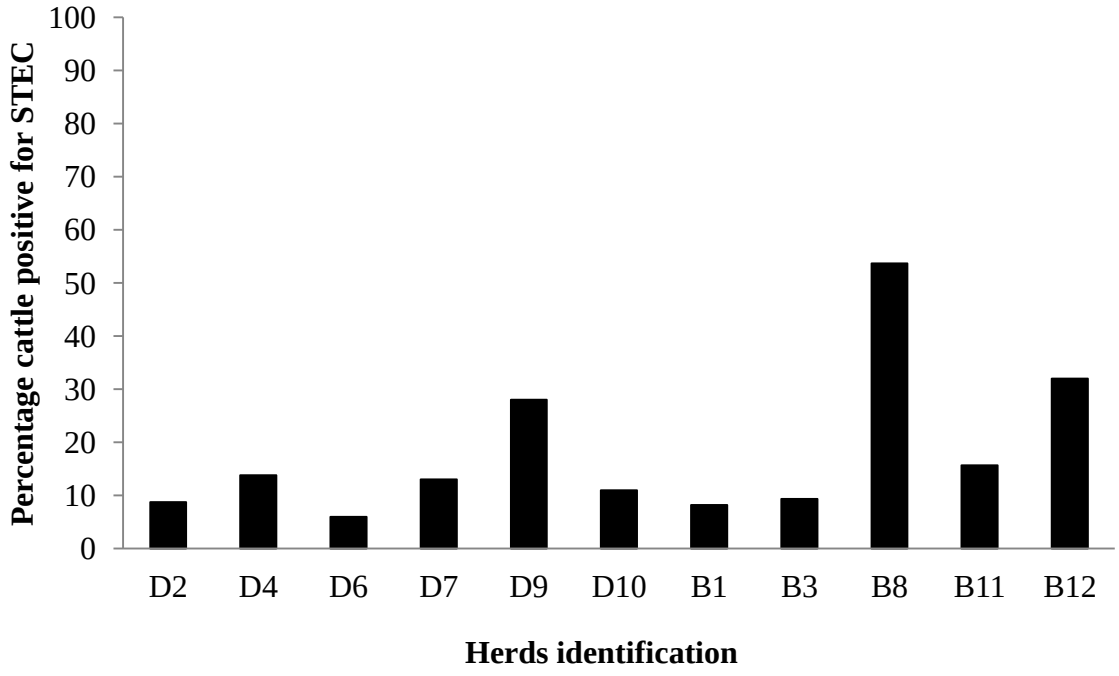
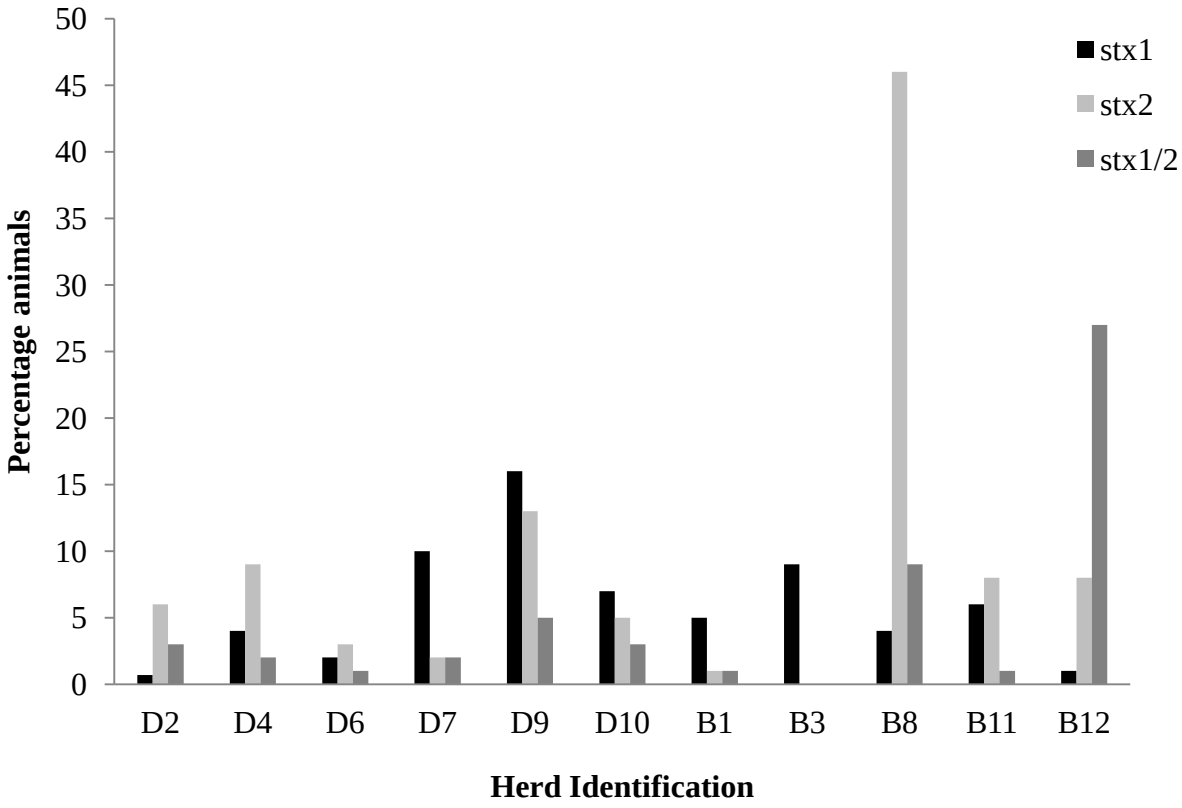


Table 2.3. Prevalence of genes *stx1*, *stx2* and *eaeA* by total of animals, dairy animals and beef animals.

Gene prevalence	**Total animals (n=175)**	**Dairy animals (n=95)**	**Beef animals (n=80)**
*stx*1 (%)	52 (29.7)	35 (37)	17 (21)
*stx*2 (%)	73 (41.7)	38 (40)	35 (44)
*stx*1/2 (%)	33 (18.9)	11 (12)	22 (28)
stx1 and stx2	4 (2.3)	4 (4)	0
stx1 and stx1/2	3 (1.7)	2 (2)	1 (1)
stx2 and stx1/2	8 (4.6)	3 (3)	5 (6)
stx1, stx2 and stx1/2	2 (1.1)	2 (2)	0
eaeA positive (%)	108 (62)	48 (51)	60 (75)
eaeA negative (%)	67 (38)	47 (49)	20 (25)

Figure 2.2. Prevalence of *stx1, stx2 and stx1/2* by herd. D= dairy, B=beef. The vertical axis has been set up at 50% to improve visibility. B= beef and D= dairy



stx1= Shiga toxin 1 gene
stx2= Shiga toxin 1 gene
stx1/2= Shiga toxin 1 gene and Shiga toxin 2 gene

Figure 2.3. Prevalence of enterohemorrhagic *Escherichia coli* and Shiga toxin-producing *E. coli* strains by herd. D= dairy, B=beef. The vertical axis has been set up at 50% to improve visibility.

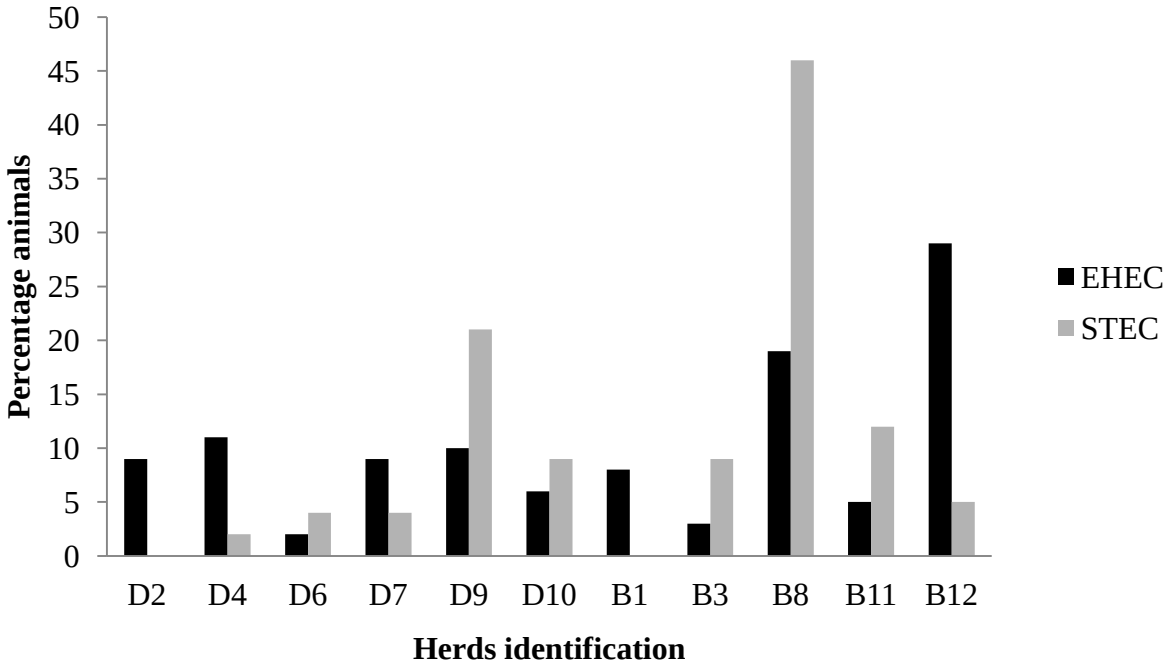


Table 2.4. Univariable analysis of dairy herd variables for risk of STEC shedding with herd as a random effect

Characteristic	**No. (%) with characteristic**	**No. (%) with STEC**	**p-value**	**OR**	**95% CI**
Year					
2011	417 (58.1)	43 (10.3)	0.0925	0.534	0.258- 1.109
2012	301 (41.9)	52 (17.3)		ref	ref
Season					
Spring	100 (14)	13 (13)	0.9685	1.024	0.309- 3.395
Summer	618 (86)	82 (13.3)		ref	ref
Temperature Aver.					
> 20.6F	301 (41.9)	52 (17.3)	0.0925	1.871	0.902- 3.883
≤ 20.6F	417 (58.1)	43 (10.3)		ref	ref
Temperature Max					
> 27.8F	201 (28)	39 (19.4)	0.0631	1.990	0.963- 4.111
≤ 27.8F	517 (72)	56 (10.8)		ref	ref
Temperature Min					
> 15.6	301 (41.9)	52 (17.3)	0.0925	1.871	0.902- 3.883
≤ 15.6	417 (58.1)	43 (10.3)		ref	ref
Temperature Aver5 days					
>19.4	395 (55)	58 (14.7)	0.6062	1.265	0.517- 3.099
≤19.4	323 (45)	37 (11.5)		ref	ref
Temperature Max5 days					
>28.9	375 (52.2)	63 (16.8)	0.0519	2.002	0.994- 4.031
≤28.9	343 (47.8)	32 (9.3)		ref	ref
Temperature Min5 days					
>15	475 (66.2)	76 (16)	0.0299	2.299	1.085- 4.873
≤15	243 (33.8)	19 (7.8)		ref	ref

Table 2.4. (cont'd)

Longitude					
< -85.241836	274 (38.2)	52 (19)	0.0128	2.592	1.378- 4.875
> -85.241837 < -84.829245	100 (13.9)	13 (13)		1.573	0.666- 3.715
> -84.54	344 (47.9)	30 (8.7)		ref	ref
Lactation					
1st	279 (39.57)	49 (17.56)	0.0346	1.636	1.036- 2.584
2nd or higher	426 (60.43)	44 (10.33)		ref	ref
Days in milk					
0	54 (7.68)	5 (9.26)	<.0001	1.091	0.397- 3.003
1-30d	70 (9.96)	21 (30)		3.778	2.069- 6.900
>= 31d	579 (82.36)	64 (11.05)		ref	ref
Dry					
Yes	53 (7.42)	4 (7.55)	0.4124	0.635	0.214- 1.882
No	661 (92.58)	90 (13.62)		ref	ref
Breed					
Jersey	94 (13.1)	6 (6.4)	0.2461	0.378	0.116- 1.237
Mixed	101 (14.1)	11 (10.9)		0.686	0.240- 1.961
Holstein	523 (72.8)	78 (14.9)		ref	ref
Herd type					
Closed	149 (20.8)	13 (8.7)	0.3539	0.59	0.197- 1.789
Open	569 (79.3)	82 (14.4)		ref	ref
Calves-Replacements proportion					
<5.1	94 (13.1)	6 (6.4)	0.3230	0.395	0.117- 1.328
>48.4	100 (14)	13 (13)		0.878	0.305- 2.528
From 46.9 to 48.3	524 (73)	76 (14.5)		ref	ref
Proportion herd lactating					
>50	94 (13.1)	6 (6.4)	0.1396	0.404	0.122- 1.345
<31.7% to 49%	624 (86.9)	89 (14.3)		ref	ref

Table 2.4. (cont'd)

Proportion herd dry					
1.7- 4%	200 (27.9)	41 (20.5)	0.0133	2.025	1.109- 3.699
>7.6%	94 (13.1)	48 (11.3)		0.537	0.196- 1.476
6.2- 7.5%	424 (59.1)	6 (6.4)		ref	ref
Herd Size					
>1000	274 (38.2)	37 (13.5)	0.8411	1.099	0.435- 2.782
<1000	444 (61.8)	58 (13.1)		ref	ref
Adding Cow/Replacements					
At least 5 animals	149 (20.8)	13 (8.7)	0.3539	0.594	0.197- 1.789
0%	569 (79,3)	82 (14.4)		ref	ref
Adding Bulls					
4 animals	101 (14.1)	11 (10.9)	0.7197	0.802	0.241- 2.674
0%	617 (85.9)	84 (13.6)		ref	ref
Culling Rate					
Low level	195 (27.2)	17 (8.7)	0.1452	0.525	0.221- 1.250
High level	523 (72.8)	78 (15)		ref	ref
N° Milkings					
3- 4 times	274 (38.2)	37 (13.5)	0.8411	1.099	0.435- 2.782
2-3 times	444 (61.8)	58 (13.1)		ref	ref
Loose Housing					
No	624 (86.9)	89 (14.3)	0.1396	2.473	0.743- 8.227
Yes	94 (13.1)	6 (6.4)		ref	ref
Tie stanchion					
No	569 (79.3)	82 (14.4)	0.3539	1.684	0.559- 5.073
Yes	149 (20.8)	13 (8.7)		ref	ref
Free stall					
Yes	718 (100)	95 (13.2)	N/A		
No	0	0			
Access pasture/dry lot					
No	524 (73)	61 (11.6)	0.3961	0.687	0.288- 1.637
Yes	194 (27)	34 (17.5)		ref	ref

Table 2.4. (cont'd)

Lactation access pasture					
No	524 (73)	61 (11.6)	0.3961	0.687	0.288- 1.637
Yes	194 (27)	34 (17.5)		ref	ref
Transition pen separate					
No	201 (28)	39 (19.4)	0.0631	1.990	0.963- 4.111
Yes	517 (72)	56 (10.8)		ref	ref
Sick animals penned separated					
Yes	368 (51.6)	43 (11.7)	0.4421	0.718	0.308- 1.672
No	350 (48.8)	52 (14.9)		ref	ref
1st lactations animals penned separated					
Yes	274 (38.2)	37 (13.5)	0.8411	1.099	0.435- 2.782
No	444 (61.8)	58 (13.1)		ref	ref
Cow/Heifers Raised					
Another farm	94 (13.1)	6 (6.4)	0.1396	2.473	0.743- 8.227
Off-site/ On main farm	624 (876.9)	89 (14.3)		ref	ref
Feeders clean Year					
<365	201 (28)	39 (19.4)	0.0631	1.990	0.963- 4.111
365	517 (72)	56 (10.8)		ref	ref
Washed					
No	469 (65.3)	54 (11.5)	0.2509	0.614	0.267- 1.413
Yes	249 (34.7)	41 (16.5)		ref	ref
Spray					
No	569 (79.3)	82 (14.4)	0.3539	1.684	0.559- 5.073
Yes	149 (20.8)	13 (8.7)		ref	ref
Lime					
No	524 (73)	76 (14.5)	0.2067	1.619	0.654- 4.005
Yes	194 (27)	19 (9.8)		ref	ref
Tx Respiratory Disease					
Yes	618 (86.1)	67 (10.8)	<.0001	0.313	0.189- 0.518
No	100 (13.9)	28 (28)		ref	ref

