IDENTIFICATION OF CONTAMINATION SOURCES AND OCCURRENCE OF SHIGATOXIGENIC *E. COLI* ON SMALL-SCALE COW/CALF OPERATIONS IN OKLAHOMA AND LOUISIANA

By

BUDDHINI PIYUMIKA KUMARI JAYASUNDERA

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Oklahoma State University

Stillwater, Oklahoma

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Thesis Approved:

Dr. Divya Jaroni

Thesis Adviser

Dr. Peter Muriana

Dr. Li Maria Ma

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Abstract: *Escherichia coli* O157:H7 and the big six non-O157 *E. coli* serogroups (O26, O45, O103, O111, O121, and O145) have been declared adulterants in meat. Cattle and other ruminants are common reservoirs of these shigatoxigenic *E. coli* (STEC). However, little is known about the prevalence of STEC in beef cattle, especially on cow-calf operations. Foodborne outbreaks involving STEC have been repeatedly traced back to farms, indicating the need to understand the prevalence of these microorganisms at the pre-harvest level. In this study, fecal, water, sediment, and equipment swab samples were collected from several cow-calf operations in Oklahoma and Louisiana to determine the occurrence of *E. coli* O157:H7 and the big six non-O157 *E. coli* as well as identify potential on-farm contamination sources. Positive samples were screened for the presence of *stx* and *eae* genes for confirmation as STEC. Results from the study indicate a 4.4% and 21.4% positive occurrence of *E. coli* O157:H7 in Oklahoma and Louisiana, respectively and a 14% occurrence of non-O157 STEC in both states. The serogroups O26, O45, and O103 were the most prevalent in both states. In Louisiana, the use of municipal water significantly increased prevalence of non-O157 in comparison to the use of well water as a water source ($P<0.05$). A combination of water sources, predominantly streams and runoff, were used on the Oklahoma farms, which significantly increased ($P<0.05$) the prevalence of *E. coli* O157:H7 and non-O157 serogroups. Additionally, results indicate that other factors such as type of feed, animal density, and the frequency of cleaning particular common cattle contact areas like trailers, chutes, and alleyways, may serve as potential contamination sources in the farm environment.

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CHAPTER 1

INTRODUCTION

The shigatoxigenic *Escherichia coli* (STEC) are a group of common foodborne pathogens that cause a wide spectrum of disease, ranging from diarrhea, hemorrhagic colitis (HC) and to more life threatening manifestations such as hemolytic uremic syndrome (HUS) and sometimes, even death. According to the latest Centers for Disease Control and Prevention (CDC) estimates, STEC may cause about 265, 000 foodborne illnesses in the United States (US) every year (CDC, 2014). Of this group of pathogens, *E. coli* O157:H7 is probably the best known. Due to the severity of illnesses that may result from *E. coli* O157:H7 infections, the USDA-FSIS declared it an adulterant in ground beef in 1994. Recently, non-O157 STEC serotypes have also been implicated in foodborne illnesses associated with consumption of meat (CDC, 2012a, 2012b). Non-O157 STEC infections do not usually occur with the same severity as *E. coli* O157:H7 infections (Johnson et al., 2006), however, because the non-O157 STEC share many similarities with *E. coli* O157:H7 in virulence properties, their potential for causing similar disease as *E. coli* O157:H7 cannot be overlooked. As a result, six non-O157

STEC serogroups (O26, O45, O103, O111, O121, and O145) were also declared adulterants in ground beef in 2012. Therefore, undertaking measures to lower the pathogen load from entering the food chain has become a prerogative.

Cattle have been identified as the primary reservoirs for STEC (Elder et al., 2000; Smith et al., 2001). There is also considerable evidence that on-farm practices may affect pathogen loads on cattle that enter slaughter facilities, resulting in cross contamination at the post-harvest level (Elder et al., 2000). However, the pathogenic mechanisms of *E. coli* O157:H7 and other STEC, as well as on-farm reservoirs that are responsible for colonization in cattle are poorly understood. Reservoirs that may serve as potential contamination sources have been identified in the feedlot areas. These include: feces (0.8%), feed bunks (1.7%), water troughs (12%), and incoming water supplies (4.5%) (Van Donkersgoed et al., 2001; Sargeant et al., 2003; Dodd et al., 2003).

Studies so far have been able to show that fecal shedding of STEC by cattle may be seasonal, demonstrating a peak in prevalence during the summer months (Chapman et al. 1997; Hancock et al., 1997; Van Donkersgoed et al., 1999; Elder et al., 2000; Smith et al., 2001). The diet of cattle may also influence fecal shedding of *E. coli* O157:H7 as evidenced in several studies (Buchko et al., 2000; Tkalcic et al., 2000). Additionally, contaminated water sources and equipment may also contribute to the dissemination and persistence of *E. coli* O157:H7 on the farm environment. Cattle water troughs are known to harbor *E. coli* O157:H7 for extended periods of time (Hancock et al., 1998; Murinda et al., 2004; Polifroni et al., 2012; LeJeune et al, 2001). Studies have also been conducted on various breeds of cattle in order to determine whether particular breeds of cattle were more susceptible to heat stress than others (Brown-Brandl et al., 2006a, 2006b), and

results from these studies have shown that heat stress, being a type of physical stress to the host animal (Rostagno, 2009), may have an effect on the shedding patterns of gastrointestinal pathogenic microbes such as *E. coli* O157:H7 and *Salmonella spp*. (Brown-Brandl et al., 2006; Edrington et al., 2004).

However, most studies to date have concentrated on beef feedlots and large ranches (Laegrid et al., 1999). In the case of cow/calf operations, which are the points of origin of beef products, limited information on the impact of production practices on prevalence of STEC exists. It is therefore important to understand the factors that affect *E. coli* O157:H7 burden in these cow/calf operations as it can be a critical path in the farm-to-fork continuum. This can, in the long run, help with the development of risk management strategies and mitigation of the pathogens in the environment.

In this study, the various management practices in place on small-scale cow/calf operations in the states of Oklahoma and Louisiana were identified. The prevalence of these STEC on these farms and their association to farm management practices were then analyzed in order to determine potential on-farm contamination sources. Identification of such contamination sources may aid in the mitigation of these pathogens at the pre-harvest level.

CHAPTER II

REVIEW OF LITERATURE

A. *Escherichia coli*

Escherichia coli is a rod shaped facultative anaerobic bacterium that is commonly found in the mammalian gastrointestinal tract (Drasar and Barrow, 1985). The microorganism was first described in 1885 by Dr. Theodor Escherich as a result of his investigations of children's feces from cases of infantile diarrhea (Escherich, 1885). Transmission and persistence of this bacterium in the mammalian population is largely due to its fecal-oral lifestyle. Up to 1% of the mammalian gastrointestinal microbial population can be composed of *E. coli*, and as a result, this bacterium is widely used as an indicator of fecal contamination in water supplies (Waghela, 2004; Winfield and Groisman, 2003). The majority of *E. coli* strains are commensal (Drasar and Barrow, 1985), and some strains are known to have beneficial effects to humans. Such benefits include playing a role in the synthesis of vitamin K_2 (Bentley and Meganathan, 1982) and preventing the colonization of pathogenic bacteria in the gut (Reid et al, 2002). However, some *E. coli* strains may become pathogenic to humans, and are harbored within

food animals (Drasar and Barrow, 1985; Waghela, 2004).

1. Pathogenic *Escherichia coli*

Pathogenic *E. coli* differ from nonpathogenic strains in that they possess an array of virulence genes whose products work together to confer pathogenic properties to the organism. These virulence genes may be located either chromosomally or extrachromosomally (Waghela, 2004). Proteins encoded by these virulence genes are involved in cellular adherence, toxin activity, and cellular invasion. It is widely accepted that nonpathogenic *E. coli* may have acquired the genes that encode these proteins as a result of genetic transfer from related enterobacteria (Waghela, 2004). Strains are differentiated based on their somatic lipopolysaccharide (O) antigens, flagellar (H) antigens, and capsular (K) antigens (Kauffmann, 1947). There are nearly 700 different antigenic types based on the different O, H, and K antigens (Robins-Browne and Hartland, 2002). *Escherichia coli* generally cause three types of infections, namely: enteric infections, urinary tract infections, and septicemic infections (Waghela, 2004). The pathogenic *E. coli* that cause enteric infections are also referred to as diarrheagenic *E. coli*, and are classified into six main pathotypes based on their pathogenic characteristics, epidemiology, clinical features, and distinct serological characteristics. The six pathotypes include: enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), diffuse-adherence *E. coli* (DAEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E.* coli (EPEC), and enterohaemorrhagic *E. coli* (EHEC) (Levine, 1987). Additionally, a new group of isolates have been classified as necrotoxigenic *E. coli* (NTEC) (Waghela, 2004).