Table 2.4. (cont'd)

Tx Foot Infection Disease					
Yes	624 (86.9)	89 (14.3)	0.1396	2.473	0.743- 8.227
No	94 (13.1)	6 (6.4)		ref	ref
Tx Metritis					
Yes	624 (86.9)	89 (14.3)	0.1396	2.473	0.743- 8.227
No	94 (13.1)	6 (6.4)		ref	ref
Feed TMR					
No	194 (27)	34 (17.5)	0.3961	1.456	0.611- 3.469
Yes	524 (73)	61 (11.6)		ref	ref
% Corn silage Diet					
No	205 (30.8)	34 (16.6)	0.8115	1.117	0.450- 2.769
Yes	461 (69.2)	48 (10.4)		ref	ref
% Distillers grains Diet					
No	496 (74.5)	67 (13.5)	0.2053	1.738	0.738- 4.092
Yes	170 (25.5)	15 (8.8)		ref	ref
% Cottonseed Diet					
No	503 (75.5)	62 (12.3)	0.7234	0.860	0.372- 1.987
Yes	163 (24.5)	20 (12.3)		ref	ref
Other SP					
Horses	94 (13.1)	6 (6.4)	0.1396	0.404	0.122- 1.345
None	624 (86.9)	89 (14.3)		ref	ref
NEL					
	717 (99.86)	95 (13.25)	0.3511	0.419	0.067- 2.612
Contact with Cats					
No	101 (14.07)	11 (10.89)	0.7197	0.802	0.241- 2.674
Yes	617 (85.93)	84 (13.61)		ref	ref
Contact with Deer					
No	100 (13.93)	13 (13)	0.9685	1.024	0.309- 3.395
Yes	618 (86.07)	82 (13.27)		ref	ref

Table 2.4. (cont'd)

Contact with Dogs					
No	374 (52.1)	65 (17.4)	0.0111	2.269	1.206- 4.267
Yes	344 (47.9)	30 (8.7)		ref	ref
Contact with Opossum					
No	101 (14.07)	11 (10.89)	0.7197	0.802	0.241- 0.7197
Yes	617 (85.93)	84 (13.61)		ref	ref
Contact with Raccoons					
Always	200 (27.9)	41 (20.5)	0.0311	2.671	1.094- 6.520
Frequent	369 (51.4)	41 (11.1)		1.257	0.528- 2.995
Rarely	149 (20.8)	13 (8.7)		ref	ref
Contact with Rodents					
Always	200 (27.9)	41 (20.5)	0.0311	2.671	1.094- 6.520
Frequent	369 (51.4)	41 (11.1)		1.257	0.528- 2.995
Rarely	149 (20.8)	13 (8.7)		ref	ref
Contact with Skunks					
No	101 (14.07)	11 (10.89)	0.7197	0.802	0.241- 2.674
Yes	617 (85.93)	84 (13.61)		ref	
ACleanliness					
Cleanest third	523 (72.8)	78(14.9)	0.1452	1.903	0.800- 4.525
Middle third	195 (27.2)	17 (8.7)		ref	ref
Bed Cleanliness					
Cleanest third	523 (72.8)	78(14.9)	0.1452	1.903	0.800- 4.525
Middle third	195 (27.2)	17 (8.7)		ref	
Rumensin					
Yes	274 (38.2)	37 (13.5)	0.8411	1.099	0.435- 2.782
No	444 (61.8)	58 (13.1)		ref	ref

Table 2.4. (cont'd)

Direct Fed Microbials					
Yes	344 (47.9)	30 (8.7)	0.0111	0.441	0.234- 0.829
No	374 (52.1)	65 (17.4)		ref	ref
Anthelmintic					
Yes	518 (72.2)	54 (10.4)	0.0123	0.443	0.234- 0.838
No	200 (27.9)	41 (20.5)		ref	ref

Table 2.5. Final multivariable model for dairy herds with herd as a random effect.

Variable	Estimate	Standard Error	p-value	Individual p-value	OR	95% CI
TempFmax5 days						
> 28.9 F	0.9079	0.3476	0.0092		2.479	1.253- 4.906
<=28.9 F	0	ref			ref	ref
Lactation						
First	0.5640	0.2426	0.0204		1.758	1.092- 2.830
2 or more	0	ref			ref	Ref
DIM						
Dry	-0.3731	0.6338	<.0001	0.5590	0.689	0.197- 2.411
1 – 30 days	1.3604	0.3108		<.0001	3.898	2.117- 7.175
>=31	0	ref			ref	Ref
Intercept	-2.0173	0.2594				

Table 2.6. Univariable analysis of beef herd variables for risk of STEC shedding with herd as a random effect

Characteristic	**No. (%) with characteristic**	**No. (%) with STEC**	**p-value**	**OR**	**95% CI**
Feedlot size capacity					
<=54	54 (14.29)	29 (53.7)	0.0050	6.560	1.771-24,306
>54	324 (85.71)	51 (15.74)		ref	Ref
Feed Annual					
<= 54	54 (14.3)	29 (53.7)	0.0050	6.560	1.771-24,306
> 54	324 (85.7)	51 (15.7)		ref	Ref
Proportion Beef breed					
< 100%	249 (65.9)	27 (10.8)	<.0001	0.168	0.079- 0.359
> 100%	129 (34.1)	53 (41.1)		ref	Ref
Breed					
Holstein	83 (21.96)	13 (15.66)	0.0194	1.033	0.258- 4.137
Angus	54 (14.29)	29 (53.70)		6.617	1.691- 25.887
Crossbreed	241 (63.76)	38 (15.77)		ref	Ref
Proportion Steers					
Mix	249 (65. 9)	27 (10.8)	<.0001	5.947	2.785- 12.702
100% Male	129 (34.1)	53 (41.1)		ref	Ref
Arrival average weight					
< 272.2 kg	54 (14.3)	29 (53.7)	0.0050	6.560	1.771-24,306
> 272.2 kg	324 (85.7)	51 (15.7)		ref	Ref
Sale average weight					
<589.7 kg	54 (14.3)	29 (53.7)	0.0050	6.560	1.771-24,306
>589. 7 kg	324 (85.7)	51 (15.7)		ref	Ref
Feedlot days					
< 200	158 (41.8)	37 (23.4)	0.7800	1.285	0.220- 7.517
> 200	220 (58.2)	43 (19.6)		ref	ref

Table 2.6. (cont'd)

Purchased from off-site					
0%	54 (14.3)	29 (53.7)	0.0050	6.560	1.771-24,306
100%	324 (85.7)	51 (15.7)		ref	Ref
Purchased from out-state					
100%	303 (80.2)	56 (18.5)	0.4546	0.462	0.061- 3.518
< 100%	75 (19.8)	24 (32)		ref	Ref
Barn post arrival					
True	75 (19.8)	24 (32)	0.4546	2.167	0.284- 16.515
False	303 (80.2)	56 (18.5)		ref	Ref
Antibiotics at arrival					
Yes	75 (19.8)	24 (32)	0.4546	2.167	0.284- 16.515
No	303 (80.2)	56 (18.5)		ref	Ref
Waterer clean Year					
Never	75 (19.8)	24 (32)	0.4546	2.167	0.284- 16.515
Yes	303 (80.2)	56 (18.5)		ref	Ref
Sanitation areas					
No	54 (14.3)	29 (53.7)	0.0050	6.50	1.771- 24.306
Yes	324 (85.7)	51 (15.7)		ref	Ref
Washed					
Never	129 (34.1)	53 (41.1)	<.0001	5.947	2.785- 12.702
Once a year	249 (65.9)	76 (59)		ref	Ref
Spray Disinfectant					
No	303 (80.2)	56 (18.5)	0.4546	0.462	0.061- 3.518
Yes	75 (19.8)	24 (32)		ref	Ref
Tx Respiratory Disease					
Several antibiotics	129 (34.1)	53 (41.1)	<.0001	5.947	2.785- 12.702
Only one type	249 (65.9)	76 (59)		ref	Ref
Tx Foot Infections					
Oxitetracycline	129 (34.1)	53 (41.1)	<.0001	5.947	2.785- 12.702
Others	249 (65.9)	76 (59)		ref	ref

Table 2.6. (cont'd)

Tx Arthritis					
No	54 (14.3)	29 (53.7)	0.0050	6.560	1.771- 24.306
Yes	324 (85.7)	51 (15.7)		ref	Ref
Feed TMR					
0%	54 (14.3)	29 (53.7)	0.0050	6.560	1.771- 24.306
100%	324 (85.7)	51 (15.7)		ref	Ref
Forage Diet					
15 %	324 (85.7)	51 (15.7)	0.0050	0.152	0.041- 0.565
100 %	54 (14.3)	29 (53.7)		ref	Ref
NEG					
High Level	54 (14.3)	29 (53.7)	0.0050	6.560	1.771- 24.306
Low Level	324 (85.7)	51 (15.7)		ref	Ref
Corn silage diet					
0 %	54 (14.3)	29 (53.7)	0.0050	6.560	1.771- 24.306
15 %	324 (85.7)	51 (15.7)		ref	Ref
Distiller grains diet					
0 %	303 (80.2)	56 (18.5)	0.4546	0.462	0.061- 3.518
20 %	75 (19.8)	24 (32)		ref	Ref
Antibiotics before sampling					
No	76 (20.1)	25 (32.9)	0.2708	2.891	0.436- 19.193
Yes	302 (79.9)	55 (18.2)		ref	Ref
Radius animals presence					
100	129 (34.13)	53 (41.09)	<.0001	3.880	1.663- 9.048
400	166 (43.92)	14 (8.43)		0.498	0.191- 1.294
500	83 (21.96)	13 (15.66)		ref	Ref
Other SP					
Yes	249 (65. 9)	27 (10.8)	<.0001	0.163	0.079- 0.359
No	129 (34.1)	53 (41.1)		ref	Ref
Pasture					
Yes	54 (14.3)	29 (53.7)	0.0050	6.560	1.771-24,306
No	324 (85.7)	51 (15.7)		ref	ref

Table 2.6. (cont'd)

Anthelmintic					
Yes	324 (85.7)	51 (15.7)	0.0050	0.152	0.041- 0.565
No	54 (14.3)	29 (53.7)		ref	Ref
Rumensin					
Yes	324 (85.7)	51 (15.7)	0.0050	0.152	0.041- 0.565
No	54 (14.3)	29 (53.7)		ref	Ref
Direct Fed Microbials					
Yes	75 (19.8)	24 (32)	0.4546	2.167	0.284- 16.515
No	303 (80.2)	56 (18.5)		ref	Ref
Area Cleanliness					
Cleanest third	303 (80.2)	56 (18.5)	0.4546	0.462	0.061-3.518
Middle third	75 (19.8)	24 (32)		ref	Ref
Contact with Opossum					
Always	129 (34.1)	53 (41.1)	<.0001	5.947	2.785- 12.702
None	249 (65.9)	76 (59)		ref	Ref
Contact with Deer					
None	249 (65. 9)	27 (10.8)	<.0001	0.163	0.079- 0.359
Yes	129 (34.1)	53 (41.1)		ref	Ref
Contact with Dogs					
Always	129 (34.1)	53 (41.1)	<.0001	5.947	2.785- 12.702
None	249 (65.9)	27 (10.8)		ref	Ref
Contact with Skunks					
Always	129 (34.1)	53 (41.1)	<.0001	5.947	2.785- 12.702
None	249 (65.9)	27 (10.8)		ref	Ref
Year					
2011	166 (43.9)	14 (8.4)	0.0077	0.194	0.058- 0.645
2012	212 (56.1)	66 (31.1)		ref	Ref
Season					
Spring	188 (49.74)	40 (49.74)	0.6438	1.508	0.263- 8.647
Summer	190 (50.26)	40 (21.05)		ref	Ref

Table 2.6. (cont'd)

Temperature Aver.					
> 20.6 C	129 (34.1)	53 (41.1)	<.0001	5.947	2.785- 12.702
≤ 20.6 C	249 (65.9)	27 (10.8)		ref	Ref
Temperature Max					
> 27.8 C	161 (42.59)	56 (34.78)	0.0582	3.497	0.957- 12.773
≤ 27.8 C	217 (57.41)	24 (11.06)		ref	Ref
Temperature Min					
> 15.6 C	212 (56.08)	66 (31.13)	0.0077	5.163	1.549- 17.203
≤ 15.6 C	166 (43.92)	14 (8.43)		ref	Ref
Temperature Aver5 days					
>19.4 C	129 (34.1)	53 (41.1)	<.0001	5.947	2.785- 12.702
≤19.4 C	249 (65.9)	27 (10.8)		ref	Ref
Temperature Max5 days					
>28.9 C	129 (34.1)	53 (41.1)	<.0001	5.947	2.785- 12.702
≤28.9 C	249 (65.9)	27 (10.8)		ref	Ref
Temperature Min5 days					
>15 C	54 (14.3)	29 (53.7)	0.0050	6.560	1.771-24,306
≤15 C	324 (85.7)	51 (15.7)		ref	Ref

Table 2.7. Description of the abbreviations used for each variable analyzed in the beef and dairy models.

Variable	**Abbreviation**
Average temperature on day of sampling	Temperature Aver
Maximum temperature on day of sampling	Temperature Max
Minimum temperature on day of sampling	Temperature Min
Average temperature during 5 days previous sampling	Temperature Aver 5 days
Maximum temperature during 5 days previous sampling	Temperature Max 5 days
Minimum temperature during 5 days previous sampling	Temperature Min 5 days
Longitude coordinates of farm	Longitude
Number of lactations	Lactation
Number of days the cow had been producing milk	Days in milk
Cow was not producing milk	Dry
Cattle's breed	Breed
Herd incorporated cattle from other farms	Herd type
Proportion of the herd that were calves and replacements cattle	Calves-Replacements proportion
Proportion of the herd that were lactating cows	Proportion herd lactating
Proportion of the herd that were dry cows	Proportion herd dry
How many cows & heifers were added in the last year	Adding Cow/Replacements
How many bulls were added in the last year	Adding Bulls
Overall culling rate (% culled per year)	Culling rate
Number of milkings per day (2x/3x/Combination, 2x-3x)	N° Milkings
Lactating cow housing included free stall	Free stall
Lactating cow housing included loose housing	Loose Housing
Did cattle have access to pasture	Pasture
Lactating cow housing included tie stall/stanchion	Tie stanchion
Do lactating cows have access to pasture/dry lot	Lactation access pasture
Were first lactation animals penned separately	1st lactation animals penned separated
Were transition (post-calving) animals penned separately	Transition pen separate
Were sick cows penned separately	Sick animals penned separated
Where were cows and heifers mostly raised	Cow-Heifers Raised
How often were feedbunks cleaned per year	Feeders clean Year
How often were the processing/animal handling areas washed/power washed per year	Washed
How often were the processing/animal handling areas sprayed with disinfectant per year	Spray Disinfectant

Table 2.7. (cont'd)

How often was lime spread on the processing/animals handling areas per year	Lime
Were treatments for respiratory disease administered	Tx Respiratory Disease
Were treatments for foot infections administered	Tx Foot Infection Disease
Were treatments for arthritis/swollen joints administered	Tx Arthritis
Were treatments for metritis administered	Tx Metritis
Percentage of cattle receiving TMR	Feed TMR
Percentage of Forage in diet	Forage Diet
Percentage of Corn silage in diet	% Corn silage Diet
Percentage of Distiller's grain in diet	% Distillers grains Diet
Percentage of Cottonseed meal in diet	% Cottonseed Diet
Was Rumensin included in ration	Rumensin
Were Direct fed microbials used in the ration	Direct Fed Microbials
Were Antiparasitic agents used	Anthelmintic
How many cattle did reside within a 2-mile radius of the farm	Radius animal presence
Did Opossum have contact with cattle environment/feed	Contact with Opossum
Did cats have contact with cattle environment/feed	Contact with Cats
Did Deer have contact with cattle environment/feed	Contact with Deer
How frequent was the contact of Raccoons with cattle environment/feed	Contact with Raccoons
Did Dogs have contact with cattle environment/feed	Contact with Dogs
Did Skunks have contact with cattle environment/feed	Contact with Skunks
How frequent was the contact of Rodents with cattle environment/feed	Contact with Rodents
Did Other species of animals have contact with cattle environment/feed	Other SP
Cattle cleanliness score in thirds	ACleanliness
Cattle bedding cleanliness score in thirds	Bedcleanliness
Did cattle receive antibiotics any route during one month previous sampling	Antibiotics before sampling
What was the capacity of animals that feedlot can hold	Feedlot size capacity
How many cattle were fed/marketed annually	Feed Annual
Proportion of cattle that were breed beef	Proportion Beef breed
Proportion of cattle that were steers	Proportion Steers
In the last year, Average arrival weight of the cattle fed	Arrival average weight
Average sale weight of the cattle	Sale average weight
Average length of time cattle were on your feedlot (days)	Feedlot days
What percentage of incoming cattle were purchased from off-site	Purchased from off-site

Table 2.7. (cont'd)

Proportion purchased out-of-state (%)	Purchased from out-state
Were cattle housed in a separate barn post-arrival	Barn post arrival
Were antibiotics used in the feed or water at arrival	Antibiotics at arrival

Figure 2.4. Dairy and beef questionnaires used to collect information from the herds.

Michigan State University
STEC Project
Dairy Producer Questionnaire

Herd Code:	Farm Name:
Owner/Mngr:	Appointment:
Address:	City/Town:
County:	Cell Phone:
Office Phone:	Email:
Interviewer:	Date:
Veterinarian:	Interviewee:

Figure 2.4. (cont'd)

Herd Information:

What is the usual herd population?		
Calves & heifers (#):	Lactating Cows (#)	Dry Cows (#):
How many animals were added from off the farm in the last year?		
Cows & heifers (#):	Bulls (#):	☐ Closed herd
Overall culling rate (% culled per year):		
Number of milkings per day (2x/3x/Combination, 2x-3x):		
Which best describes your type of lactating cow housing (check all that apply):		
☐ Free Stall	Proportion of herd:	
☐ Loose Housing	Proportion of herd:	
☐ Access to pasture/dry lot	Proportion of herd:	
☐ Tie stall/stanchion	Proportion of herd:	
Do lactating cows have access to pasture (Yes [in season] / Rarely / No):		
Are first lactation animals penned separately (Yes / Sometimes / No):		
Are transition (post-calving) animals penned separately (Yes / Sometimes / No):		
Are sick cows penned separately (Yes / Sometimes / No):		
Are cows and heifers mostly raised (on main farm / off-site / contracted from another farm):		

Figure 2.4. (cont'd)

How often feedbunks are cleaned (times)?	Day / Week / Month:
How often waterers are cleaned (times)?	Day / Week / Month:
What methods are used to clean areas animals are housed? ☐ ☐ Scrape ☐ Wash/Power Wash ☐ Spray a Disinfectant ☐ Spread Lime None	
How often is each type of cleaning done and where? Scrape:	
Wash/Power Wash:	
Spray a disinfectant:	
Spread Lime:	

What are the common antibiotics/remedies used for each of the following purposes?:

Dry Cow treatment:	
Clinical Mastitis:	

Figure 2.4. (cont'd)

Metritis:	
Respiratory Disease:	
Foot Infections (including footbaths):	

Are dewormers agents used?	☐ Yes	☐ No
If yes, what is used?		
What percentage of the cows receive a total mixed ration (TMR)?		
Indicate if the lactating herd receives the following as part of their diet and at what proportions:		
☐ Forage	Percentage of diet (%):	
☐ Concentrate	Percentage of diet (%):	

Figure 2.4. (cont'd)

	Total: 100%
☐ Corn silage	Percentage of diet (%):
☐ Distiller's grain	Percentage of diet (%):
☐ Cottonseed meal	Percentage of diet (%):
List the principle ingredients and approximate percentages for the most recent ration (if possible, get a copy ration for targeted pen):	
Is Rumensin included in your ration?	☐ Yes ☐ No
Are any direct fed microbials used in the ration?	☐ Yes ☐ No
If yes, what products are used?	
Do you use antimicrobials in feed?	☐ Yes ☐ No
If yes, what antimicrobials are used and for what purpose?	

Figure 2.4. (cont'd)

Which best describes methods of fly control (check all that apply, circle most common):				
☐ None	☐ Pour-on insecticide	☐ Premise spray	☐ Back Rub/Duster	☐ Eat tags
☐ Feed Larvicide	☐ Predator Insects	☐ Fly Bait	☐ Fly sticky strips	
☐ Other Describe:				
About how many cattle reside within a 2-mile radius of farm (Excluding your farm)?				
What is the frequency the following animals have contact with cattle environment or cattle feed (None, Rarely, Frequently, Always):				
Opossums:	Cats	Deer		
Raccons:	Dogs	Skunks		
Rodents:	Birds			
If birds, which species:				
Other (describe):				

Figure 2.4. (cont'd)

Pen/Group Number:		Description:				
Average lactating cow stock density (Average cow per stall or cows per sq. ft if not free stall):						
How many animals in the past month have had any of the following symptoms (new cases):						
Diarrhea:	Bloat:	Respiratory:				
Clinical Mastitis:	Metritis:	Displaced abomasum:				
Ketosis:						
List unusual health observations for this pen:						
What was the annual mortality rate of the adult cows (%) ?						
What is the primary housing method for cows?						
☐ Loose/group housing	☐ Freestall dry lot					
Principle bedding type:						
☐ None	☐ Sand	☐ Shavings	☐ Straw	☐ Mattress	☐ Pack	☐ Hulls
Other (describe):						

Figure 2.4. (cont'd)

Which best describes the water source (never / sometimes / usually / always):	
Continuos flow water tank:	Ritchie type water
Nose operated water cups/bowls	Surface Water
If surface water, describe:	
Other (describe):	

Figure 2.4. (cont'd)

Michigan State University
STEC Project
Beef Producer Questionnaire

Herd Code:	Farm Name:
Owner/Mngr:	Appointment:
Address:	City/Town:
County:	Cell Phone:
Office Phone:	Email:
Interviewer:	Date:
Veterinarian:	Interviewee:

Figure 2.4. (cont'd)

Herd Information:

How many cattle pens are there?	
How many cattle pens contain animals that will be here at least 90 days?	
How many cattle pens can be sampled?	
Feedlot Capacity:	Capacity of pen(s) to be sampled:
How many cattle are fed/marketed annually?	
In the last year, what proportion of the cattle fed are:	
Holstein (#):	Beef type (#):
In the last year, what proportion of the cattle fed are:	
Steers (#):	Heifers (#):
In the last year, what is the average arrival weight of the cattle fed (lb)?	
What is the average sale weight of the cattle (lb)?	
What is the average length of time cattle are on your feedlot (days)?	
What proportion of incoming cattle are purchased:	
In-State (%):	Out-of-State (%):
What is the most common out-of-state location cattle came from?	

Figure 2.4. (cont'd)

Are the cattle housed in a separate barn for 30-45 days post-arrival: Yes ☐ No ☐
Upon arrival, are antibiotics used in the feed or water of the new cattle? : Yes ☐ No ☐
How often are feedbunks cleaned (time(s) per day/week/month)?_____ Day/Week/Month
How often are waterers cleaned (time(s) per day/week/month)? _____ Day/Week/Month
What methods are used to sanitize the processing/animal handling areas? ☐ None ☐ Scrape ☐ Wash/Power Wash ☐ Spray a Disinfectant ☐ Spread Lime
How often is each done? Scrape:
Wash/Power Wash:
Spray a disinfectant:
Spread Lime:
What are the common antibiotics/remedies used for each of the following purposes?:

Respiratory Disease:	
Foot Infections:	

Figure 2.4. (cont'd)

Arthritis/Swollen Joints:		
Are dewormers agents used?	☐ Yes	☐ No
If yes, what is used?		
What percentage of the cattle receive a total mixed ration (TMR)?		
Indicate if the herd receives the following as part of their diet and at what proportions:		
☐ Forage	Percentage of diet (%):	
☐ Concentrate	Percentage of diet (%):	
	Total: 100%	
☐ Corn silage	Percentage of diet (%):	
☐ Distiller's grain	Percentage of diet (%):	
☐ Cottonseed meal	Percentage of diet (%):	
List the principle ingredients and approximate percentages for the most recent ration (if possible, get a copy ration for targeted pen):		
Is Rumensin included in your ration?	☐ Yes	☐ No

Figure 2.4. (cont'd)

Are any direct fed microbials used in the ration?	☐ Yes ☐ No
If yes, what products are used?	
Do you use antimicrobials in feed?	☐ Yes ☐ No
If yes, what antimicrobials are used and for what purpose?	
Do you use anticoccidials?	☐ Yes ☐ No
If yes, what anticoccidials are used?	
Which best describes methods of fly control (check all that apply, circle most common):	
☐ None ☐ Pour-on insecticide ☐ Premise spray ☐ Back Rub/Duster ☐ Eat tags	
☐ Feed Larvicide ☐ Predator Insects ☐ Fly Bait ☐ Fly sticky strips	
☐ Other Describe:	
About how many cattle reside within a 2-mile radius of farm (Excluding your farm)?	

Figure 2.4. (cont'd)

What is the frequency the following animals have contact with cattle environment or cattle feed (None, Rarely, Frequently, Always):		
Opossums:	Cats	Deer
Raccons:	Dogs	Skunks
Rodents:	Birds	
If birds, which species:		
Other (describe):		

Test Group Information:

Pen Number:	
Characterize the cattle in the pen (check all that apply):	
Holstein: ☐	Beef type: ☐
Steer: ☐	Heifer: ☐
What was the origin of the pen?	
What is the average purchase weight (lb)?	
What proportion of cattle in the pen were (%):	

Figure 2.4. (cont'd)

- Commingled by an order buyer?				
- Commingled after arrival?				
- All from a single source?				
How many animals in the past month have had any of the following symptoms (new cases):				
Diarrhea:	Bloat:	Respiratory (shipping fever):		
List unusual health observations for this pen:				
What was the sickness rate of this pen during the first 45 days in the feedlot (%)?				
What was the mortality rate of this pen (%)?:				
What antibiotics have been used in feed or water within the last two months?				
Which best describe the type of housing for the test pen:				
☐ Bedded pack	☐ Pasture	☐ Slotted floor barn		
☐ Outside lot	Other (describe):			
Principle bedding source (check all that apply):				
☐ None	☐ Sand	☐ Corn stocks	☐ Straw	☐ Sawdust

Figure 2.4. (cont'd)

Other (describe):	
Which best describes the water source (never / sometimes / usually / always):	
Continuos flow water tank:	Ritchie type water
Nose operated water cups/bowls	Surface Water
If surface water, describe:	
Other (describe):	

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CHAPTER 3

ASSOCIATION OF BOVINE LEUKEMIA VIRUS AND *MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS* WITH SHEDDING OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI*

Abstract

Bovine leukemia virus (BLV) is a retrovirus that causes enzootic bovine leukosis in cattle and *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the etiologic agent of Johnes' disease in cattle. Both diseases are chronic in nature that leads to disruption of normal immunological or physiological processes. Cattle are the major reservoir of Shiga toxin-producing *Escherichia coli* (STEC), a major cause of foodborne illness in humans. We tested the hypothesis that cattle infected with BLV and/or MAP are more likely to shed STEC. We conducted a cross-sectional study during the summers of 2011 and 2012 in 11 Michigan cattle herds. A fecal sample from each animal was collected for STEC culture and multiplex PCR for *stx1, stx2,* and *eae*A was used to screen suspect colonies for STEC confirmation. Antibody detection ELISA assays for BLV and MAP were used to screen serum from each animal. Blood samples were collected from a subsample (n=497) to quantify the percentage of lymphocytes, monocytes and neutrophils using flow cytometry. Of the animals sampled, 34.9% were BLV positive while 2.7% were MAP positive and 16% were shedding STEC. Dairy herds had a higher frequency of BLV and MAP than did beef herds, but beef herds had more STEC. Neither BLV nor MAP was associated with STEC shedding. We also observed no association between

percentage of white blood cells and STEC status. Although controlling both BLV and MAP is important for overall herd health and productivity, controlling BLV and MAP will not likely have an impact on STEC shedding in cattle.

Introduction

Bovine leukemia virus (BLV) and *Mycobacterium avium subsp. paratuberculosis* (MAP) have been associated with a suppressed immune response in cattle. Secondary health issues are often associated with BLV and MAP because of their chronic and potentially debilitating nature (Bartlett, et al 2014; Gonda, et al 2007).

Bovine leukemia virus (BLV) is a retrovirus that causes enzootic bovine leukosis. Most animals infected with BLV never develop clinical signs, but 30% of BLV carriers will develop a persistent lymphocytosis and less than 5% will develop malignant lymphosarcoma (Erskine, et al 2012). BLV affects host defense mechanisms by disrupting the homeostasis of normal lymphocyte proliferation and programmed cell death, in both B-cells and T-cells, which can increase susceptibility to infectious diseases (Bartlett, et al 2014). According to USDA surveys, 83% of US dairy herds have at least one infected animal (USDA 2010). The within-herd BLV prevalence ranges from 23% to 46% in affected dairy herds (Ott, et al 2003; Sargeant, et al 1997; Trono, et al 2001).

Mycobacterium avium subsp. *paratuberculosis* (MAP) causes Johnes' disease (JD). Initial MAP infection most likely occurs at a very early age (<6 months) yet clinical disease does not normally occur until after 2 years of age (Blood, et al 1989). This pathogen becomes localized in the mucosa of the small intestine and associated lymph nodes. MAP elicits T-cell activation and clonal expansion that causes alterations in the intestine's histology and physiology (Manning and Collins 2001) and produces changes in the intestinal microbiota composition. An estimated 50% of Michigan dairy herds, and 68% nationally, have MAP-infected animals (Pillars, et al 2009).

Shiga toxin-producing *Escherichia coli* (STEC) is an important cause of foodborne illness and hemolytic uremic syndrome (HUS) in children (Bolton 2011) which can result in kidney failure and death in some cases. Cattle are a major STEC reservoir (Bolton 2011; Ferens and Hovde 2011; Gyles 2007), though it does not typically cause symptomatic infections (Kuhnert, et al 2005) except for contributing to diarrhea/dysentery in a subset of calves (Gyles and Fairbrother 2010; Vande Walle, et al 2013). Food and water contaminated with feces is the most common source of human exposure. For this reason, the USDA-FSIS considers STEC an adulterant of all raw non-intact beef and raw intact beef intended for use in raw non-intact products under the Federal Meat Inspection Act (21 U.S.C. 601(m)(1)).

Because of the chronic nature of both BLV and MAP infections and the immunosupresion and gastrointestinal disruption, we hypothesized that both infections may have an impact on STEC colonization and shedding in cattle. Therefore, the objective of this study was to determine if cattle infected with MAP and/or BLV are at higher risk for shedding STEC in their feces and thus could increase STEC contamination in the human food chain.