a. Enterotoxigenic E. coli

Enterotoxigenic *E. coli* are known to cause a cholera-like syndrome where acute diarrhea has been observed in humans as well as in animals (Waghela, 2004). Enterotoxigenic *E. coli* infections are common in developing countries, and may have an impact on the morbidity and mortality of young children in these regions of the world (Waghela, 2004). In developed countries like the United States (US), this syndrome is known as traveler's diarrhea, and is often associated with recent travel to developing countries and consumption of contaminated food and water from these regions (Waghela, 2004). Colonizing factors (CF) enable ETEC to attach to the surface of intestinal epithelial cells, multiply, and produce either a heat-stable toxin (ST) or a heat-labile toxin (LT) or both (Mol and Oudega, 1996; Gaastra and Svennerholm, 1996). Both the structure and mode of action of the LT toxin bear similarities to the cholera toxin (Tauschek et al, 2002; Sears and Kaper, 1996).

b. Enteroaggregative E. coli

The EAEC attach to intestinal epithelial cells in an aggregative pattern that resembles stacked bricks, and are accompanied by the presence of a thick mucus on the epithelium once this aggregative adherence is formed (Tzipori et al, 1992). This thick mucus may play an important role in the persistence of infection (Tzipori et al, 1992). Initial attachment to the intestinal epithelium may be brought about with the help of factors such as aggregative adherence fimbriae types I, II, and III, a class of adhesins (Okeke and Nataro, 2001; Bernier et al, 2002). Most EAEC strains produce three toxins that may stimulate intestinal secretion (Sears and Kaper, 1996). Enteroaggregative *E. coli*

are generally associated with watery diarrhea in young children, especially in regions with poor hygiene (Okeke and Nataro, 2001). Unlike with ETEC, however, bloody diarrhea may result (Waghela, 2004) and may also result in growth retardation in infants (Steiner et al, 1998; Nataro and Kaper, 1998).

c. Diffuse-adherence E. coli

Diffuse-adherence *E. coli* may cause disease in malnourished children and immunocompromised individuals and may be regarded as an important cause of chronic diarrhea (Nataro and Kaper, 1998). The diffuse-adherence *E. coli* entirely cover the epithelial cell surface, and this pattern of adherence is what gave rise to its namesake (Scaletsky et al, 1984).

d. Enteroinvasive E. coli

The EIEC exhibit an invasive nature and pathogenesis that is very similar to the pathogenicity of *Shigella*, producing an illness that resembles dysentery, which can be fatal in young children (Dupont et al, 1971). The clinical syndrome may present as vomiting, fever, and watery diarrhea, where watery diarrhea may also develop into mucoid and bloody stools in some cases (Waghela, 2004).

e. Enteropathogenic E. coli

This group of pathogenic *E. coli* cause watery diarrhea that is characterized by the presence of attaching and effacing (A/E) lesions on the intestinal epithelium (Moon et al., 1983). This group of *E. coli* form microcolonies in localized regions in the intestinal epithelium by coming into close contact with the enterocyte surface and resulting in the

loss of microvilli (Waghela, 2004; Moon et al., 1983). Following attachment, EPEC may translocate either into or onto the enterocyte several proteins involved in signal transduction for the formation of A/E lesions via a Type II Secretion System (TTS) (Jarvis et al., 1995; Frankel et al., 1998). The genes for these proteins are located as a gene cluster known as the locus of enterocyte effacement (LEE) that occurs on a 35kb chromosomal pathogenicity island (Kenny, 2002). The LEE contains about 41 genes and it is presumed that EPEC may have acquired them as a result of horizontal gene transfer (Deng et al., 2001).

f. Enterohemorrhagic E. coli

Enterohemorrhagic *E. coli* are the causative agents of diarrhea and hemorrhagic colitis which may progress to severe life threatening complications involving acute renal failure and central nervous system damage (Tesh, 2004). This group of *E. coli* produces cytotoxins, and is capable of attaching to and changing the cellular morphology of enterocytes (Tesh, 2004)

The EHEC fall under the STEC grouping, and of this group, *E. coli* O157:H7 is the best known and studied. Approximately 75% of *E. coli* O157:H7 outbreaks have been linked to bovine-derived products with ground beef being the most implicated food source for *E. coli* O157:H7 outbreaks (Vugia et al, 2007; USDA-APHIS, 1997). *Escherichia coli* O157:H7 was declared an adulterant in ground beef in 1994 following the Jack-in-the-Box outbreak that killed four individuals. However, in addition to *E. coli* O157:H7, six STEC serogroups have gained prominence as emerging foodborne pathogens. This group of STEC, known as the ‘big six’, includes the O26, O45, O103,

O111, O121 and O145 serogroups. The big six non-O157 serogroups were declared adulterants in meat by the USDA-FSIS in June 2012 (USDA-FSIS, 2011).

The EHEC produce a variety of potent toxins that cause a severe form of disease known as hemorrhagic colitis (HC). An estimated ten percent of patients with HC may go on to develop a complication known as hemolytic uremic syndrome (HUS), a life-threatening condition that is characterized by renal failure, thrombocytopenia, and hemolytic anemia. These sequelae are generally more serious in the elderly and younger individuals, mostly because of the compromised or underdeveloped immune system of these categories of individuals. Hemorrhagic colitis and HUS are characteristic complications of *E. coli* O157:H7 infections and, in 1982, *E. coli* O157:H7 was recognized as a human pathogen. Since then, there has been a steady increase in *E. coli* O157:H7 associated foodborne illnesses worldwide (CDC, 2014).

By definition, the STEC carry one or both shiga toxin genes – shiga toxin 1 and shiga toxin 2 (designated *stx*1 and *stx*2 respectively). Most *E. coli* O157 isolates produce *Stx*2 only, while isolates producing *Stx*1 only are rare (Griffin and Tauxe, 1991). *Stx*1 and *Stx*2 producing isolates have been found occasionally, but with more frequency than isolates producing *Stx*1 only (Griffin and Tauxe, 1991).

The genes that encode for shiga toxins are encoded on bacteriophages (Scotland et al., 1983). This suggests that *E. coli* of any serotype is able to acquire these toxin genes. However, current opinion suggests that the ability of an organism to produce the toxins alone is not the only deciding factor when it comes to the organism being able to produce disease (Tarr and Neill, 1996). Appropriate transmission and colonization factors may

also play a role in enabling the organism to cause disease. The acquisition of the toxin genes, therefore, is only likely to confer pathogenicity upon an organism as long as that organism contains a background of appropriate complementary virulence factors (Tarr and Neill, 1996).

2. Microbiology

a. Escherichia coli O157:H7

Of all the STEC serotypes, *E. coli* O157 has been the best studied. The shiga toxin-producing strains of this serogroup are clonal in origin and therefore share many similarities both phenotypically and genotypically. The main toxin-producing serotype isolated from this group is *E. coli* O157:H7, although non motile variants (H-) have also been occasionally isolated. The biochemical reactions of *E. coli* O157 are very similar to that of other *E. coli* serotypes with a few important exceptions. *E. coli* O157:H7 isolates typically do not ferment sorbitol within 24 hrs. They also do not produce β–glucoronidase. These characteristics are usually exploited in differential media used for their isolation. For example, selective and differential media such as sorbitol MacConkey agar (SMAC), Rainbow® Agar O157, and R&F® *E. coli* agar are currently used by the United States Food and Drug Administration (FDA) when testing for the pathogen in food (FDA, 2011).

b. Non-O157 STEC

While *E. coli* O157:H7 has been associated with bovine-product related outbreaks, over the years other non-O157 STEC serotypes have recently been implicated in disease (CDC, 2012a, 2012b). There are more than a hundred serotypes of *E. coli* that

are capable of producing shiga toxins (Nataro and Kaper, 1998). An estimated 20-50% of STEC infections are caused by non-O157 serogroups, which amounts to about 37, 000 annual cases of illness in the US (Brooks et al., 2005; Johnson et al., 2006). Out of the STEC, the big six group of non-O157 STEC have been identified as major emerging pathogens due to their frequent association with hemolytic colitis (HC) and hemorrhagic uremic syndrome (HUS) (Brooks et al., 2005; Johnson et al., 2006). This group of non-O157 STEC was declared adulterants of meat in June 2012 by the United States Department of Agriculture (USDA-FSIS, 2011).

Of the pathogenic non-O157 STEC, virulence gene profiles vary from strain to strain (Brooks et al., 2005) and considerable variability has been shown to exist between the non-O157 serogroups and their association with disease severity and outbreaks (Hedican et al., 2009; Wickham et al., 2006; Tarr et al., 2005). Between the years 1982-2002, it was found that the serogroup O111 accounted for the most number of non-O157 associated HUS cases (Brooks et al., 2005). Additionally, STEC O26 and O103:H2 have also been associated with HUS (Caprioli et al., 1994; Luzzi et al, 1995). RAPD patterns of STEC belonging to the serogroups O26, O103, and O111 show different, but clustered, patterns indicative of a close and unique clonal relationship among these pathogens of the respective serotypes (Schmidt et al., 1999). However, Shmidt et al (1999) were able to show that certain *E. coli* O103 strains may have acquired variant intimin (*eae*) genes that differ from those seen in O26, O111, and O157 isolates. Meanwhile, the O121 serogroup has been frequently associated with bloody diarrhea (Brooks et al., 2005). A study by Hedican et al (2009) was able to show that although non-O157 STEC are capable of

causing HC and HUS, O157 cases were more likely to involve these sequelae (Hedican et al., 2009).

Among the non-O157 serogroups, isolates with virulence gene profiles that show only *stx*$_1$ amounted to 61%, while those with only *stx*$_2$ amounted to only 22%, and 17% possessed both *stx*$_1$ and *stx*$_2$ (Brooks et al., 2005). However, while the presence of *stx*$_2$ in *E. coli* O157:H7 increases the probability of diarrheagenic illnesses progressing to HUS, with the pathogenic non-O157 STEC, differences in illness severity between cases involving isolates that only had *stx*$_1$ and those that had at least *stx*$_2$ were not significant (Hedican et al., 2009).

Biochemically, differences between sugar fermenting patterns within the non-O157 serogroups exist (Possè et al., 2008). Unlike *E. coli* O157:H7, the non-O157 STEC do ferment sorbitol (Eklund et al., 2001; Brooks et al., 2005; Johnson et al., 2006) and therefore cannot be easily distinguished on agar-based culture media such as sorbitol MacConkey's agar (SMAC). Additionally, due to the diversity of the non-O157 serogroups, direct or indirect identification of shiga-toxins or the genes that encode them has been adopted as the practical approach to detect these bacteria (Griffin et al., 2003; Blanco et al., 2001).

3. Epidemiology

Most STEC foodborne outbreaks have been associated with the consumption of raw or undercooked meat. Cattle are considered to be the primary reservoirs of *E. coli* O157:H7 (Elder et al., 2000; Smith et al., 2001). There is considerable evidence that on-

farm practices can affect pathogen loads on cattle that enter slaughter facilities, resulting in cross contamination at the post-harvest level (Elder et al., 2000).

Out of the human STEC infections, the most severe form of illness is caused by *E. coli* O157:H7. However, its isolation rate from food and animal feces is considerably lower than that of the non-O157 STECS in the group. Studies conducted in Canada found evidence of non-O157 STEC isolated in 17 and 45% of cattle, although the incidence of *E. coli* O157:H7 in the two studies conducted was less than 1% (Johnson et al., 1996). A German study found that out of the 259 cattle surveyed in the study, 26 were positive for non-O157 STEC, while only 2 were positive for *E. coli* O157 (Montenegro et al., 1996). An epidemiological study by Rangel et al. (2005) demonstrated that, between the years 1982-2002, the transmission route for 52% of cases was foodborne, 21% unknown, 14% person to person contact, 9% waterborne, 11% through animal contact, and 0.3% were laboratory related. Of the foodborne cases, 41% of the outbreaks occurred as a result of contaminated ground beef and 21% as a result of contaminated produce (Rangel et al., 2005).

In a study by Brooks et al (2005) where clinical isolates from persons with sporadic illnesses from the years 1982-2002 were tested for STEC confirmation and serotyping, the six most common non-O157 serogroups and their prevalence were identified. These included O26 (22%), O111 (16%), O103 (12%), O121 (8%), O45 (7%), and O145 (5%). More recently, Gould et al (2013) conducted an epidemiological study of non-O157 STEC infections during the years 2000-2010, where 2006 cases of non-O157 STEC infection were reported to FoodNet. It was also found that, within this decade, the number of non-O157 STEC infections increased from 0.12 per 100,000 population in

2000 to 095 per 100,000 per population, with a parallel decrease in O157 STEC infections during this period (Gould et al, 2013). The prevalence of the most common serotypes was as follows: O26 (26%), O103 (22%), O111 (19%), O121 (6%), O45 (5%), and O145 (4%). These findings suggest that, over the course of three decades, the order of prevalence of the non-O157 STEC associated with human illness has changed little.

A UK study by Smith et al. (1991) demonstrated that 25% of pork sausages contained non-O157 STEC, but *E. coli* O157:H7 was not detected. In another study, 17% of raw beef samples were shown to contain non-O157 STEC, but once again, *E. coli* O157:H7 was not isolated (Willshaw et al., 1992). Gould et al. (2013) also found that fewer non-O157 STEC infections were associated with outbreaks in comparison to O157 STEC infections reported between the years 2000-2010. These findings suggest that humans are exposed to non-O157 STEC more frequently from food and environmental sources that *E. coli* O157:H7. However, the incidence of non-O157 STEC in infections is lower than that of *E. coli* O157:H7 infections (Johnson et al., 1996). It may therefore be concluded that either *E. coli* O157:H7 is more virulent and transmissible than the other STEC, or that a milder form of illness that is rarely brought to medical attention is produced by the non-O157 STEC. At the same time, it must be understood that the non-O157 STEC are not all equally pathogenic. Certain serogroups, such as O26, O103, and O111, seem to predominate in cases of human illness (Goldwater et al., 1994; Brooks et al., 2005).

4. Pathogenicity and virulence factors

In general, infections with pathogenic strains of non-O157 shiga toxin-producing *E. coli* are fewer and clinically less severe than infections with *E. coli* O157:H7. However, the clinical manifestations of non-O157 STEC disease are similar to that of *E. coli* O157:H7 disease, posing a substantial dilemma for the clinician since these symptoms are nearly indistinguishable from O157-induced disease, as well as many other foodborne enteric infections (Johnson et al., 2006). The similarities in clinical manifestations indicate that the non-O157 STEC may also employ similar mechanisms of pathogenesis as *E. coli* O157:H7. Additionally, because most of the genes encoding for virulence factors found in *E. coli* O157:H7 are located on lambda bacteriophages, horizontal transfer of these genes to the non-O157 STEC may explain why a majority of these strains also possess similar virulence genes (Donnenberg and Whittam, 2001; Croxen and Finlay, 2010; Ogura et al,. 2009). Genetic profiling has confirmed the presence of the locus of enterocyte effacement (LEE), shiga-like toxins stx_1 and stx_2, intimin (*eae*), and other genes shared with *E. coli* O157:H7 (Coombes et al, 2008; Frankel et al., 2008;).

One of the most important characteristics, from the pathogenesis standpoint, of STEC O157 and some STEC non-O157 is the ability to produce attaching and effacing (A/E) lesions. These lesions may be produced on a variety of cell types. The production of A/E lesions enhances the pathogen's ability to colonize the intestine. The genes that are required for the formation of A/E lesions are encoded in the chromosomal pathogenicity island LEE (McDaniel and Kaper, 1997; Elliott et al., 1998; Perna et al., 1998). Characteristics of the A/E lesions include: degeneration and effacement of the

intestinal epithelial cell microvilli, the adherence of bacteria to the epithelial cells, and the assembly of cytoskeletal structures such as actin, talin, erzin, and α-actinin beneath the attached bacteria (Knutton et al., 1989; Kaper et al., 1998a). A variety of signal transduction pathways are induced following attachment to the eukaryotic cell. These signals are responsible for the formation of these A/E lesions, ion secretion, and subsequent bacterial invasion. It has been found that serogroups producing these lesions, including isolates of O157, O26, and O111 STEC, contain the pO157 plasmid.

However, in order to form A/E lesions, *eae* must be secreted first to form intimate attachment to the epithelial cells. Therefore, STEC strains that have genes for both *stx* as well as *eae* would be expected to be associated with human infection and disease. The incidence of *eae* in bovine STEC isolates identical to those isolated from human infections was found to be higher than those found in STEC serotypes not frequently associated with human illness (Barret et al., 1992; Willshaw et al., 1992; Beutin et al., 1995; Johnson et al. 1996; Gyles et al., 1998; Kaper et al., 1998b). Intimin plays an important role in the formation of A/E lesions by initiating intimate attachment to follicle-associate epithelial cells of the ileal Peyer's Patches prior to the release of effector molecules during the Type III secretion system (Phillips and Frankel, 2000). Intimin-γ is associated with *E. coli* O157:H7 (Tzipori et al., 1995) and has been shown to be tissue-specific, targeting follicle-associated epithelium cells of the Peyer's Patches in the ileum (Phillips and Frankel, 2000). Schmidt et al. (1999) were able to show that some strains of O103 may have variant *eae* sequences unique from the other STEC, indicating that considerable differences exist between STEC serogroups as well. Additionally, while several non-O157 STEC serotypes are *eae*-positive, they are associated with only

sporadic cases of human illness. This suggests that other properties exist that contribute to the organism's pathogenicity. A study by Chase-Topping et al. (2012) demonstrated that the number of STEC strains that were both *stx*$^{+}$ and *eae*$^{+}$ decreased considerably at the farm and animal levels, which may account for why disease with non-O157 STEC strains are not as prevalent as those with the O157 serogroup. The fewer the number of virulent strains in the environment, the lower the risk for human illness (Chase-Topping et al., 2012). However, the severity of disease caused by some virulent non-O157 STEC strains does not allow for these serogroups to be overlooked.