Materials and methods

1. Animal selection

A total of eleven Michigan cattle herds (6 dairy farms and 5 beef feedlots) were sampled for STEC, BLV and MAP. The herds were chosen based on convenience and willingness to participate in the study and were sampled during the spring-summer months of 2011 and 2012. The number of cattle sampled was based on the type and size of herds. In the beef feedlots, all of the animals present in each feedlot were sampled, while the quantity of the animals sampled in the dairy herds depended on the size of the herd. All adult cows were sampled in each dairy herd with less than 175 animals. For herds with more than 175 animals, a convenience sample of 175 animals was selected from cattle in the different management groups. This study was approved by the Michigan State University Institutional Animal Care and Use Committee (AN12/10-223-00).

2. Fecal sample collection and analysis

Fresh fecal samples were collected per rectum from 1,108 animals; a total of 724 dairy and 384 beef cattle were sampled. For the first four herds, samples were transported to the laboratory on ice where they were stored at 4°C and then processed within 48 hours. For the other remaining seven herds, samples were transported to the laboratory in a cooler without ice and processed immediately. This change in protocol was done to optimize our ability to detect STEC in the fecal samples.

Samples were cultured for STEC by first enriching in *Escherichia coli* (EC) broth (Oxoid Ltd.; Waltham, MA) supplemented with novobiocin (8mg/l), rifampin (2mg/l) and potassium tellurite (1mg/l) for 25 hours at 42°C (Jason, et al 2009) and then plating on STEC CHROMagar™ (CHROMagar, Paris, France) ans sorbitol MacConkey (SMAC) agar. The EC broth was also used to perform immunomagnetic separation (IMS) targeting *E.coli* O157:H7 using Dynabeads® MAX E.coli O157 (Invitrogen Corporation, California, USA). The IMS O157 protocol was followed by subculture to O157 CHROMagar (CHROMagar, Paris, France) and SMAC agar. Up to 20 suspect colonies were selected from each of the three agar plates for multiplex PCR targeting the Shiga toxin genes (*stx1, stx2*) *and eae*A (intimin) for STEC confirmation.

Bacterial colonies with at least one *stx* gene were considered to be STEC, and individual animals were considered positive if any STEC was recovered from the fecal sample. A total of 1,096 animals (718 dairy, 378 beef) were included in the final analysis, 12 animals were excluded because the STEC culture was missing.

3. Blood collection and analysis

At least three milliliters of blood were collected into serum separator vaccutainer tubes from the coccygeal or jugular vein. Serum was separated by centrifugation and submitted to the Diagnostic Center for Population and Animal Health at Michigan State University. Antibody detection ELISA assays specific for BLV (Bovine Leukemia Virus Antibody Test Kit, VMRD, Pullman, WA) and MAP (Paracheck, Prionics, USA Inc, Omaha, NE) were used to screen serum from each animal. Animals were classified as either positive or negative based on the

manufacturer's suggested criteria, and the optical density was recorded.

Whole blood samples (n=497) were collected in vaccutainer tubes containing Acid Citrate Dextrose (ACD) from six herds in year two, which included 290 dairy cattle and 207 beef cattle. These samples were used to quantify the percentage of lymphocytes, monocytes and neutrophils, using a Becton Dickinson FACSCalibur™ flow cytometer (San Jose, CA, USA). Cell profiles were analyzed based on size and granularity, and the level of fluorescence caused by indirect immunofluorescence with primary monoclonal antibodies (Davis and Hamilton 1993).

4. Data collection and analysis

The daily average, maximum, and minimum ambient temperatures 1-5 days before sampling were collected from the closest weather station (Quality Controlled Local Climatological Data (NOAA). For dairy cattle, production data including the lactation number (parity) and days in milk (DIM) were also recorded.

All data was analyzed using SAS 9.3 (SAS Institute Inc., Cary, NC, USA). The dependent variable was the STEC status (negative or positive) of each animal. The independent variables were BLV status (binary and continuous) and MAP status (binary and continuous); percentage of neutrophils, lymphocytes and the lymphocyte to monocyte ratio.

The distribution of the independent variables was explored as well as the presence of confounders. The point of significance to be incorporated into the initial multivariable model was 1.5 with a correlation coefficient of 0.9 (Dohoo, et al 2010). Herd was also incorporated in the multivariable models as a random effect as herds varied in management strategies and

geographic location. Both univariate and multivariate associations were examined using logistic regression and generalized linear mixed models (GLIMMIX). The point of significance to be incorporated in the final multivariable model was 0.05. Odds ratios (OR) and 95% confidence intervals (95% CI) were estimated for each variable. A backward process was used to evaluate the significance of each variable, and biologic rationale was considered prior to introducing variables into the final model. See **Table 2.7** for explanation of abbreviations used for variables.

Results

1. Descriptive statistics

The within-herd animal STEC prevalence ranged from 6% to 54%. The STEC prevalence was 13% (95/715) in dairy cattle and 21% (80/378) in beef cattle. Feedlot cattle had a higher risk of being STEC positive than did dairy cattle (OR: 1.8; 95% CI: 1.25-2.47). For BLV, the within-herd prevalence ranged from 0% to 79%. Two of the beef herds (n=129 cattle) were negative for BLV. The individual animal prevalence of BLV among dairy and beef herds was 48% (344/717) and 10% (38/378), respectively. Dairy cattle were 8.3 times (95% CI: 5.68- 12.22) more likely to be positive for BLV than beef cattle (P=<0.0001). The within-herd MAP prevalence was 0% to 8%; three beef herds (n=161 animals) were negative for MAP. The individual animal prevalence of MAP for dairy and beef herds was 3% (23/717) and 2% (7/378), respectively. Dairy cattle had an increased frequency of MAP, however, this difference was not significant (OR: 1.76; 95% CI: 0.72- 4.91; P=0.2434). BVDV was not detected in any of the cattle tested. Data is summarized in **Table 3.1**.

1.2 Immune system cells

When looking at the association between BLV status, STEC status and the percentage of neutrophils, lymphocytes and the lymphocyte to monocyte ratio (L:M), only the ratio was significantly associated with BLV status. As the L:M increased per unit, the likelihood of being BLV positive also increased (OR: 1.2; 95% CI: 1.09- 1.24; p-value: < 0.0001). By contrast,

neither MAP nor STEC status was associated with the percentage of neutrophils, lymphocytes and L:M (**Table 3.2**).

2. Univariable models

Using univariable models, there was no association between STEC and BLV when cattle herds were analyzed together (p-value: 0.5406) or separately as dairy herds (p-value: 0.7228) or beef herds (p-value: 0.9084). Similarly, there was no association between STEC and MAP (p-value: 0.3126) (**Table 3.3**). Also, there was no association between STEC status and the percentage of neutrophils, lymphocytes or the L:M ratio after including herd as a random effect **(Table 3.4)**. There was a significant association between STEC and average maximum temperature 1-5 days before sampling and between STEC and year.

3. Multivariable models

There was no association between STEC and either BLV or MAP status. When BLV or MAP status was examined as a continuous variable represented by the optical density of the ELISA assays, there was still no association between STEC status and either BLV or MAP. Additionally, production system type, maximum average temperature 1-5 days before sampling, and county were examined in the model as potential confounders (**Table 3.5**). When analyzed separately by production type, there was no association between STEC and MAP in dairy herds either between STEC and BLV (p-value: 0.5936) (**Table 3.6**). The model did not converge in the

case of beef herds because few herds and few animals within herds were positive for both BLV and MAP, so no conclusion could be made.

Discussion

In this study, we examined a population of beef and dairy cattle in Michigan to determine whether there was an association between STEC shedding and two common chronic infections that affect the immune system and the gastrointestinal function. Although immune suppressive effects have been widely reported for both BLV and MAP, we did not find a higher rate of STEC colonization among this population of BLV- or MAP-positive cattle.

A possible explanation for the lack of association could be that the immune disruption due to BLV and MAP is generally only present in a subset of infected animals, so an effect may have been diluted by a large number of immune competent cattle in our study, as the cattle sampled in this study were cattle with not visual clinical signs of disease.

The lack of association between the percentage of neutrophils, lymphocytes and L:M ratio and STEC (**Table 3.4**) in our study differ in part with previous studies (Hoffman, et al 2006; Vande Walle, et al 2013), which may be because we were examining natural STEC infections. Animals infected with STEC did not have a noticeably different white blood cell profile, further supporting the notion that STEC colonization does not stimulate major immune responses in cattle. Because STEC is believe to be a commensal with totally asymptomatic infection in cattle (Wells, et al 1991).

However, based on histological changes in the intestine of the colonized cattle, Vande Walle et al. (2013) have suggested that *E. coli O157:H7* represent a bovine pathogen. There is also some evidence of innate and adaptive bovine immune response to *E. coli O157:H7*, such as the production of pro-inflammatory cytokines and antibodies against secreted proteins (Vande Walle, et al 2013). It has also been suggested that *E. coli O157:H7* may cause immunosupresion

in cattle, preventing the onset of an antigen-specific cellular immune response. For example, calves infected with Stx2^{+} *O157* did not develop a lymphoproliferative response to heat-killed Stx2^{+} *O157* (Hoffman, et al 2006). Nevertheless, the pathogenic properties of STEC in cattle appear to be minimal. It is therefore possible that immune suppression due to either BLV or MAP may be largely inconsequential as a determinant of STEC colonization and shedding. More studies in this area will help to understand the interaction between STEC and the bovine immune system.

In summary, the lack of association between STEC and these two chronic nature diseases could be due to the interaction between STEC, a commensal, and the immune system of cattle; the immune system is no affected by the presence of BLV/MAP. Also could be that the cattle we sampled was at a stage of BLV/MAP infection that does not compromise or disrupt their immune system and the gastrointestinal function, yet. As consequence STEC have a normal interaction with the immune system and cannot take advantage of them.

Because diet, health, and management practices vary widely among herds, herd was included in the univariable and multivariable models as a random effect. Using herd as a random effect allowed us to control for several known confounders and unknown confounders (Dohoo, et al 2010). The established association between warm temperatures and STEC shedding (Cobbold, et al 2004; Dunn, et al 2004; Gautam, et al 2011; Kondo, et al 2010; Smith, et al 2005) was the main reason to incorporate maximum average temperature 1-5 days before sampling as a confounder.

In the univariate and multivariable analyses, the significant difference in STEC shedding between 2011 and 2012 may have been because that 2012 was warmer. According with NOAA, “In 2012, the contiguous United States average annual temperature of 55.3°F was 3.2°F above

the 20th century average, and was the warmest year in the 1895-2012 period of record for the nation" (NOAA National Climatic Data Center). This finding confirms the association between STEC shedding and warm temperature/seasonality reported by several studies (Dunn, et al 2004).

Due to the high prevalence of BLV and MAP in Michigan cattle herds and their known immune suppression effects, reducing BLV and MAP was seen as potential interventions to control STEC shedding in the human food chain. Although controlling BLV and MAP is important for overall herd health and productivity, based on this study, it does not appear that controlling BLV and MAP would be an effective way to reduce STEC shedding in cattle.

APPENDIX

Table 3.1. Prevalence of bovine leukemia virus (BLV), *Mycobacterium avium subsp. paratuberculosis* (MAP) and STEC by herd. * B=beef, D=dairy

Herd*	Total animals sampled	BLV Positive (%)	MAP Positive (%)	STEC Positive (%)
B1	134	9 (7)	2 (1)	11 (8)
D2	148	43 (29)	3 (2)	13 (9)
B3	32	5 (16)	0	3 (9)
D4	174	56 (32)	1 (1)	24 (14)
D6	94	53 (56)	4 (4)	6 (6)
D7	100	54 (54)	5 (5)	13 (13)
B8	54	0	0	29 (54)
D9	100	58 (58)	2 (2)	28 (28)
D10	101	80 (79)	8 (8)	11 (11)
B11	83	24 (29)	5 (6)	13 (16)
B12	75	0	0	24 (32)
Total	1,095	382 (35)	30 (3)	175 (16)

Table 3.2. Mean and Standard deviation (SD) for the percentage of neutrophils, lymphocytes and L: M in cattle positive and negative to bovine leukemia virus (BLV) and *Mycobacterium avium subsp. paratuberculosis* (MAP) according to STEC status.

Cell type	**BLV positive**	**BLV negative**	**MAP positive**	**MAP negative**
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
Neutrophils (%)				
STEC positive	15.56 (14.27)	18.53 (12.58)	16.55 (4.65)	17.58 (13.27)
STEC negative	16.85 (14.81)	20.81 (25.16)	7.79 (0.28)	19.49 (21.61)
Lymphocytes (%)				
STEC positive	67.87 (16.06)	60.74 (12.84)	66.01 (8.29)	63.01 (14.39)
STEC negative	64.13 (16.43)	62.30 (39.80)	68.17 (14.79)	62.84 (32.14)
L: M				
STEC positive	6.8 (5.21)	4.73 (2.64)[a]	7.60 (6.15)	5.36 (3.76)[a]
STEC negative	5.58 (4.92)	3.96 (1.97)[b]	7.85 (10.19)	4.54 (2.98)[b]
TOTAL	208	289	477	20

Table 3.3. Univariable analysis to evaluate the association between risk factors and the dependent variable for STEC shedding.

Characteristic	No. (%) with characteristic	No. (%) with STEC	p-value	OR	95% CI
MAP					
Negative	30 (2.75)	2 (6.67)	0.5012	1.672	0.373- 7.504
Positive	1062 (97.25)	173 (16.29)		ref	ref
BLV					
Negative	712 (65.20)	125 (17.56)	0.5406	1.139	0.750- 1.730
Positive	380 (34.8)	50 (13.16)		ref	ref
Beef					
Beef	378 (34.58)	80 (21.16)	0.2356	1.832	0.673- 4.986
Dairy	715 (65.42)	95 (13.29)		ref	Ref
Temperature max 1-5 days					
>28.9 C	502 (45.93)	116 (23.11)	0.0073	3.055	1.349- 6.827
≤ 28.9 C	591 (54.07)	59 (9.98)		ref	Ref
Year					
2011	581 (53.16)	57 (9.81)	0.0095	0.333	0.145- 0.764
2012	512 (46.84)	118 (23.05)		ref	Ref

Table 3.4. Univariable analysis to evaluated ELISA for bovine leukemia virus (BLV) status, *Mycobacterium avium subsp. paratuberculosis* (MAP) status and percentage of neutrophils, lymphocytes, and lymphocytes monocytes ratio for their association with STEC shedding in animals.

Characteristic	**No. (%) with characteristic**	**No. (%) with STEC**	**p-value**	**OR**	**95% CI**
MAP					
Negative	492 (96.09)	116 (23.58)	0.5012	1.672	0.373- 7.504
Positive	20 (3.91)	2 (10)		Ref	Ref
BLV					
Negative	297 (58.01)	80 (26.94)	0.7811	0.925	0.532- 1.608
Positive	215 (41.99)	38 (17.67)		Ref	Ref
Neutrophils			0.3565	0.993	0.979- 1.008
Lymphocytes			0.8422	0.999	0.991- 1.007
L:M			0.1800	1.042	0.981- 1.106

Table 3.5. Final multivariable model for both beef and dairy herds to evaluate bovine leukemia virus (BLV) and Mycobacterium avium subsp. paratuberculosis (MAP) status as determinants of STEC shedding.

Characteristic	Estimate	Standard Error	p-value	OR	95% CI
Intercept	-3.2813	0.7935	0.0033		
MAP					
Negative	0.7214	0.7466	0.3341	2.057	0.475- 8.902
Positive	Ref	Ref		Ref	Ref
BLV					
Negative	0.08792	0.2158	0.6838	1.092	0.715- 1.667
Positive	Ref	Ref		Ref	Ref
Temperature max 1-5 days					
> 28.9 C	1.1547	0.3533	0.0011	3.173	1.586- 6.346
< 28.9 C	Ref	Ref		Ref	Ref
Beef					
Beef	0.6778	0.3663	0.0645	1.970	0.960- 4.041
Dairy	Ref	Ref		Ref	Ref

Table 3.6. Final multivariable model for dairy herds to evaluated bovine leukemia virus (BLV) and *Mycobacterium avium subsp. paratuberculosis* (MAP) status as determinants of STEC shedding.

Characteristic	Estimate	Standard Error	p-value	Individual p-value	OR	95% CI
MAP						
Negative	0.1383	0.7695	0.8575		1.148	0.457- 8.604
Positive	ref	Ref			ref	Ref
BLV						
Negative	-0.2822	0.2653	0.2879		0.754	0.695- 1.640
Positive	ref	Ref			ref	Ref
Temperature max 1-5 days						
> 28.9 C	0.8761	0.4005	0.0291		2.401	0.460- 7.319
< 28.9 C	ref	Ref			ref	Ref
Lactation						
First	0.6285	0.2545	0.0138		1.875	1.137- 3.090
2 or more	ref	Ref			ref	Ref
DIM						
0	-0.3612	0.6334	<.0001	0.5687	0.697	0.201- 2.417
1-31	1.4126	0.3149		<.0001	4.107	2.213- 7.621
>31	ref	Ref			ref	Ref

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CHAPTER 4

SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* ACQUISITION, LOSS AND PERSISTENCE IN CATTLE

Abstract

Shiga toxin-producing *Escherichia coli* (STEC) are one of the most common foodborne pathogens in the USA and other developed countries. Cattle are consider the main reservoir for STEC and food and water contaminated with cattle feces are the most common sources of human infections with STEC. In order to develop intervention strategies for STEC in cattle at the pre-harvest level, understanding the factors that influence STEC shedding are necessary. However, the dynamics of shedding are poorly understood and potential risk factors that influence shedding over time are unclear. The objective of this study was to generate information about the dynamics of STEC shedding in cattle over time and to identify factors associated with acquisition, persistence and loss of the bacteria.

Fecal STEC shedding of 149 individual cattle (46% dairy and 54% beef) from 11 different herds were analyzed four consecutive times separated by an average of 19 days. Information on potential risk factors was collected at each time point. STEC prevalence, loss rate and acquisition rate were calculated for each visit and an analysis for risk factors was conducted. STEC shedding was intermittent; only 5 animals shed STEC continuously throughout the study. Twenty- seven cattle shed STEC for at least two consecutives sample times. The average STEC

duration of shedding was 24 days. On the other hand, 28 cattle were negative throughout the study period. The rate of STEC loss was higher than the rate of STEC acquisition in all visits. STEC acquisition rate and STEC loss rate did not vary between visits; however, it varied between herds.

The percentage of animals that at any time during the study lost STEC was significantly different between years and herds, while the percentage of animals that at any time during the study acquired STEC was significantly different only by type of production system.

We found herd as the only factor significantly associated with being continuously STEC negative through the study period in dairy herds. Herd was the only factor significantly associated with the rate of STEC new infections in dairy herds as well.

In summary STEC shedding is intermittent and our study did not clearly identify any specific factor that influence STEC shedding over time. Herd, year, and type of production system (beef or dairy) appear to have the largest influence on STEC dynamics. Understanding the specific factors that influence STEC dynamics will allow designing effective intervention strategies at preharvest to prevent and reduce STEC human infections.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) is one of the most common foodborne pathogens in the USA and other developed countries and has a significant impact on public health. Cattle are the primary reservoir for STEC and food or water contaminated with cattle feces is the most common source of infection for humans (Kuhnert, et al 2005).The dynamics of STEC shedding remains poorly understood (Robinson, et al 2009). A better understanding of the dynamics of STEC shedding by cattle, and determining management and individual factors that influence this dynamic would help to design intervention strategies at the pre-harvest level aimed to reducing STEC shedding and ultimately human food contamination.

Different research groups have performed longitudinal prospective studies in cattle, but the studies have not been consistent in their findings. Among the established findings are the seasonality and intermittence or transient shedding of STEC in cattle (Callaway, et al 2013; Hussein 2007; Smith, et al 2013; Widiasih, et al 2004).

Our hypothesis was that there are factors that influence the dynamics of STEC shedding. The objectives of this study were to determine the rates of STEC acquisition, persistence and loss in these individual cattle. Additionally, we aimed to identify herd management practices and individual cattle characteristics for STEC acquisition or persistence in dairy.

Materials and methods

1. Herd selection

Dairy farms and beef feedlots were contacted and selected for inclusion in the study based on proximity to MSU, adequate animal handling facilities and willingness to participate in all phases of the study. It was also required that the farms had completed and detailed records for each animal, regarding management and health history, such as antibiotic administration and diseases. From twelve herds contacted initially, eleven agreed to participate; one herd chose not to participate because of concerns regarding animal welfare. The farm owners provided written informed consent to participate in the study. This study was approved by the Michigan State University Institutional Animal Care and Use Committee (AN12/10-223-00).

2. Study design

The study design was composed of three phases. Phase I involved completing a questionnaire designed to collect demographic information and data related to potential STEC risk factors. Phase II focused on sampling a representative number of animals within each herd and culturing feces for STEC isolation. Phase III consisted on a longitudinal sampling of a subset of animals, that were also sampled in Phase II. The first two phases have been described in detail in Chapter II of this dissertation. This study was performed during years 2011 and 2012. The herds were visited and sampled between May 11th and October 18th of 2011 (n=5) or

between May 29th and October 16th of 2012 (n=6). The time between Phases II and III had a range between 10 and 33 days and mean 19 days. Phase III consisted of three visits: first, second and third, that were in a range between 14 to 28 days (mean 19 days), 7 to 29 days (mean 18 days), and 28 to 60 days (mean 17 days) apart, respectively. The total period of the study was between 35 to 89 days (mean 53 days).

2.1 Sampling

In Phase II, fecal samples were collected from adult cattle within the herd. The number of cattle sampled was based on the type of herd and number of cattle. In dairy herds with fewer than 175 animals, all adult cattle were sampled. In dairy herds with greater than 175 animals, a convenience sample of 175 cattle was selected from different management groups. In the beef feedlots, a "herd" consisted of a pen or management group and all cattle in that group were sampled. In Phase III, fecal samples were collected from a subsample of the animals sampled in Phase II. An equal number of Phase II STEC positives and negatives animals were chosen from each herd for Phase III; their STEC status was determined based on PCR performed on raw feces at the beginning and then from culture isolates. When a positive animal was selected a negative animal from the same pen was also selected if not from a near pen; the total number of animals was 10 to 15 per herd. In one feedlot we were able to sample all the animals due to the easy accessibility to the animals.

Fresh fecal samples were collected by rectal palpation using individual obstetrical sleeves and placed in whirl-pak bags. For the first four herds (sampled in 2011), samples were transported to the laboratory on ice where they were stored at 4°C and then processed within 48 hours. For the other seven herds (one in 2011 and six in 2012), samples were transported to the

lab in a cooler without ice and processed immediately. This change in protocol was made because a prior study found that ice storage decreased the likelihood of STEC recovery from feces (Mindy Brashears, personal communication). Some of the STEC isolates and raw sample feces from 2011 sampling visits were lost; which made impossible to determine the STEC status in 45 individual cattle during some specific sample points.

When each farm was sampled, the date, time, latitude and longitude were recorded. In addition, the maximum, minimum and average temperatures from the day of each sampling and the preceding five days during Phase II were recorded using data from the closest weather station (Quality Controlled Local Climatological Data (NOAA)).

3. Laboratory protocol for STEC detection and isolation

Five grams of feces were inoculated in 2X EC broth (Oxoid Ltd.; Waltham, MA) supplemented with novobiocin (8mg/l), rifampin (2mg/l) and potassium tellurite (1mg/l) for 24 hours at 42°C (Jason, et al 2009) followed by subculture on STEC CHROMagar™ (CHROMagar, Paris, France) and sorbitol MacConkey (SMAC) agar. A portion of the EC culture was also processed by immunomagnetic separation using Dynabeads® (Invitrogen Corporation, California, USA) specific for *E .coli O157:H7* followed by subcultured to O157 CHROMagar (CHROMagar, Paris, France) and SMAC agar. Up to 20 presumptive STEC single colonies were selected from each plate, inoculated into Luria-Bertani (LB) broth for growth overnight at 37°C, and confirmed by PCR using a previously described protocol (Tarr, et al 2002) with either the Taq 2x MeanGreen Master Mix or Kappa2G Multiplex Master Mix (Kapa Biosystems, Massachusetts). The multiplex PCR used to confirm STEC single colonies detects

the presence of *stx1*, *stx2* and *eaeA* (intimin). Individual colonies with at least one *stx* gene were considered to be STEC, and fecal samples from individual cattle were considered positive if at least one STEC isolate from the sample was recovered.

4. Data analyses

The data was collected, input and analyzed using SAS 9.3 (SAS Institute Inc., Cary, NC, USA). STEC persistence and rates of acquisition and loss were based on whether or not the animal had a least one STEC isolate. "PERSISTENT STEC POSITIVE" (PP) shedding animal was defined as an animal that shed STEC for two consecutive visits. A "CONTINUOUS STEC NEGATIVE" (CN) animal was defined as an animal that was not detected as shedding STEC throughout the four sequential samples during the entire study period. When an animal was positive in one visit, but negative in the next one, this was called "STEC LOSS". In contrast, when an animal was negative in one visit, but positive in the next one, this was called "STEC ACQUISITION". The "Rate of STEC Acquisition" (RA) was calculated by dividing the number of animals that acquired STEC by the number of cattle at risk of acquiring infection, i.e. the number negative in the previous sampling. The "Rate of STEC Loss" (RL) was calculated by dividing the number of animal that lost STEC by the number of cattle at risk of losing an infection, i.e. the number of positives in the previously sampling. We also determined the number of animals that had lost STEC at any point during the study; this was called ANY STEC LOSS (AL). Also we determined the quantity of animals that had acquired STEC at any point during the study; this was called ANY STEC ACQUISITION (AA). These AL and AA variables have a dichotomous outcome and these were analyzed by Chi-square.

We also calculated the RATE of NEW STEC INFECTIONS (RNI) for all 149 animals. This rate is the result of dividing the number of new infections by the number of times that animal was at risk of acquiring STEC. Thus an individual animal could be at risk of a new infection 1, 2, 3 or 4 times during the study. These variables were analyzed with general linear models (GLM).

Univariate analyses for each variable were performed to identify herd and individual animal factors associated with STEC shedding in dairy herds. Each variable was initially examined in a univariate analysis to identify factors to be included in a multivariable model, using a backward manual selection procedure. Variables with non-normal distributions and potential confounding were identified. The point of significance was $P < 0.15$ for inclusion in the first multivariable model; however, the point of significance for the final multivariable model was $P < 0.05$. Odds ratios (ORs) and their 95% confidence intervals (95% CI) were estimated for each variable in the univariable analysis.

In the first model, the dependent variable was CONTINUOUS NEGATIVE (CN) and the animal was considered a random effect. Those cattle that were negative at all sample points were classified as CN. In this model there were three events. The first event is the time between Phase II and Phase III.1, the second event is the time between Phase III.1 and Phase III.2 and the third event is the time between Phase III.2 and Phase III.3. In those animals where the result was missing between two visits, the next consecutive result was used instead, this happened in 18 animals.

In the second model, the dependent variable was the RNI. This rate was calculated by dividing the number of new infections by the times the animal was at risk of a new infection. In this way, we were able to use all the animals even if we were missing some STEC results. The

dependent variable was binary (STEC new infection yes or no). The model had herd as a random effect. See **Table 2.7** for explanation of the abbreviations used for variables.

Results

1. Descriptive statistics

A total of 149 animals were sampled in this longitudinal study. During 2011 80 (54%) cattle were sampled, whereas 69 (46%) cattle were sampled during 2012. Of the animals sampled, 46% were dairy cattle and 54% were beef cattle. However, STEC culture results were available for all four sampling times for only 104 cattle (36 dairy and 68 beef).

From the 104 cattle with no missing data, a total of 28 (27%) animals were STEC negative throughout the study period. Nine of these were dairy cattle and 19 were beef cattle. One beef herd and two dairy herds had zero CN cattle.

From the cattle with no missing data a total of 27 (26%) cattle were PP. Except for one animal, all PP were sampled during year 2012. Of the PP cattle, 16 (59 %) were beef and 11 (41%) were dairy cattle. The PP animals were found in three dairy and four beef herds (**Figure 4.1**). The average duration of shedding in the PP during our study period animals was 24 days; the range was between 14 to 43 days. Only five animals, all from the same beef herd, were STEC positive at all sampling periods.

There were a total of 45 (30%) animals missing STEC results from at least one sample point. These cattle were not used in the calculation of the PP or STEC negative analysis. Of the animals missing STEC results, 33 were dairy cattle and 12 were beef cattle. As previously mentioned, all the animals missing STEC results belonged to year 2011. Of these animals 14 had at least one STEC positive result. Thus 60% of the 149 animals were positive at least one time during the study. The percentage of cattle missing STEC results was calculated in each sample point. The percentages of missing results were 17%, 7% and 17%, respectively.

Using all culture results for each of the four visits, the STEC point prevalence at the first visit was 30% (45/149 cattle), at the second visit 26% (32/124), at the third visit 24% (34/139), and at the fourth visit 24% (29/123).

2. STEC Loss and Acquisition Rate

RL and RA were calculated from the 104 animals with complete data. RL between each of the first three visits and the subsequent one were 64%, 54% and 54%, respectively. In contrast, the RA between each of the first three visits and the next one were 21%, 20% and 21%, respectively. RL and RA varied by herd between each visit, as can be observed (**Figure 4.2** and **Figure 4.3)**.

3. ANY STEC LOSS and ANY STEC ACQUISITION

AL and AA were determined using all 149 animals in the study. A total of 60 (40%) animals lost STEC at least one time, whereas 54 (36%) animals acquired STEC at least one time.

There was a significant different between herds ($P < 0.0001$) (**Figure 4.4**) and between years ($P < 0.0001$) for AL. A total of 19% of animals lost STEC in 2011, whereas 65% of animals lost STEC in 2012. There was no significant difference in AL between beef and dairy ($P = 0.2815$); AL occurred in 36% of beef animals and in 45% of dairy animals. There was no difference between herds ($P = 0.1004$) (**Figure 4.5**) nor between years ($P= 0.3063$) for AA. AA occurred in 33% of animals in 2011 and in 41% of animals in 2012. AA was significantly

different between beef and dairy herds (P < 0.0021); any acquisition occurred in 48% of beef animals and in 23% of the dairy animals.

4. Rate of new STEC infections

The RNI was significant different between herds (P <0.0001) **Figure 4.6**. This rate was also significant different between beef and dairy system (P =0.0036) and between years (P <0.0001). The RNI was 27% for beef herds and 16% for dairy herds; whereas for years the rate of STEC infection was 13% for 2011 and 33% for 2012 (P <0.0001).

5. Univariate analysis of herd level management risk factors

With the two models, CN and RNI, we did not identify any management or individual-cow factor significantly associated with these outcomes. In the first model, CN, 24 variables had a P-value less than 0.15 but when the variable HERD was included in the model, these factors became not significant or the model did not converge (**Table 4.2**). In the RNI model, 10 variables had a P-value less than 0.15 but when trying to build the multivariable model, none of these variables became significant or the value of the estimated covariance parameter estimate was 0 (**Table 4.3**). As a consequence, we could not build a multivariable model with this data.

Discussion

In this study we determined that STEC shedding is intermittent in dairy and beef cattle and that RL is higher than RA. Only a minority of animals shed STEC constantly and that HERD is the most important factor that influences RNI.