Besides the presence of the *eae* gene, Chase-Topping et al. (2012) were able to demonstrate the relationship between severe human disease and the presence of the *stx*$_{2}$ and *tccP2* combination in the O26 serogroup. TccP2 is an important effector molecule in the Type III secretion system also used by virulent non-O157 STEC strains (Madic et al., 2011). The Type III secretion system plays an important role in the formation of A/E lesions, a selection pressure that selects for Type III competent STEC variants would increase the likelihood of zoonotic transmission of more virulent strains in humans (Coombes et al., 2008).

Having adhered to the intestinal epithelia, the bacteria grow and then release an array of extracellular products. Of these products, the cytotoxins known as shiga toxins are an important component. There are two antigenetically distinct forms of the toxin: *stx*1 and *stx*2. Both toxins are compound toxins made up of a 32 kDa A subunit and a pentameric B subunit made of 7.7 kDa monomers. By definition, STEC have the ability to produce one, or both, shiga toxins. The incidence of STEC has seen an increase over

recent years, although the trigger for this shift in virulence is unknown (Zhang et al., 2000).

In an *in vivo* study with rabbits, the presence of shiga toxins was shown to produce more severe illness with more serious histological lesions, edema, and severe inflammation than the non-toxigenic isolate (Sjogren et al., 1994). Isolates of the serogroup O26 have been found to usually produce *stx*1 (Scotland et al., 1990) and isolates of O111 produce *stx*1 and some produce *stx*2 in addition (Willshaw et al., 1992; Gyles et al., 1998). Epidemiological evidence suggests that STEC isolates producing *stx*2 alone are more commonly associated with producing more serious disease than isolates producing *stx*1 only, or *stx*1 and *stx*2 (Boerlin et al., 1999; Käppeli et al., 2011). Louise and Obrig (1995) were able to show that *stx*2 was a thousand times more cytotoxic than *stx*1 toward human renal microvascular cells. These cells are the target of Shiga toxins in the development of HUS.

Unlike with the O157 serogroup, however, the prevalence of stx^+ non-O157 STEC at the farm and animal levels is seen to decrease from the total number of non-O157 STEC serogroup isolated from these samples (Chase-Topping et al., 2012). Schmidt et al. (1999) demonstrated that pathogenic STEC (specifically O26, O103, and O111 serogroups) belong to their own lineages different from *E. coli* O157:H7 and have unique genetic profiles and virulence traits, with over 80% genetic identity within a serogroup. Clustered groupings from RAPD results have shown that there is genetic relatedness among the non-O157 STEC serogroups as well (Schmidt et al., 1999).

Acid resistance may also play a role in the virulence of this group of pathogens though this feature has been studied mostly in *E. coli O157:H7*. In order for STEC to cause gastrointestinal disease, they must be able to pass through the acidic stomach environment once consumed. Several mechanisms that enable *E. coli* to resist acidic conditions in the gastric environment have been identified. These include the acid-induced oxidative system, acid-induced arginine-dependent system, and a glutamate-dependent system. Lin et al. (1996) was able to show that the arginine-dependent system provided more protection in EHEC strains than in commensal *E. coli* strains at pH 2.0, while the glutamate-dependent system was equally effective in all strains. In a survival comparison study of *E. coli* O157 and non-O157 STEC at pH 2.5, Waterman and Small (1996) were able to show that there was little difference between the two groups with regards to acid resistance.

The ability of *E. coli* O157 to cause disease from low inocula may be associated with their ability to demonstrate acid resistance. In water-borne infections, the organisms may be directly exposed to stomach acid upon consumption, and acid tolerance may have a greater significance here. For infections acquired through food, however, acid resistance may be of lesser significance since food components may provide protection for the bacteria in the gastric environment. Acid tolerance may, however enhance the survival of these organisms in food purposely acidified with the intention to reduce microbial growth. One of the concerns at the pre-harvest level is that these organisms may develop acid tolerance in the gut of cattle fed high forage diets (Tkalcic et al., 2000).

Besides factors such as *eae* that help initiate intimate attachment with the target cell, other adhesion factors may also play a role in the virulence of this group of

pathogens. Peristaltic flow may remove microorganisms from the intestinal epithelial mucosa. Therefore, following passage through the stomach, viable organisms must be able to adhere to the intestinal mucosa. A number of possible adhesion factors have been identified for the Enterohemorrhagic *E. coli*. Fimbrial adhesins found on the surface of these microorganisms are thought to be chromosomally encoded. Burland et al. (1998) were able to demonstrate that a fimbrial gene cluster was not encoded on the pO157 plasmid.

B. STEC on beef cattle operations

1. Types of beef cattle operations

In the US, cow/calf operations and feedlots comprise of the two main types of beef cattle operations. Both types differ greatly in the way animals are reared, including space provided per animal, diet, and water sources. All of these factors may have an impact on the bovine gastrointestinal microflora (Gillespie et al., 2007).

Cow/calf operations may be categorized into seedstock operations that focus mainly on the production of purebred or registered cattle with the goal of making genetic improvements in cattle that benefit the beef industry. Typical (non-specialized) cow/calf operations contain a breeding herd of cows, bulls, replacement heifers, and beef calves. Animals graze in herds on large pastures, and their diet is typically forage-based. Calves are weaned at 6-10 months of age, with steer calves and heifers being sold afterwards. Some calves may be selected to enter the breeding herd. Calves sold from cow/calf operations may then enter stocker operations, which serve as an intermediate between cow/calf operations and feedlots. Alternatively, some cow/calf operations may raise cattle

until they reach a market-ready weight, after which the cattle are transported directly to feedlots before they enter the slaughterhouse.

Stocker operations are further categorized into background and grower stocker operations. Calves are sent to background stocker operations if they are either too small or underweight and need to be grown before entering a feedlot, or if the feedlots they otherwise would have entered have reached maximum occupancy at the time. Calves in background stocker operations are grown in dry-lots and fed high roughage diets. Grower stocker operations, on the other hand, raise animals as pastured cattle and utilize a grazing program. At the end of the grazing season, these cattle are then marketed or transported to feedlots.

Feedlots are designed to meet the feed, water, and care requirements of large numbers of cattle, a practice known as intensive rearing (Gillespie et al., 2007). Feed grains and by-products are typically used to feed large numbers of cattle, and animal rations comprise 70-90% grain in order to provide the necessary energy requirements to reduce the time required to reach market weight.

2. Prevalence of STEC on beef cattle operations

A number of epidemiological studies on the prevalence of *E. coli* O157:H7 have been done. However, these studies have centered mainly on feedlots. Results from these studies have shown a strong correlation between feedlot cattle and *E. coli* O157:H7 (Hancock et al., 1997, 1999; Laegreid et al., 1999; Rice et al., 1999). A study conducted at 73 feedlots across 11 states (Idaho, Iowa, Nebraska, South Dakota, Washington, Colorado, Kansas, Oklahoma, California, New Mexico, and Texas) showed that 11.0% of

fecal samples collected from these feedlots were positive for *E. coli* O157:H7 (APHIS, 2001). An earlier study conducted in the same 11 states, however, showed a lower prevalence of *E. coli* O157:H7 (1.8%) in the fecal samples that were obtained from these feedlots (Hancock et al., 1997). The results from these studies suggest the possibility that there has been an increase in the population of infected cattle within the United States. The prevalence of non-O157 serogroups in one study conducted by Menrath et al. (2010) demonstrated that 24.7% of 1,646 fecal samples obtained during the study tested positive for non-O157 *E. coli.*

Most studies to date have concentrated on beef feedlots and large ranches (Laegrid et al., 1999) while there is limited information on the impact of production practices on small-scale cow/calf operations where in cow/calf operations, calves are raised primarily on pastures until they are transported to feeder cattle finishing sites or directly to the abattoir. It is therefore important to understand the factors that affect *E. coli* O157:H7 burden in these cow/calf operations as it can be a critical path in the farm-to-fork continuum and can, in the long run, help with the development of risk management strategies.

3. On-farm reservoirs

Important pathogenic mechanisms of *E. coli* O157:H7 and other STEC, as well as the identification of the reservoirs that are responsible for colonization in cattle, are poorly understood. Also, the reported prevalence of *E. coli* O157:H7 within the feedlot and farm environments seem to vary greatly (Rasmussen and Casey, 2001). Reservoirs that have been identified in the feedlot areas include feces (0.8%), feed bunks (1.7%),

water troughs (12%), and incoming water supplies (4.5%) (Van Donkersgoed et al., 2001; Sargeant et al., 2003; Dodd et al., 2003). Buchko et al. (2000) found that, when cattle were inoculated with 10^{10} CFU/ml *E. coli* O157:H7, the organism could be isolated from 17% of feed samples, 10% of water trough biofilm swabs and drinking water samples, and from 100% of the manure samples taken from feedlot pens. Furthermore, they also found that 17% of mouth swab samples were positive for *E. coli* O157:H7.

Being enteric pathogens, STEC are shed in the feces of animals (Caprioli et al., 2005; Elder et al., 2000; Smith et al., 2001). Considering the proximity of these animals in feedlot pens where vertical integration practices are common, dissemination of STEC throughout a herd becomes easier. With the exception of young calves (who may show symptoms of diarrhea) cattle infected with STEC are generally asymptomatic even during periods of shedding (Cray and Moon, 1995; Gansheroff and O'Brien, 2000). Fecal shedding of *E. coli* O157:H7 and other STEC has also been shown to be seasonal, generally showing a peak in prevalence during the summer months and dipping to low levels in the colder winter season (Chapman et al. 1997; Hancock et al., 1997; Van Donkersgoed et al., 1999). During a study involving controlled artificial lighting, Edrington et al. (2006) found that increased day length may be a contributing factor to fecal shedding in cattle, supporting the theory that fecal shedding is higher in the summer months than in fall or winter.

Besides the length of day and environmental conditions, it has also been suggested that the strain of *E. coli* O157:H7 may also influence fecal shedding patterns. A study conducted by Gautam et al. (2012) was able to provide evidence of this when they identified shedding patterns in groups of cattle inoculated with particular strains of

E. coli O157:H7. These findings indicate that the frequency, level, pattern, and duration of fecal shedding may not only be associated with environmental conditions, but also depend on the strain of bacteria. Furthermore, in order for STEC to be shed in the feces over a period of time, the microorganism must be able to colonize and persist in the gastrointestinal tract of cattle. In a study conducted by Dopfer et al. (2011) to determine the dynamics of STEC and their virulence factors in cattle, it was found that once *E. coli* encoding the shiga toxins in combination with enterohemolysin were transmitted and established in a calf, they were eliminated less efficiently in comparison to *E. coli* without this combination of virulence markers. The presence of particular combinations of virulence factors coincided with the persistence of *E. coli* in the bovine gastrointestinal tract. It has also been suggested that supershedders that shed high concentrations of STEC in their feces ($>10^4$ CFU/g feces) may play a key role in the persistence and transmission of STEC between cattle and their environment. This in turn could increase the level of contamination at harvest and the risk of human infection (Ayscue et al. 2009; Chase-Topping et al., 2007, 2008; Cobbold et al., 2007).

Cattle that become infected with *E. coli* O157:H7 and other STEC can cross infect each other on the farm. In one study by Hancock et al. (1997) it was found that herds that tested positive for *E. coli* O157:H7 after two years had a higher prevalence (median = 1.9%) in comparison to herds that tested negative in the previous sampling (median = 0.2%). Furthermore, Laegreid et al. (1999) were able to determine from a serological study conducted in the states of Montana, Missouri, Kansas, Nebraska, and South Dakota, that 83% of calves and 100% of all herds had been exposed to *E. coli* O157:H7.

The diet of cattle may also influence the fecal shedding of *E. coli* O157:H7 as evidenced in several studies (Buchko et al., 2000; Tkalcic et al., 2000). High roughage diets were shown to prevent shedding of large populations of *E. coli* O157:H7 in the feces of calves when compared with high-concentrate diets (Tkalcic et al. 2000; Lowe et al., 2010). Roughage-rich diets, however, tended to cause *E. coli* O157:H7 to become more acid tolerant compared to concentrate-rich diets (Tkalcic et al., 2000). In another study conducted by Buchko et al. (2000), three groups of six yearling steers were inoculated with 10^{10} CFU/ml *E. coli* O157:H7 and then placed on three separate diets. The cattle fed barley showed an increased shedding of *E. coli* O157:H7, which subsequently resulted in a higher isolation rate of *E. coli* O157:H7 from the environment.

Contaminated water sources and equipment may also contribute to the dissemination and persistence of *E. coli* O157:H7 on the farm environment. Previous studies have found that *E. coli* O157:H7 may be more frequently isolated from the sediments and biofilms of the water troughs used to supply drinking water to cattle (Zottola, 1994). Cattle water troughs are known to harbor *E. coli* O157:H7 for extended periods of time (Hancock et al., 1998; Murinda et al., 2004; Polifroni et al., 2012; Wetzel and LeJeune, 2006). It has also been shown that approximately 25% samples of cattle water supply contain *E. coli* O157:H7 (Sanderson et al., 2006). These results suggest that common-use troughs can function as vectors for horizontal transmission of *E. coli* O157:H7 within a group of animals.

The age of cattle has also been shown to be an important factor in the fecal shedding of *E. coli* O157:H7 (Wells et al., 1991; Zhao et al., 1995). In one study, Blanco et al. (1996) found that 20% of cows and 23% of calves tested positive for shiga toxin-

producing *E. coli*. Furthermore, weaned heifers had a higher prevalence of *E. coli* O157:H7 (1.8%) than un-weaned calves (0.9%) or adults (0.4%) on dairy farms located in Washington, Oregon, and Idaho (Hancock et al., 1997).

C. Isolation and detection of STEC from environmental samples

1. Culture methods

Culture methods in place today for isolating and detecting STEC in environmental samples involve an enrichment step and immunomagnetic separation (IMS) for specific serotypes prior to plating on selective and differential media. Either of the two selective enrichment strategies for the primary isolation of STEC from environmental sources are used. One method is to use a selective enrichment broth, and the other is to use a nonselective enrichment medium. The use of selective enrichment broth uses parameters such as pH or specific carbon sources to favor the replication of *E. coli*. Combining these selective factors with antibiotics helps restrict the growth of competing and background microflora (Davies et al. 2005; Comstock et al., 2012; Durso and Keen, 2007; Hussein and Bollinger, 2008). For the isolation of non-O157 STEC, however, care must be taken in selecting antibiotics to be added to selective media as they may respond differently than STEC O157. For example, STEC O157 has been shown to be significantly more resistant to novobiocin (20 mg/L) than non-O157 STEC (Vimont et al., 2007). *Escherichia coli* broth (EC broth) has been used to enrich fecal samples for non-O157 isolation with successful results (Paddock et al., 2013).

The availability of serotype-specific monoclonal antibodies for *E. coli* O157:H7 and non-O157 STEC in combination with magnetic beads provides improved sensitivity

in isolation of STEC from complex environmental matrices. This technique of selective isolation is known as immunomagnetic separation. Immunomagnetic separation techniques may be entirely manual or automated. The necessity of including an IMS step for primary culture from complex environmental samples (such as feces or soil) has been demonstrated repeatedly in outbreak situations. In such cases, the use of an IMS step resulted in the isolation of the outbreak strain after standard culture methods, such as direct plating onto differential media, had failed (Durso et al., 2005; Davies et al., 2005; Goode et al.. 2009; Comstock et al., 2012).

Following IMS, plating is carried out on differential media, employing unique colony morphology on select agar to identify STEC. Sorbitol MacConkey agar (SMAC) was one of the first differential mediums used for STEC detection and is still used today, especially in clinical and regulatory settings, and for the screening of ground and water surfaces (LeJeune et al., 2001; Sargeant et al., 2004; Shelton et al., 2004, Heijnen and Medema, 2006; Mull and Hill, 2009). Sorbitol MacConkey agar is also the agar medium recommended by the FDA for the detection of *E. coli* O157:H7 from food (FDA, 2011). Lately, CHROMagar O157 in combination with IMS has proven to be superior to SMAC for the detection of STEC O157 from environmental sources (Durso et al., 2005; Davies et al., 2005; Goode et al., 2009; Comstock et al., 2012). Rainbow agar O157 has also provided successful isolation of STEC O157 from environmental samples (Fratamico et al., 2011; Grant, 2008). For the isolation of non-O157 STEC, CHROMagar STEC, Rainbow agar, and modified Rainbow agar have been used (Tillman et al., 2012; Wylie et al., 2012; Kalchayanand et al., 2013).

2. Molecular methods

Many modern detection methods employ molecular methods such as multiplex PCR, real-time PCR, and quantitative real-time PCR either partially or exclusively. Some methods combine an initial enrichment step and a PCR step for the rapid detection of target cells (Sen et al., 2011; Yoshitomi et al., 2012; Heijnen and Medema, 2006; Jacob et al., 2012). Enrichment cultures that are combined with a PCR step may also be used in a most probable number procedure as a means of obtaining quantitative information for specific samples (Heijnen and Medema, 2006). Genes targeted in these molecular methods for isolation of STEC include those for the Shiga toxins (*stx*1, *stx*2), O-antigen transporter gene (*rfb*E), flagella (*fli*C), and intimin (*eae*) (Jacob et al., 2012; Ibekwe and Grieve, 2002; Paddock et al., 2011).