Two dairy herds that provided access to pasture for their cattle and one beef herd that kept its cattle permanently in pasture had zero negative animals thorough the study; this result is in agreement with data that indicates cattle in pasture-based production systems may have an increased risk for STEC shedding (Hancock, et al 1994; Jay, et al 2007; Kondo, et al 2010). Similarly, the five animals that shed STEC throughout the study period were all from the one herd that kept its cattle on pasture. This same herd also had the highest RNI and one of the highest rates of RA among herds. Raising cattle in pasture is thought to increase the exposure to sources of STEC such as water runoff and wildlife activity (Jay, et al 2007; Kondo, et al 2010; Laegreid, et al 1999). In contrast, other studies have reported that cattle in confinement-based production systems have higher risk of STEC shedding (Gannon, et al 2002; Ogden, et al 2004; Synge, et al 2003) or even that there is no difference between the two production systems (Hancock, et al 1997b). It is important to mention that only one of the 11 herds sampled was 100% pasture based, so generalization to all pasture based systems should be done with caution. However, based on our findings and those of others (Gannon, et al 2002; Hancock, et al 1994; Jay, et al 2007; Kondo, et al 2010; Ogden, et al 2004; Synge, et al 2003), studies designed to specifically explore STEC shedding in pasture-based production systems should be undertaken.

The only significant variable in the two univariable models that we investigated was HERD regardless of whether the variable HERD was included as a random effect or as fixed effect. An explanation for this finding could be that all the management practices variables were

at the herd level. In line with the differences in management practices among dairy herds was our finding that AL was significantly different between herds. Based on our statistical model we know that herd characteristics, such as management practices, influence the dynamics of STEC in dairy cattle, but we were not able to identify which specific practices or factors account for this difference.

We also found a significant difference in AA and RNI between beef and dairy productions systems. These two production systems have different management practices. For example dairy farms divided their animals by milk production levels and gestation length, as well as separated calf from cows. While beef farms or feedlots keep their cattle in groups by age or weight usually until finishing, depending of their production system. Also there are differences in diet as net energy requirements are different between beef and dairy cattle. In our study, beef animals presented a higher risk of RA and a higher RNI. In contrast, some studies have reported dairy cattle production systems with a higher likelihood for STEC shedding (Cobbaut, et al 2009; Cobbold, et al 2004).

A possible explanation for the difference between production systems in AA could be that the beef cattle were younger than the dairy cattle. Younger cattle have been reported to have a higher risk for STEC shedding (Cho, et al 2009; Dopfer, et al 2006; Gannon, et al 2002; Hancock, et al 1997a; Stanford, et al 2005). In addition, younger cattle usually have a less developed immune system, anatomic and physiologic differences and are often fed different diets, which may explain the differences observed in our study (Gannon, et al 2002).

The percentage of negative cattle was almost double in herds sampled during 2011 than during 2012. Also the herds sampled during 2012 had higher rates of RA compared to herds sampled in 2011. One possible explanation for this difference could be that 2012 was a warmer

year than 2011. The 2011 average global surface temperature was between 0.07 and 0.16 degrees Celsius warmer than the 1981-2010 average while 2012 was between 0.14 to 0.17 degrees Celsius above, depending on the analysis (Lindsey 2012; Osborne and Lindsey 2013). Warm temperatures are considered one of the most important risk factors for STEC shedding (Berry and Wells 2010; Ogden, et al 2004; Stanford, et al 2005; Widiasih, et al 2004). This is in line with the finding that all the PP animals except for one were sampled during 2012. Also 2012 had the highest RNI, plus AL and RL were significantly higher in 2012.

Our findings that herd, age and warm temperatures influence the dynamics of STEC shedding in cattle, are similar to those reported by Dopfer, et al (2006). In this study, beef cattle were followed during 2 years and a strong farm and age effect for the first detection of STEC and EHEC was found. In addition, a significant seasonal effect for the first STEC detection was found. Finally as age increased, EHEC and STEC were detected less frequently (Dopfer, et al 2006).

Another possible explanation for the difference we just described in STEC dynamics between years is stress, as warmer temperatures can produce heat stress. Stress affects the capacity of the animal's body to produce milk or gain weight. Stress can also affect its immunity system (Berry and Wells 2010; Rostagno 2009). With a weaker immune system, some bacteria can reproduce or colonize easier in their host; STEC is an example of that (Dean-Nystrom, et al 2008). Also warmer temperatures influence the environment where the animal lives. Warmer temperatures enhance the conditions for the survival and replication of bacteria, such as STEC, outside of the animal and in the environment, thus providing greater opportunities for animal exposure. So it could be possible that animals sampled during 2012 had more chance to have heat stress and as a consequence were more susceptible to STEC colonization and shedding.

The range of duration of STEC shedding has been reported to be from < 1 week to <1 month for O157 and from < 1 week to 3 weeks for O26 (Besser, et al 1997; Khaitsa, et al 2003; Widiasih, et al 2004). Although we did not identify the O-typing of our isolates, our findings are consistent with these studies. A common finding among all studies is the intermittent shedding of STEC by cattle (Besser, et al 1997; Laegreid, et al 1999; Robinson, et al 2004; Shere, et al 1998; Widiasih, et al 2004). Some studies proposed that exposure to periods of stress could be the cause of intermittent STEC shedding (Stanford, et al 2005). For example, Stanford et al (2005) found that *E. coli* O157:H7 shedding is transitory and sporadic after weaning. In our study only five animals were constantly STEC positive at all sampling points. Our finding that only a minority of cattle shed STEC constantly is in agreement with other studies (Robinson, et al 2004).

One weakness of our study is that we determined the presence of STEC but we did not determine the O-typing of the isolates. As a consequence, we could not differentiate between a persistent infection and a re-infection; there is the possibility that an animal could be positive to one strain at one sampling point, and positive to a different strain at the next sampling point.

From one visit to another, more than 50% of all the cattle STEC positive became negative by the next sampling (RL). However, the RA was almost always 20%. One possible explanation for this finding could be that as the time past by the cattle developed an immune response against STEC, which stop STEC shedding and as a consequence decreased STEC transmission. Another possibility could be that an animal was actually shedding STEC at very low levels below our laboratory detection levels. The detection limits for bacteriological cultures are between10^2 to 10^6 cfu/grams depending on enrichment techniques and if immunomagnetic separation is used (Dopfer, et al 2012). Factors such as the inoculated serotype, the inoculation level, and the initial

concentration of the target organism in the sample, can also influence the sensitivity of the different techniques implemented (LeJeune, et al 2006; Verstraete, et al 2010). Our finding of high rate of loss and low rate of acquisition is in agreement with a report that individual animals can have short periods of increased intensity of STEC shedding (Robinson, et al 2009), although we did not evaluate STEC quantitatively in our samples.

The limitation of finding temperature and age as risk factors is that they are not easily modified by human interventions (Cho, et al 2009). Possible intervention strategies would be to separate young from older animals and avoid hot temperatures in confined-production systems. These measures may not be practical or feasible to implement, though.

A limitation from our study was the incomplete STEC results from 30% of the cattle. This limitation potentially precluded the design of a multivariable model and also could explain the differences found between years 2011 and 2012 as most of the missing data came from cattle sampled during 2011. Also it could influence the lack of association between some independent variables and the dependent variable in both statistical models. For example, some of the management and individual factors analyzed have been reported to increase or decrease STEC shedding; such as temperature, distiller's grains, contact with birds and age. However, these variables did not present an effect in the RNI per se or once other variables were included into the model, as they became not longer significant or the statistical model did not work properly. RL and RA were calculated using only the cattle with complete data, so there is the possibility that these rates could be different if we had complete data for all the cattle. As a consequence, we approached this limitation by calculating AL and AA which took into account all data for all cattle including those with missing samples. Another limitation was the change in methodology in the handling of the samples, after the first four herds sampled during 2011 (change from 4C to

ambient temperature). It could be possible that in the samples handling at 4C we were able to recover less isolates as the reason for the change in methodology was to improve the recovery of STEC isolates.

In addition, a bigger sample size of herds would allow more power to identify the specific management practices influencing CN animals and the RNI or corroborate that they do not influence them.

In summary, we found that STEC dynamics varies by herd, type of production system temperature and exposure to pasture. Temperature appears to be an important variable that affects STEC shedding dynamics and should be taken into consideration when implementing or testing new control measures. STEC shedding dynamics also appear to differ by type of production systems, although specific factors responsible for this could not be identified in this study. Finally, we described differences in STEC dynamics between cattle in pasture-based systems and cattle in confinement-based systems. These differences should be investigated further because it can have relevant influence in the implementation of control strategies in different production systems.

APPENDIX

Figure 4.1. Percentage of cattle that were culture positive for STEC on two, three or four consecutive sampling points for a period of time between 35 to 89 days.

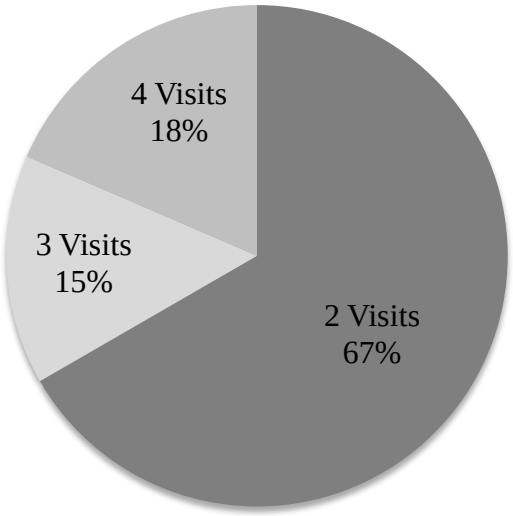

Figure 4.2. Rate STEC LOSS over time by herd. Event represents the time between one sampling and the next one. Event 1= Phase II to Phase 3.1, Event 2= Phase 3.1 to 3.2 and Event 3= Phase 3.2 to 3.3. B= beef and D= dairy.

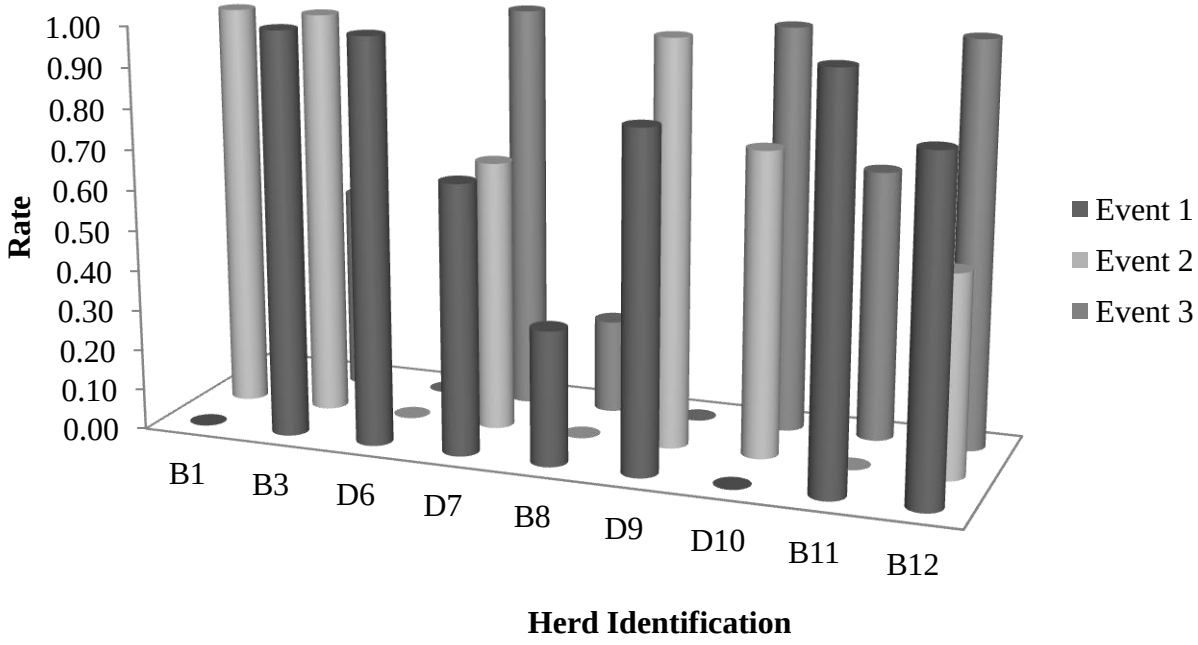

Figure 4.3. Rate of STEC ACQUISITION over time by herd. Event represents the time between one sampling and the next one. Event 1= Phase II to Phase 3.1, Event 2= Phase 3.1 to Phase 3.2 and Event 3= Phase 3.2 to 3.3. B= beef and D= dairy.

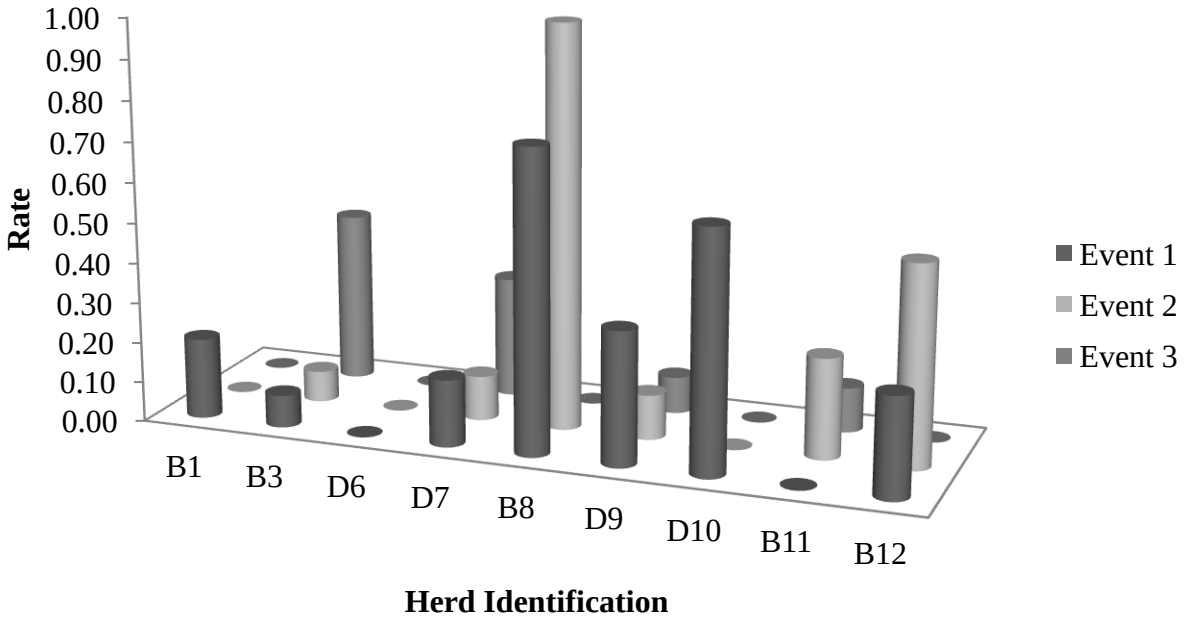

Figure 4.4. Percentage of animals that lost STEC at any time during the study by herd. B= beef and D= dairy

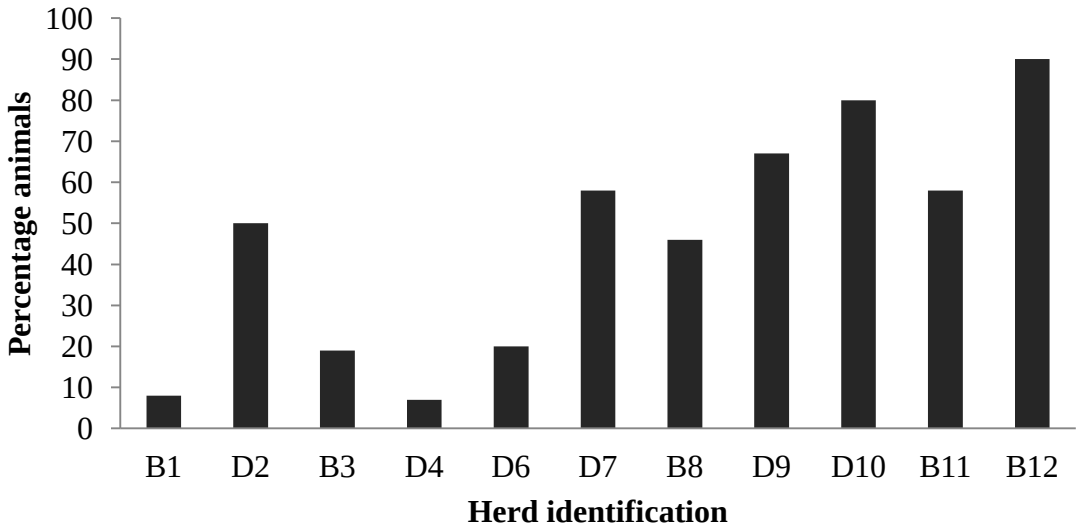

Figure 4.5. Percentage of cattle that acquired STEC at any time during the study by herd.