Studies to detect the seven STEC serogroups in cattle fecal samples using multiplex PCR have been conducted (Paddock et al., 2012; Bai et al, 2012; Bai et al., 2010). In these studies, fecal samples were spiked in order to establish a standard curve for detection of the *E. coli* serogroups using the multiplex PCR methods described. In addition to fecal samples, Ibekwe and Grieve (2003) were able to detect and quantify *E. coli* O157:H7 in soil and water samples using real-time PCR.

CHAPTER III

METHODOLOGY

A. Experimental design

Samples were obtained from small-scale cow/calf operations (<50 cattle) in Oklahoma and Louisiana. Twenty seven cow/calf pastures from Oklahoma, and 18 from Louisiana were sampled over two years (2013-2014). Pastures from Kay, Osage, Payne, Creek, and Logan counties from Oklahoma (Northern and Western Oklahoma counties), and East Baton Rouge, Lafourche, Morehouse, and St. Landry parishes from Louisiana were sampled for this study. Sampling was carried out during the summer, between the months of May and August in Oklahoma, and until October in Louisiana. Each pasture was visited twice each year over the course of the sampling period and the temperature, humidity, and wind conditions recorded during each visit. Samples taken from each pasture included fecal, water, sediment, and equipment swab samples for *E. coli* O157:H7, and only fecal samples were analyzed for non-O157.

Each pasture was considered an experimental unit. Pastures that reported at least one sample positive for a particular STEC serogroup were regarded as positive for carrying the corresponding STEC serogroup. A pasture was reported as having positive occurrence for a particular serogroup based on the percentage of positive samples obtained from each pasture for that serogroup. These values were then used in combination with the results from the survey to determine an association between farm management practices and occurrence of STEC on the farm.

B. Occurrence of STEC on small-scale cow/calf operations

1. Sample processing and enrichment

a. Fecal samples

Fifteen fecal samples were collected from each pasture. Samples were aseptically obtained from fresh fecal pats, taking care to scoop approximately 100 g from the center of each pat without coming in contact with the soil. In order to obtain maximum variability in fecal samples collected, the color, consistency, and distance between fecal pats was taken into consideration. If feces were freshly deposited by the animals during sampling, these samples were given priority during collection. Fecal samples were placed in labeled sterile fecal cups (McKesson Corporation, McKesson Medical-Surgical, Richmond, VA 23228, USA). The samples were then transported on ice so as to minimize microbial growth post-collection during this time.

For the detection and isolation of *E. coli* O157:H7, 10 g of each fecal sample was placed in an appropriately labeled Whirl-pak™ bag (NASCO, Fort Atkinson, WI, USA). To each bag, 90 ml of Gram Negative broth supplemented with Vancomycin Cefixime

and Cefsoludin (GNVCC; GN: Becton, Dickinson and Company, Sparks, MD 21152, USA; VCC: Sigma-Aldrich Co., 3050 Spruce Street, St. Louis, MO 63103, USA) was added. The samples were homogenized thoroughly by stomaching for 1 minute. Excess air in the sample bag was then expelled and the bag folded securely closed. Samples were incubated at 25 ˚C for two hours, followed by incubation at 42 ^{o}C for 12 hours.

For the non-O157 samples, 10 fecal samples out of the 15 that were collected from each pasture were chosen at random. Using a sterile tongue depressor, approximately 1 g of feces was added to labeled test tubes containing 9 ml of *E. coli* Broth (EC Broth; Oxoid Ltd., Basingstoke, Hampshire, England). Samples were mixed using a vortex and then incubated for 6 hours at 37 ^{o}C. Fecal samples for non-O157 analysis taken from the first sampling year were frozen prior to processing. These samples were processed the following year, and were thawed at 4 ^{o}C for 24 hrs prior to processing.

Each day that samples would come in, a positive and negative control was also run in order to check performance of media during each experiment. For the positive control, *E. coli* O157:H7 ATCC 43888 was used, while *Salmonella gaminara* strain was used as the negative control. Cultures for the controls were prepared by inoculating 100 µl of cryopreserved cells in TSB and incubating overnight (18-20 hours) at 37 °C. A single colony was then picked, inoculated in 9 ml TSB, and incubated at 37 °C for 12-18 hours to obtain an overnight culture. A loopful of this overnight culture was then streaked onto TSA and incubated at 37 °C for 18-20 hours. Control cultures were maintained on TSA until use. On the day of the experiment, appropriately labeled control plates were used to transfer a swab of colonies to appropriately labeled Whirl-Pak™ bags containing

90 ml of GNVCC. The entire cotton swab was left in the bag and the bags closed securely thereafter. This was followed by gentle massaging, and the control bags were added with the fecal samples for incubation. Positive and negative controls were also run for the non-O157 samples. Bacterial strains used for the positive controls include: *E. coli* O26:H11 CDC 03-3014, *E. coli* O45:H2 CDC 00-3039, *E. coli* O103:H11 CDC 06-3008, *E. coli* O111:H8 CDC 2010C-3114, *E. coli* O121:H19 CDC 02-3211, and *E. coli* O145:NM CDC 99-3311. On the day of the experiment, a swab from the non-O157 control plates was taken and then added to 9 ml of EC broth in appropriately labeled tubes. Swabs were left in the tubes. Controls were incubated with the rest of the non-O157 samples for 6 hours at 37 °C.

b. Water samples

Approximately 120 ml of water samples were collected in appropriately labeled screw capped medical-grade fecal cups. A total of 3-5 water samples were collected from each pasture. Five water samples were collected from the pasture if water troughs were not present. However, in the presence of water troughs, only a total of three water samples were obtained from surrounding water bodies (ponds, runoff, creeks, etc.) as well as water troughs. Samples were stored on ice during transportation.

For each sample, 30 ml of the water sample was transferred into an appropriately labeled sterile, Whirl-pak™ bag and combined with 30 ml of a 2X concentrate of TSB. The contents of the bag was then mixed thoroughly and incubated at 25 °C for two hours, followed by incubation at 42 °C for 12 hours.

c. Sediment samples

Sediment samples were collected from water troughs. To collect these samples, a long ladle was used to scoop up sediment from the bottom and sides of the trough (different locations of the trough). Samples were collected in labeled screw capped medical grade fecal cups. The ladle was wiped clean with 70% ethanol after each collection, and samples were transported on ice from the farm to the lab for processing.

Samples were then transferred into a sterile labeled Whirl-pak bag. Depending on the samples size of the sediment collected, 20-50 ml of a 2X concentrated TSB was added in a 1:1 ratio. The contents of the bag were then mixed thoroughly by massaging. Excess air was expelled from the bag and the bag closed tight before incubating at 25 °C for two hours, followed by incubation at 42 °C for 12 hours.

d. Water equipment swabs

Equipment swabs were collected from the outer sides of the water troughs, especially where there was evidence of bird droppings, mud, and fecal matter. A sterile sponge hydrated with 10 ml of BPW was used to obtain these samples. Sponges were added to labeled Whirl-pak™ bags and transported on ice for processing.

To each equipment swab sample bag, 90 ml of mTSB was added. Samples were then stomached (Stomacher 400 Circulator, Seward, Davie, Florida, USA) for 1 minute at 230 rpm. Once excess air was expelled from the bags and the bags closed tight, the samples were incubated at 25 °C for two hours, followed by incubation at 42 °C for 12 hours.

2. Immunomagnetic separation (IMS)

Prior to real time PCR, an immunomagentic separation was carried out on all the samples in order to concentrate the pathogen in the sample tube. This step also involves washing steps that help minimize the amount of fecal matter, hay, or other debris that might otherwise be present in the sample and may interfere with the detection of the pathogen.

For all *E. coli* O157:H7 samples, IMS was carried out using anti-*E. coli* O157:H7 Dynabeads®. All steps were carried out according to manufacturer's instructions (Appendix B), except for the washing step, which was carried out three times in this study as opposed to the suggested two times by the manufacturer. Washing the sample three times yielded a cleaner product in comparison to washing the sample only twice. The additional wash step did not interfere with the concentration of the pathogen-bead mix at the end of the procedure.

For all non-O157 samples, IMS was carried out using anti-*E. coli* non-O157 STEC Dynabeads® (Invitrogen Dynal AS, Oslo, Norway). All steps were carried out according to the manufacturer's instructions (Appendix B)). However, appropriate aliquots of each dynabead mixture was pooled into a single microcentrifuge tube to obtain a mixed dynabead sample containing anti-non-O157 beads for each strain.

3. Detection and confirmation of STEC using RT-PCR

a. *Escherichia coli* O157:H7

For *E. coli* O157:H7 detection, the BAX® System Real-time PCR Assay for *E. coli* O157:H7 (DuPont Nutrition and Health, Wilmington, DE) was used. Following manufacturer's instructions, 20 µl of the bead-pathogen mix was used as the sample for screening. Samples that tested positive during this step were also noted down as STEC positive.

A 50 µl aliquot of all *E. coli* O157:H7 samples were also plated on Sorbitol MacConkey agar supplemented with Cefixime and Tellurite (CT-SMAC; SMAC, Remel, Thermo Fisher Scientific, Lenexa, Kansas, USA; CT, 77981 CT supplement, Fluka, Sigma Aldrich, Switzerland). This step was carried out along with the RT-PCR detection step so as to not miss detecting the pathogen on any sample that turned out negative on the RT-PCR but positive on traditional agar-based culture. The CT-SMAC plates were incubated at 37 °C for 18-20 hours. Following incubation, up to three suspect colonies were picked using a sterile toothpick and transferred (streaked) onto SMAC. These plates were then incubated for 18-20 hours at 37 °C. Following incubation, isolated colonies were then transferred onto Blood agar (TSA plates with 5% sheep's blood, Teknova, Hollister, CA, USA) and incubated for 18-20 hours at 37 °C. The resulting colonies were then subject to presumptive identification using latex agglutination (*E. coli* O157:H7 Latex Test, Thermo Scientific, Remel, Rim, Lenexa, KS, USA). Isolates that turned positive using latex agglutination were then confirmed for *E. coli* O157:H7 as described above.

b. Non-O157 STEC

The BAX® System Real-Time PCR STEC Suite (DuPont Nutrition and Health, Wilmington, DE) was used to detect the non-O157 serogroups (unconfirmed for *stx* or *eae*). As with the *E. coli* O157:H7 samples, manufacturer's instructions were followed, with an additional IMS step prior to RT-PCR detection. A 20 µl of all non-O157 samples (bead-pathogen complex) that turned out positive on the RT-PCR were then plated on CHROMagar™ STEC (DRG International, Springfield, NJ, USA) and incubated at 37 °C for 18-20 hours. Suspect colonies were picked and tested for the corresponding serogroup using the appropriate anti-*E. coli* non-O157 antisera (Statens Serum Institut, S Artillerivej, DK-2300 Cph. S, Denmark). Individual colonies were then plated onto Blood agar and incubated at 37 °C for 18-20 hours to be confirmed for STEC.

In order to confirm non-O157 isolates STEC, the BAX® System STEC Screening Assay (DuPont Nutrition and Health, Wilmington, DE) was used. This assay tests for both the *eae* and *stx* (*stx*$_1$ and *stx*$_2$) genes. Using a sterile metal loop, a single colony was picked from the Blood agar plate and transferred to 100 µl of sterile double distilled water. The suspension was vortexed lightly for a few seconds and 20 µl of the resulting suspension was used as the sample in the BAX® System STEC Screening Assay. Samples that tested positive for both *stx* and *eae* genes were recorded as STEC positive isolates.

4. Preparation of stock cultures

A freezer stock solution comprising Brain Heart Infusion broth (BHI: Remel Inc., Lenexa, KS 66215, USA) and glycerol (Sigma-Aldrich, Co, Spruce Street, St. Louis, MO

63103, USA) in 85:15 ml ratio was used for *E. coli* O157:H7 stocks. For preparation of non-O157 stocks, a freezer stock solution of 7:3 ml of TSB and glycerol was used. Colonies were transferred from the respective Blood agar plates to correspondingly labeled freezer stock solution tubes. All stock cultures were then stored at -80 °C.

C. Determination of on-farm management practices and identification of contamination sources

On-farm management practices were identified by conducting a production practices survey. The potential contamination sources investigated in this study included: water source, water access/water container, type of feed, breed, and animal density (animals per acre). Additionally, the relationship between the prevalence of STEC and the frequency of cleaning on-farm equipment and other frequently used areas where cattle come into contact was also investigated.

A four-page questionnaire was developed as a means of assessing these factors (Appendix A). The questionnaire comprised a set of yes/no questions relating to the presence or absence of particular types of water containers, water sources, feed, and breed. The cleaning frequency of common cattle contact areas such as trailers, chutes, alleyways, heavy equipment, water troughs, and feed bins was assessed using a 5-point Likert scale, where: 1 = never cleaned, 2 = cleaned rarely (once in few months), 3 = monthly cleaning, 4 = weekly cleaning, and 5 = cleaned more than one day a week. Questionnaires were filled out during each visit to the pastures. Farmer demographics, although included in the survey, was not used in the analysis.

D. Data analysis

All responses from the surveys were pooled and analyzed using SAS version 9.3. For the univariate analyses for water access, water source, feed type, and breed, an ANCOVA using the PROC GLM method followed by Tukey's Test was carried out. Statistical significance was compared between pastures that had a particular category (e.g. water source: municipal water) to those that did not. Each serogroup was analyzed separately. Analysis of the cleaning regimen was done using an ANOVA by the PROC GLM method. Statistical significance was compared for cleaning frequencies of particular common cattle contact areas within a particular serogroup. A 95% confidence limit was used in both cases. In order to establish a relationship between animal density and the positive occurrence of the respective STEC strains, a regression analysis using the CORR method was used to determine a correlation. This method was used for the animal density category because animal density was reported as a real number, and not as a univariate (yes/no) value or graded according to a Likert scale. Pearson's correlation coefficient was used, with positive correlation coefficient indicating an increase in STEC prevalence with increasing animal density, and a negative correlation coefficient indicating a decrease in STEC prevalence with increasing animal density. Results were identified as statistically significant when $|r|>0.5$. No cross-comparison between serogroups was carried out.

CHAPTER IV

RESULTS AND DISCUSSION

A. Occurrence of STEC on small-scale cow/calf operations

1. Oklahoma

A total of 1224 samples were collected from Oklahoma, out of which 885 were fecal, 251 were water samples, 44 were sediments, and 44 were equipment swabs. Fifty five samples in total tested positive for *E.coli* O157:H7, resulting in a total positive occurrence of 4.4% for this pathogen (Table 1). Thirty three of the 885 fecal samples tested positive for *E. coli* O157:H7 (3.7% positive occurrence among fecal samples). Likewise, 16 water samples tested positive (6.4% positive occurrence), four sediment samples tested positive (9.1% positive occurrence among sediments), and only one equipment swab tested positive for the pathogen (2.3% positive occurrence among equipment swabs).

Table 1. Occurrence of *E. coli* O157:H7 in fecal, water, sediment, and equipment samples on Oklahoma cow/calf operations

Sample type	No. of samples collected	Percent positive
Fecal	885	3.7
Water	251	6.4
Sediment	44	9.1
Equipment	44	2.3
Total	1224	4.4

A total of 590 samples were taken to test for non-O157. Some samples were positive for multiple non-O157 serogroups. Overall, a total of 310 samples tested positive for at least one non-O157 serogroup, resulting in a total positive occurrence of 53% (Table 2). Of these positive samples, the most prevalent serogroups were O26, O45, and O103. Eighty samples tested positive for O26, 110 were positive for O45, and 73 were positive for O103. However, only 26 samples within the O26 group confirmed as STEC (33% positive occurrence within the serogroup), 25 confirmed as STEC for O45 (23% positive occurrence within serogroup), and 30 confirmed as STEC for O103 (41% positive occurrence). Twenty six samples tested positive for O121 out of which only 1 (4%) confirmed as STEC, while 20 samples tested positive for O145 with only 2 (10%) confirmed as STEC. Only one sample tested positive for the O111 group but the isolate did not have the *stx* or *eae* gene and therefore did not confirm as a true STEC. Overall, 89 fecal samples tested positive for true STEC, amounting to a14% prevalence in the state of Oklahoma.

Table 2. Occurrence of non-O157 STEC in fecal samples on cow/calf operations in the state of Oklahoma

Serogroup	Serogroup Positive Samples (n=590)	STEC Positive Samples	STEC (%)
O26	80	26	4.4
O45	110	25	4.2
O103	73	30	5.1
O111	1	0	0
O121	26	1	0.2
O145	20	2	0.3
Total	313	84	14

2. Louisiana

Out of a total of 564 samples taken, total positive samples amounted to 121, resulting in a 21.4% total positive occurrence of *E. coli* O157:H7 (Table 3). Of the total samples taken, 390 were fecal, 78 were water, 50 were sediment, and 46 samples were equipment swabs. Eighty eight of the fecal samples were positive for *E. coli* O157:H7 (23% positive occurrence). Sixteen of the water samples were also positive for this pathogen (21% positive occurrence) and 14 sediment samples were positive, resulting in a 28% positive occurrence. Only 3 equipment swabs were positive for *E. coli* O157:H7, amounting to a 7% positive occurrence within this category.