B= beef and D= dairy.

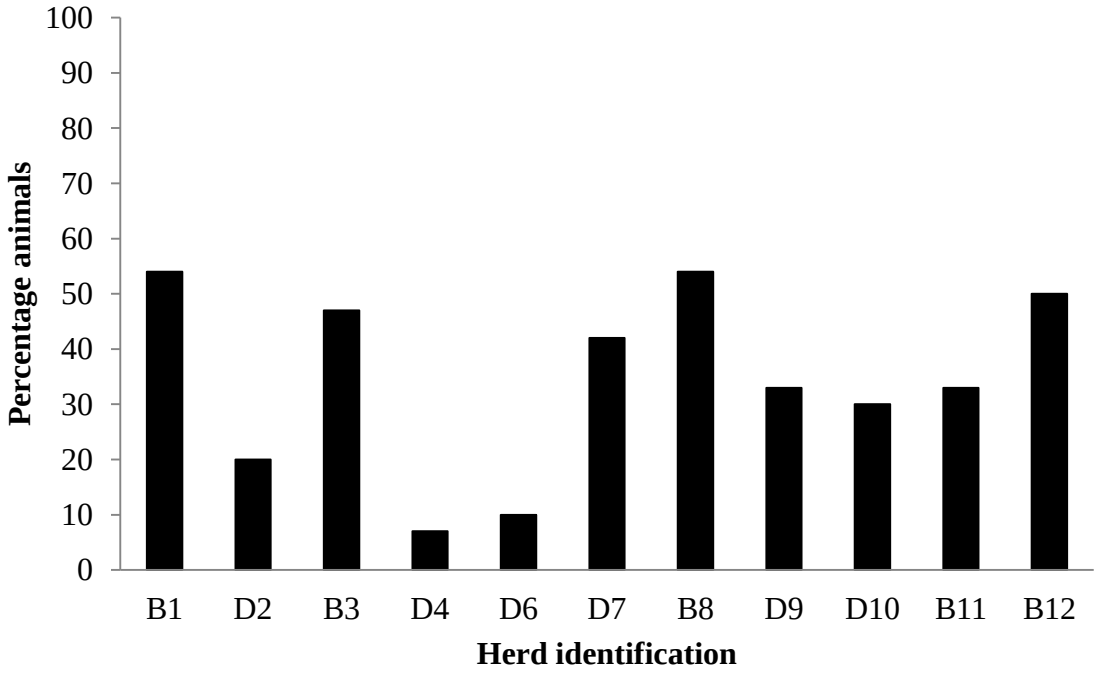

Figure 4.6. Rate of STEC NEW INFECTIONS (number of new infections in a cattle divided by the number of times cattle was at risk or susceptible to new infection) by herd. B = beef and D = dairy.

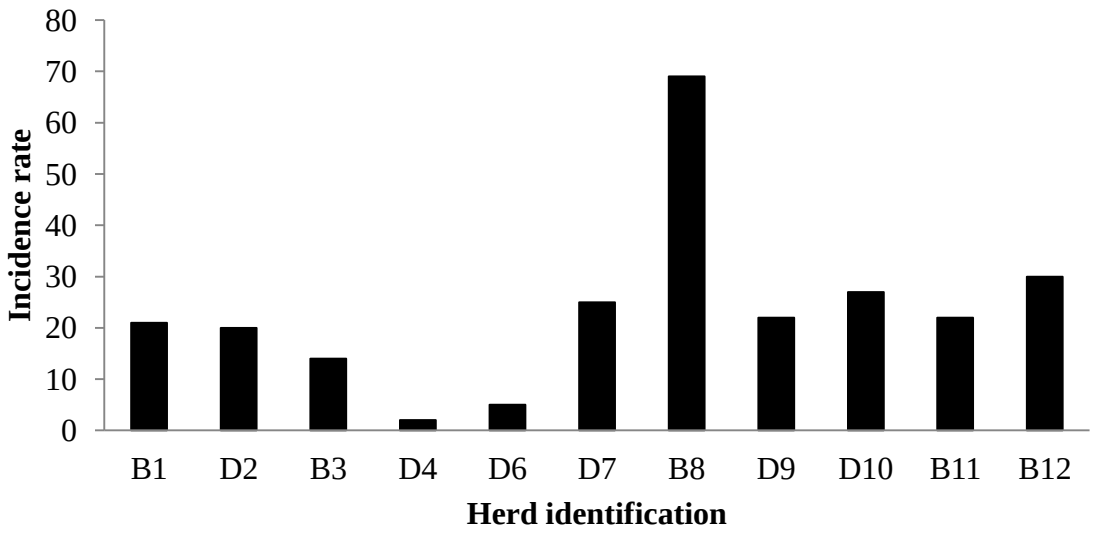

Table 4.1. Univariate analysis of dairy herd variables associated with persistently STEC-negative cattle.

Characteristic	**N° (%) with characteristic**	**N° (%) Constant STEC Negative**	**p-value**	**OR**	**95% CI**
Herd					
2	11 (7.1)	9 (82)	0.0178	6.5	0.94- 44.44
4	22 (14.3)	21 (95)		31.6	3.23- 309.78
6	19 (12.3)	16 (84)		8.7	1.63- 45.99
7	36 (23.4)	20 (56)		1.9	0.55- 6.69
9	36 (23.4)	22 (61)		2.4	0.70- 8.49
10	30 (19.5)	12 (40)		ref	Ref
Temperature at sampling date on Phase III			0.0252	0.9	0.86- 0.99
Lactating					
No	8 (5)	3 (38)	0.8682	0.86	0.15- 5.02
Yes	145 (95)	51 (35)		ref	Ref
Antibiotics used 2 weeks prior to the Phase III samplings					
Yes	3 (2)	2 (67)	0.2714	0.223	0.02- 3.32
No	119 (98)	34 (29)		Ref	Ref
Rumensin					
No	96 (62)	59 (61)	0.2988	0.63	0.26- 1.53
Yes	58 (38)	41 (71)		Ref	Ref

Table 4.1 (cont'd)

Year					
2011	52 (34)	46 (89)	0.0004	6.9	2.44- 19.32
2012	102 (66)	54 (53)		Ref	ref
Season					
Spring	36 (23)	20 (56)	0.2350	0.54	0.20- 1.50
Summer	118 (77)	80 (68)		Ref	ref
Temperature Av					
≤ 20.6 C	52 (34)	46 (88)	0.0004	6.9	2.44- 19.32
> 20.6 C	102 (66)	54 (53)		Ref	ref
Temperature Max					
≤ 27.8 C	88 (57)	66 (75)	0.0109	3.1	1.31- 7.32
> 27.8 C	66 (43)	34 (51)		Ref	ref
Temperature Min					
≤ 15.6 C	52 (34)	46 (88)	0.0004	6.9	2.44- 19.32
> 15.6 C	102 (66)	54 (53)		Ref	ref
Temperature Av5 days					
≤ 19.4 C	33 (21)	30 (91)	0.0052	6.9	1.81- 26.13
> 19.4 C	121 (79)	70 (58)		Ref	ref
Temperature Max5 days					
≤ 28.9 C	66 (43)	45(68)	0.5907	1.3	0.53- 3.00
> 28.9 C	88 (57)	55(63)		Ref	ref
Temperature Min5 days					
≤15C	30 (19)	25 (83)	0.0582	3.1	1- 10.2
>15C	124 (81)	75 (60)		ref	ref

Table 4.1. (cont'd)

Lactation					
1st	48 (31)	30 (63)	0.7878	0.9	0.35- 2.22
2nd or higher	106 (69)	70 (66)		Ref	ref
Days in milk					
0	4 (3)	2 (50)	0.1245	0.46	0.04- 5.38
1-30d	14 (11)	5 (36)		0.21	0.05- 0.98
>= 31d	106 (85)	74 (70)		Ref	ref
Dry					
Yes	4 (3)	2 (50)	0.6449	0.6	0.05- 6.22
No	150 (97)	98 (65)		Ref	ref
Breed					
Crossbreed	30 (19)	12 (40)	0.0204	0.28	0.1- 0.82
Jersey	19 (12)	16 (84)		2.45	0.55- 10.94
Holstein	105 (68)	72 (69)		Ref	ref
Herd type					
Closed	11 (7)	9 (82)	0.3735	2.2	0.38- 13.10
Open	143 (93)	91 (64)		Ref	ref
Calves-Replacements proportion					
<5.1	19 (12)	16 (84)	0.2036	2.9	0.63- 13.58
>48.4	36 (23)	20 (56)		0.6	0.22- 1.78
From 46.9 to 48.3	99 (64)	64 (65)		Ref	ref
Proportion herd lactating					
>50	19 (12)	16 (84)	0.1176	3.3	0.74- 14.60
<31.7% to 49%	135 (88)	84 (62)		Ref	ref
Proportion herd dry					
1.7- 4%	72 (47)	42 (58)	0.1831	0.6	0.25- 1.58
>7.6%	19 (12)	16 (84)		2.6	0.53- 12.73
6.2- 7.5%	63 (41)	42 (67)		Ref	ref
Herd Size					
>1000	58 (38)	41 (71)	0.2988	1.6	0.65- 3.92
<1000	96 (62)	59 (61)		Ref	ref

Table 4.1 (cont'd)

Adding Cow/Replacements					
0%	11 (7)	9 (82)	0.3735	2.2	0.38- 13.10
At least 5 animals	143 (93)	91 (64)		Ref	ref
Adding Bulls					
0%	124 (81)	88 (71)	0.0099	4.0	1.41- 11.52
4 animals	30 (19)	12 (40)		Ref	ref
Culling Rate					
Low level	49 (32)	28 (57)	0.2842	0.6	0.25- 1.51
High level	105 (68)	72 (69)		Ref	ref
N° Milkings					
3- 4 times	58 (38)	41 (71)	0.2988	1.6	0.65- 3.92
2-3 times	96 (62)	59 (61)		Ref	ref
Loose Housing					
No	135 (88)	84 (62)	0.1176	0.3	0.07- 1.36
Yes	19 (12)	16 (84)		Ref	ref
Tie stanchion					
No	143 (93)	91 (64)	0.3735	0.5	0.08- 2.65
Yes	11 (7)	9 (82)		Ref	ref
Access pasture/dry lot					
No	99 (64)	62 (63)	0.5508	0.8	0.31- 1.87
Yes	55 (36)	38 (69)		Ref	ref
Lactation access pasture					
No	99 (64)	62 (63)	0.5508	0.8	0.31- 1.87
Yes	55 (36)	38 (69)		Ref	ref
Transition pen separate					
No	66 (75)	34 (51)	0.0109	0.3	0.14- 0.77
Yes	88 (57)	66 (43)		Ref	ref
Sick animals penned separated					
Yes	77 (50)	57 (74)	0.0409	2.5	1.04- 5.85
No	77 (50)	43 (56)		ref	ref

Table 4.1 (cont'd)

1st lactations animals penned separated					
Yes	58 (38)	41 (71)	0.2988	1.6	0.65- 3.92
No	96 (62)	59 (61)		Ref	ref
Cow/Heifers Raised					
Off-site/ On main farm	135 (88)	84 (62)	0.1176	0.3	0.07- 1.36
Another farm	19 (12)	16 (84)		Ref	ref
Feeders clean Year					
<365	66 (43)	34 (52)	0.0109	0.3	0.14- 0.77
365	88 (57)	66 (75)		Ref	ref
Washed					
No	107 (69)	69 (64)	0.9848	1	0.40- 2.56
Yes	47 (31)	31 (66)		Ref	ref
Spray					
No	143 (93)	91 (64)	0.3735	0.5	0.08- 2.65
Yes	11 (7.14)	9 (82		Ref	ref
Lime					
No	99 (64)	64 (65)	0.9514	1	0.40- 2.39
Yes	55 (36)	36 (65)		Ref	ref
Tx Respiratory Disease					
Yes	118 (77)	78 (66)	0.5592	1.6	0.49- 3.74
No	36 (23)	22 (61)		Ref	ref
Tx Foot Infection Disease					
Yes	135 (88)	84 (62)	0.1176	0.3	0.07- 1.36
No	19 (12)	16 (84)		Ref	ref
Tx Metritis					
Yes	135 (88)	84 (62)	0.1176	0.3	0.07- 1.36
No	19 (12)	16 (84)		Ref	ref
Feed TMR					
No	42 (30)	21 (32)	0.5508	1.3	0.53- 3.22
Yes	141 (68)	66 (32)		Ref	ref

Table 4.1 (cont'd)

% Corn silage Diet					
No	55 (36)	38 (69)	0.5508	1.3	0.53- 3.22
Yes	99 (64)	62 (63)		Ref	ref
% Distillers grains Diet					
No	108 (70)	73 (68)	0.4323	1.4	0.57- 3.64
Yes	46 (30)	27 (59)		Ref	ref
% Cottonseed Diet					
No	118 (77)	80 (68)	0.2350	1.8	0.67- 5.08
Yes	36 (23)	20 (56)		Ref	ref
Contact with Other SP					
Horses	19 (12)	16 (84)	0.1176	3.3	0.7- 14.6
None	135 (88)	84 (62)		Ref	ref
Contact with Cats					
No	30 (19)	12 (40)	0.0099	0.2	0.09- 0.71
Yes	124 (81)	88 (71)		Ref	ref
Contact with Deer					
No	36 (23)	20 (56)	0.2350	0.5	0.20- 1.50
Yes	118 (77)	80 (68)		Ref	ref
Contact with Dogs					
No	94 (61)	63 (67)	0.5945	1.3	0.53- 3.01
Yes	60 (39)	37 (62)		Ref	ref
Contact with Opossum					
No	30 (19)	12 (40)	0.0099	0.2	0.09- 0.71
Yes	124 (81)	88 (71)		Ref	ref
Contact with Raccoons					
Always	72 (47)	42 (58)	0.2954	0.3	0.05- 2.16
Frequent	71 (46)	49 (69)		0.6	0.10- 3.9
Rarely	11 (7)	9 (82)		ref	ref

Table 4.1 (cont'd)

Contact with Rodents					
Always	72 (47)	42 (58)	0.2954	0.3	0.05- 2.16
Frequent	71 (46)	49 (69)		0.6	0.10- 3.9
Rarely	11 (7)	9 (82)		Ref	ref
Contact with Skunks					
No	30 (19)	12 (40)	0.0099	0.2	0.09- 0.71
Yes	124 (81)	88 (71)		ref	ref
Area Cleanliness					
Cleanest third	105 (68)	72(69)	0.2842	1.6	0.66- 4.02
Middle third	49 (32)	28 (57)		ref	ref
Bed Cleanliness					
Cleanest third	105 (68)	72(69)	0.2842	1.6	0.66- 4.02
Middle third	49 (32)	28 (57)		ref	ref
Rumensin					
Yes	58 (38)	41 (71)	0.2988	1.6	0.65- 3.92
No	96 (62)	59 (61)		ref	ref
Direct Fed Microbials					
Yes	60 (39)	37 (62)	0.5945	0.8	0.33- 1.89
No	94 (61)	63 (67)		ref	ref
Anthelmintic					
Yes	82 (53)	58 (71)	0.1383	1.9	0.81- 4.60
No	72 (47)	42 (58)		ref	ref
BLV					
Positive	77 (50)	48 (62)	0.5565	0.8	0.33- 1.83
Negative	77 (50)	52 (68)		ref	ref

Table 4.2. Univariate analysis to identify risk factors for rate of new STEC infections in dairy cattle

Characteristic	**No. (%) with characteristic**	**No. (%) with STEC**	**p-value**	**OR**	**95% CI**
Season					
Spring	12 (17)	9 (75)	0.2966	2	0.55- 7.04
Summer	57 (83)	25 (44)		ref	ref
Temperature Max					
≤ 27.8 C	47 (68)	17 (36)	0.1812	0.5	0.17- 1.41
> 27.8 C	22 (32)	17 (77)		ref	ref
Temperature Aver5 days					
≤ 19.4 C	25 (36)	6 (24)	0.0851	0.4	0.14- 1.14
> 19.4 C	44 (64)	28 (64)		ref	ref
Temperature Max5 days					
≤ 28.9 C	32 (46)	16(50)	0.8523	1.1	0.35- 3.55
> 28.9 C	37 (54)	18(49)		ref	ref
Temperature Min5 days					
≤15 C	20 (29)	7 (35)	0.4852	0.6	0.19- 2.23
>15 C	49 (71)	27 (55)		ref	ref
Mean temperature longitudinal					
≤18.9 C	10 (14)	2 (20)	0.2088	0.3	0.06- 1.89
>18.9 C	59 (86)	32 (54)		ref	ref
Range temperature longitudinal					
≤10	47 (68)	23 (49)	0.9935	1	0.29- 3.48
>10	22 (32)	11 (50)		ref	ref
Temperature 2nd sampling					
≤ 20 C	45 (65)	16 (36)	0.1051	0.5	0.19- 1.18
>20 C	24 (35)	18 (75)		ref	ref
Temperature 3rd sampling					
≤18.9 C	46 (67)	29 (63)	0.0552	2.9	1- 8.46
>18.9 C	23 (33)	5 (22)		ref	

Table 4.2 (cont'd)					ref
Temperature 4th sampling					
≤18.3 C	38 (55)	13 (34)	0.0961	0.5	0.20- 1.14
>18.3 C	31 (45)	21 (68)		ref	ref
Lactation					
1st	21 (30)	12 (57)	0.2612	1.6	0.69- 3.91
2nd or higher	48 (70)	22 (46)		ref	ref
Days in milk					
0	3 (5)	1 (33)	0.3901	1.6	0.14- 19.13
1-30d	6 (10)	5 (83)		2.2	0.66- 7.55
>= 31d	50 (85)	21 (42)		ref	ref
Dry					
No	66 (96)	33 (50)	0.7470	1.5	0.13- 17.10
Yes	3 (4)	1 (33)		ref	ref
Breed					
Crossbreed	10 (14.5)	8 (80)	0.3220	4.4	0.63- 31.37
Holstein	49 (71)	24 (49)		2.8	0.49- 15.51
Jersey	10 (14.5)	2 (20)		ref	ref
Herd type					
Closed	10 (14)	5 (50)	0.7885	1.2	0.25- 6.26
Open	59 (86)	29 (49)		ref	ref
Calves-Replacements proportion					
<5.1	10 (15)	2 (20)	0.2720	0.4	0.07- 2.03
>48.4	12 (17)	9 (75)		1.7	0.56- 4.97
From 46.9 to 48.3	47 (68)	23 (49)		ref	ref
Proportion herd lactating					
>50	10 (14)	2 (20)	0.2088	0.3	0.06- 1.89
<31.7% to 49%	59 (86)	32 (54)		ref	ref
Proportion herd dry					
1.7- 4%	24 (35)	18 (75)	0.1448	1.8	0.77- 4.27
>7.6%	10 (14)	2 (20)		0.4	0.09- 2.32
6.2- 7.5%	35 (51)	14 (40)		ref	ref

Table 4.2 (cont'd)

Herd Size					
>1000	27 (39)	10 (37)	0.5098	0.7	0.19- 2.28
<1000	42 (61)	24 (57)		ref	ref
Adding Cow/Replacements					
0%	10 (14)	5 (50)	0.7885	1.2	0.25- 6.26
At least 5 animals	59 (86)	29 (49)		ref	ref
Adding Bulls					
0%	59 (86)	26 (44)	0.3651	0.5	0.13- 2.15
4 animals	10 (14)	8 (80)		ref	ref
Culling Rate					
Low level	20 (29)	10 (50)	0.9028	0.9	0.26- 3.24
High level	49 (71)	24 (49)		ref	ref
N° Milkings					
3- 4 times	27 (39)	10 (37)	0.5098	0.7	0.19- 2.28
2-3 times	42 (61)	24 (57)		ref	ref
Loose Housing					
No	59 (86)	32 (54)	0.2088	3.04	0.53- 17.46
Yes	10 (14)	2 (20)		ref	ref
Tie stanchion					
No	59 (86)	29 (49)	0.7885	0.8	0.16- 4.05
Yes	10 (14)	5 (50)		ref	ref
Access pasture/dry lot					
No	47 (68)	23 (49)	0.8022	1.2	0.34- 4.00
Yes	22 (32)	11 (50)		ref	ref
Lactation access pasture					
No	47 (68)	23 (49)	0.8022	1.2	0.34- 4.00
Yes	22 (32)	11 (50)		ref	ref
Transition pen separate					
No	22 (32)	17 (77)	0.1812	2	0.71- 5.88
Yes	47 (68)	17 (36)		ref	ref

Table 4.2 (cont'd)

Sick animals penned separated					
Yes	37 (54)	12 (32)	0.1472	0.5	0.16- 1.33
No	32 (46)	22 (69)		ref	ref
1st lactations animals penned separated					
Yes	27 (39)	10 (37)	0.5098	0.7	0.19- 2.28
No	42 (61)	24 (57)		ref	ref
Cow/Heifers Raised					
Off-site/ On main farm	59 (86)	32 (54)	0.2088	3	0.53- 17.46
Another farm	10 (14)	2 (20)		ref	ref
Feeders clean Year					
<365	22 (32)	17 (77)	0.1812	2	0.71- 5.88
365	47 (68)	17 (36)		ref	ref
Washed					
No	47 (68)	20 (43)	0.4365	0.6	0.19- 2.06
Yes	22 (32)	14 (64)		ref	ref
Spray					
No	59 (86)	29 (49)	0.7885	0.8	0.16- 4.05
Yes	10 (14)	5 (50)		ref	ref
Lime					
No	47 (68)	23 (49)	0.9935	1	0.29- 3.45
Yes	22 (32)	11 (50)		ref	ref
Tx Respiratory Disease					
Yes	57 (83)	25 (44)	0.4841	0.6	0.15- 2.47
No	12 (17)	9 (75)		ref	ref
Tx Foot Infection Disease					
Yes	59 (86)	32 (54)	0.2088	3	0.53- 17.46
No	10 (14)	2 (20)		ref	ref
Tx Metritis					
Yes	59 (86)	32 (54)	0.2088	3	0.53- 17.46
No	10 (14)	2 (20)		ref	ref

Table 4.2 (cont'd)

Feed TMR					
No	22 (32)	11 (50)	0.8022	0.9	0.25- 2.94
Yes	47 (68)	23 (49)		ref	ref
% Corn silage Diet					
No	22 (32)	11 (50)	0.8022	0.9	0.25- 2.94
Yes	47 (68)	23 (49)		ref	ref
% Distillers grains Diet					
No	51 (74)	25 (49)	0.7095	1.3	0.35- 4.71
Yes	18 (26)	9 (50)		ref	ref
% Cottonseed Diet					
No	57 (83)	25 (44)	0.2966	0.5	0.14- 1.83
Yes	12 (17)	9 (75)		ref	ref
Contact with other species					
Horses	10 (14)	2 (20)	0.2088	0.3	0.06- 1.89
None	59 (86)	32 (54)		ref	ref
Contact with Cats					
No	10 (14)	8 (80)	0.3651	1.9	0.47- 7.76
Yes	59 (86)	26 (44)		ref	ref
Contact with Deer					
No	12 (17)	9 (75)	0.2966	2	0.55- 7.04
Yes	57 (83)	25 (44)		ref	ref
Contact with Dogs					
No	39 (57)	19 (49)	0.9355	1	0.30- 3.07
Yes	30 (43)	15 (50)		ref	ref
Contact with Opossum					
No	10 (14)	8 (80)	0.3651	1.9	0.47- 7.76
Yes	59 (86)	26 (44)		ref	ref
Contact with Raccoons					
Always	24 (35)	18 (75)	0.1508	1.3	0.38- 4.58
Frequent	35 (51)	11 (31)		0.5	0.15- 1.95
Rarely	10 (14)	5 (50)		ref	ref

Table 4.2 (cont'd)

Contact with Rodents					
Always	24 (35)	18 (75)	0.1508	1.3	0.38- 4.58
Frequent	35 (51)	11 (31)		0.5	0.15- 1.95
Rarely	10 (14)	5 (50)		ref	ref
Contact with Skunks					
No	10 (14)	8 (80)	0.3651	1.9	0.47- 7.76
Yes	59 (86)	26 (44)		ref	ref
Area Cleanliness					
Cleanest third	49 (71)	24 (49)	0.9028	1.1	0.31- 3.78
Middle third	20 (29)	10 (50)		ref	ref
Rumensin					
Yes	27 (39)	10 (37)	0.5098	0.7	0.19- 2.28
No	42 (61)	24 (57)		ref	ref
Anthelmintic					
Yes	45 (65)	16 (36)	0.1051	0.5	0.19- 1.18
No	24 (35)	18 (75)		ref	ref
BLV					
Positive	35 (51)	18 (51)	0.8567	0.9	0.42- 2.07
Negative	34 (49)	16 (47)		ref	Ref
Flies control					
No	22 (32)	17 (77)	0.0768	2.3	0.9- 5.7
Yes	47 (68)	17 (36)		ref	Ref

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CONCLUSIONS AND FUTURE STUDIES

Conclusions

We performed a group of hypothesis-testing epidemiological studies with the aim of elucidating risk factors for STEC shedding and a better understanding of STEC dynamics to generate knowledge that could lead to the design of intervention strategies to control STEC infection in cattle and ultimately to reduce transmission to humans.

From our study results, it is evident that the intermittent shedding pattern of STEC poses a challenge to accurately identify STEC shedding in cattle. Additionally, the intermittent shedding poses a challenge in understanding the dynamics of STEC shedding especially when considering the multiple of STEC serotypes that can be present in cattle. For these reasons, longitudinal studies will most likely provide the most accurate information when trying to further elucidate epidemiology of STEC in cattle.

Our results support the previously reported association between STEC shedding and seasonality, more specifically with warm temperatures. Those cattle exposed to warmer temperatures presented higher risk for STEC shedding. Warm temperatures could not only favor STEC shedding by cattle but as well favor the survival of STEC in the environment. This could increase STEC infection and shedding in other reservoirs, as a consequence favoring the transmission and infection to other cattle farms, animal species and crops.

Our results also support previously reported associations between STEC shedding and age, more specifically that younger cattle have a higher risk of STEC shedding. This association is also supported by our finding that cows in their first lactation are at higher risk of STEC

shedding compare with cows with two lactations or more, in other words, older cows. As a consequence, younger cattle could be a target population for intervention strategies to reduce STEC colonization and shedding.

Another population that our results support as target for intervention strategies in dairy herds is cows in their first 30 days of milk production. Cattle early in lactation are under stress and usually in a negative energy balance, which may increase susceptibility to STEC shedding. Thus this specific group of cows can be also targeted for the implementation of intervention strategies at pre-harvest aim to reduce STEC infection in humans.

All the farms that we sampled were STEC positive although the prevalence and rate of STEC new infections varied between herds. As STEC is considered part of the normal gastrointestinal flora in cattle, the high farm prevalence is not surprising. The fact that it is highly likely that STEC are present in all cattle farms emphasizes the importance of designing pre-harvest interventions strategies to reduce colonization and shedding.

We not only isolated STEC from all farms but found differences in STEC shedding dynamics by the type of production system. Beef cattle had a higher risk of STEC shedding than dairy cattle. In addition, we found a diverse STEC population between and within herds, as well as by production type. We conclude that herd, and within this term we could refer to management practices, are associated with STEC dynamics, although we were not able to identify which specific practices or factors in a herd are those associated.

Different *stx* and *eaeA* genes profiles were observed between herds. The gene *stx2* was the most frequent identified among the isolates we recovered. We also found that all the herds had a least one EHEC isolate. This is an important observation from the public health perspective, as EHEC is the most common cause of outbreaks and hospitalizations.

The differences between dairy and beef herds in management practices, such as diet, age and even in genetics may influence STEC dynamics in a way that different STEC O-types/strains can be more suitable to colonize, multiply and be shed in one production system than in the other. These factors could also influence the type of virulence factors that STEC bacteria can acquire or lose inside the gastrointestinal tract and influence its evolution.

Although we did not find a significant association between STEC shedding in cattle and exposure to pasture, we did observe differences in STEC shedding, rate of new infections and number of cattle negative to STEC in those herds that provide access to pasture to their cattle. It could be that pasture-based systems provide the right mix of environmental factors for STEC to survive in the environment as well as colonized and multiply in cattle's digestive tract. These findings deserve further investigation as pasture based systems are often advocated as having attributes that make them more appealing to the public, as they are consider more environmental friendly and better for cattle welfare.

We found in our study that BLV and MAP, chronic bovine diseases that have significant long-term effects on the immune system (BLV) and the gastrointestinal tract (MAP), were not associated with STEC shedding. Thus controlling BLV and MAP will not likely have an impact on STEC shedding in cattle. However controlling both BLV and MAP is still important for overall herd health and productivity.

The body of information about STEC is composed mostly by studies that focus in *E. coli* O157:H7. For this reason, this dissertation offers information about all types of STEC and not only about *E. coli* O157:H7. It is necessary to produce more information about non-O157 STEC as they are more frequently identified as a cause of outbreaks thanks to the improvement in detection and isolation techniques.

Future studies

The studies presented here were part of a large on-going project. There are still many unanswered questions that potentially could be investigated with the information and *E. coli* isolates gathered in this study. For example, further strain classification by O-typing or other molecular techniques could allow us to possibly identify risk factors for specific strains. Also we could possibly identify differences in STEC dynamics (rate loss, rate acquisition) between different strains. Quantification (enumeration) of bacteria in the samples collected from cattle could allow for better characterization of shedding dynamics and risk factors associated with STEC shedding.

By more precisely characterizing the STEC strains isolated in this study, comparative studies with isolates from human STEC cases in Michigan could be done. This comparison would help to determine if Michigan's cattle shed the same STEC strains that caused human cases. In addition, this is the first study of STEC in cattle in Michigan, so it will be interesting to determine which virulence factors are present in the STEC isolates we collected and determine if there is any new virulence factor.

One of the questions that surfaced from results from our study was the differences in STEC dynamics observed between pasture-based systems and more conventional confinement-based management systems. Future studies should be performed to explore if any these two production systems favor STEC shedding.

The ultimate goal was to identify potential targets for intervention. From our study, we identified two groups of dairy cattle that are at higher risk for STEC shedding; first lactation heifers and cows in the first 30 days of their lactation. It would be valuable to use intervention

tools, such as vaccines or probiotics, in these two populations of dairy cattle as a targeted intervention strategy. It would be also valuable to implement other management practices to decrease stress in early lactation as this could potentially reduce STEC shedding. This is an attractive possibility as it focuses on the highest risk populations and thus utilizes valuable resources most effectively.