Table 3. Occurrence of *E. coli* O157:H7 in fecal, water, sediment, and equipment samples on Louisiana cow/calf operations

Sample type	No. of samples collected	Percent positive
Fecal	390	23
Water	78	21
Sediment	50	14
Equipment	46	7
Total	564	21.4

Results for the non-O157 samples analyzed from Louisiana are summarized in Table 4. Out of a total of 380 fecal samples, 185 were positive for at least one non-O157 serogroup, resulting in a 49% total occurrence. Some samples were positive for more than one non-O157 serogroup. Of the 185 samples that tested positive for at least one serogroup, the four most common serogroups included O26, O45, O103, and O121, with 65 samples testing positive for O26, 123 for O45, 75 O103, and 74 for O121. Of the 123 samples that tested positive for O45, only 33 were confirmed STEC by the presence of *stx* and *eae* genes. This resulted in a 27% positive occurrence of O45 within the serogroup. Likewise, only three O26 isolates were confirmed STEC (5% positive occurrence within the serogroup), and 22 of the O103 isolates were confirmed STEC (29% positive occurrence within the serogroup). None of the O121 isolates were positive for either *stx* or *eae*. Additionally, although the O111 and O145 isolates did not confirm as STEC, ten samples were positive for the O111 serogroup and 25 were positive for the O145 serogroup. Overall, 53 samples tested positive for STEC, amounting to a 14% prevalence of non-O157 STEC in the state of Louisiana.

Table 4. Occurrence of non-O157 STEC on cow/calf operations in the state of Louisiana

Serogroup	**Serogroup Positive Samples (n=380)**	**STEC Positive Samples**	**STEC (%)**
O26	65	3	0.7
O45	123	33	8.6
O103	75	22	5.7
O111	10	0	0
O121	74	0	0
O145	25	0	0
Total	185	53	14

[1] Some samples were positive for more than one serogroup

Overall, the prevalence of *E. coli* O157:H7 was higher at 21.4% in the state of Louisiana than in the state of Oklahoma (4.4%). The highest incidence of positives

occurred with the sediment samples in both states. Sediments were obtained from the bottom of water troughs. Previous research in this area has shown that water troughs can harbor *E. coli* O157:H7 for extended periods of time (Hancock et al., 1998; Murinda et al., 2004; Polifroni et al., 2012; Wetzel and LeJeune, 2006) and that an estimated 25% of samples from cattle water supplies contain the pathogen (Sanderson et al., 2006). Organic material in troughs has also been suspected to shield and protect *E. coli* O157:H7 and other EHEC in water troughs (Ahmadi et al., 2007). The high occurrence of the pathogen in sediment samples seen in this study may also be due to this protective effect.

Of the six non-O157 STEC, the O26, O45, and O103 serogroups were the most prevalent in both states. The O45 serogroup predominated in Louisiana, while the O103 serogroup predominated in Oklahoma. Overall, STEC isolates were obtained from five serogroups in Oklahoma, while only three serogroups yielded STEC isolates from Louisiana. In the case of all six non-O157 serogroups tested in this study, the percentage of isolates that confirmed as STEC (both *stx*$^+$ and *eae*$^+$) were much lower than the reported prevalence of the serogroup itself. This trend was also seen by a study conducted by Chase-Topping et al. (2012) where the number of non-O157 STEC decreased considerably at the farm and animal levels.

B. Determination of farm management practices and identification of contamination sources

The various farm management practices in place in small-scale cow/calf operations in both states were identified from the results of the production practices survey. In order to identify possible contamination sources, responses from the survey

were pooled with the prevalence results and statistically analyzed to identify any relationship between the farm management practices on cow-calf operations outlined in the survey and the prevalence of the pathogenic *E. coli* investigated in the study. A regression analysis was carried out in order to determine any relationship between animal density and prevalence of pathogens on the farm environment.

Potential sources of contamination on the farm were identified as: water source, method of water access to cattle on the farm (water container), type of feed, breed, and animal density. The relationship between cleaning frequency of common cattle contact areas such as water troughs, trailers, alleyways, chutes, farm equipment, and feed bins and prevalence of STEC serogroups was also analyzed.

1. Water source and water container

a. Determination of on-farm management practices

Various water sources were used on cow/calf operations in both states. Water sources were categorized as: rivers/streams, well water, runoff capture, and city/municipal water. The distribution of water sources used on cow/calf operations in the states of Louisiana and Oklahoma are shown in Figure 1. Likewise, methods with which water was supplied to the animals on the farm varied from pasture to pasture. The types of water container, or water access method, were categorized as: creeks, dirt-stock tanks, cement/metal water troughs, and continuous flow tanks (Figure 2).

Of the 27 pastures in Oklahoma used in this study, 21 were using a combination of water sources. In general, a river or stream provided water to the creeks on the pastures surveyed in Oklahoma. Likewise, runoff water was generally used to fill ponds or dirt

stock tanks. The continuous flow tanks or cement/metal stock tanks were either supplied with water from a well or from municipal water. Of the 18 pastures in Louisiana used in this study, only two used a river or stream as a water source. Municipal water was used by 12 pastures, while well water was used by the remaining five pastures (Figure 1). In each case, there were no overlapping sources of water.

Figure 1. Distribution of water sources used in Oklahoma and Louisiana cow/calf operations

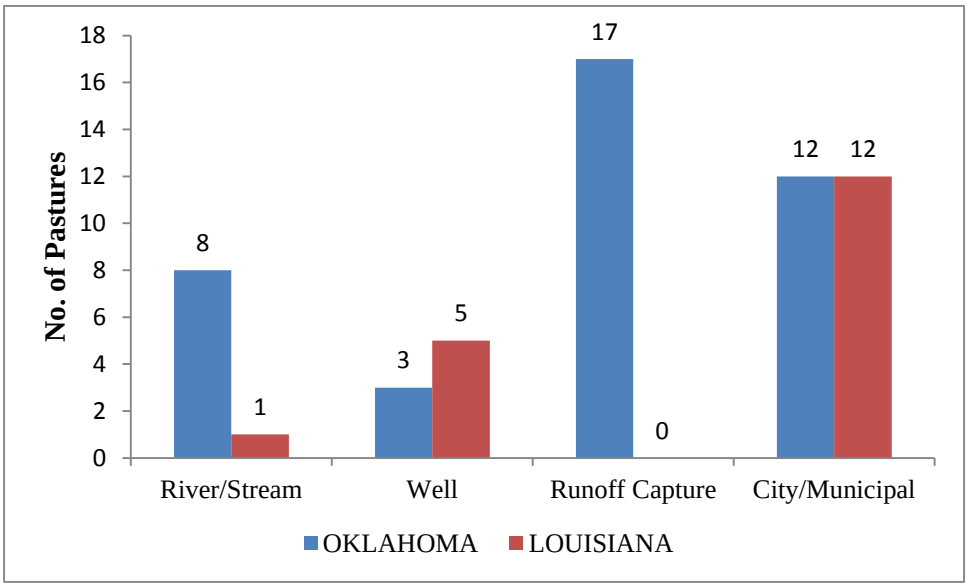
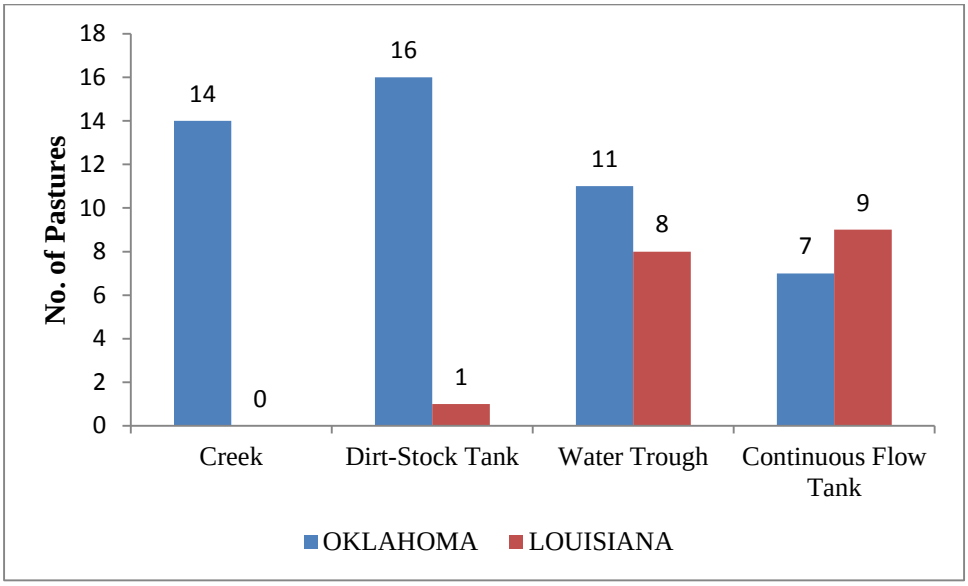


Figure 2. Distribution of water containers used in Oklahoma and Louisiana cow/calf operations



b. Identification of contamination sources

i. Oklahoma

Results from the study indicate that the type of water source and water access method may have an effect on the prevalence of *E. coli* O157:H7. The use of rivers and streams as a water source showed a statistically higher ($P<0.05$) occurrence of 27.5% of *E. coli* O157:H7 (Table 5) in comparison to only 4.3% occurrence without. A similar trend was observed with the O26 and O111 serogroups that showed a statistically significant ($P<0.05$) relationship with the use of rivers and streams as a water source. For the O26 serogroup, an occurrence of 14.3% was observed. Using rivers and streams as a water source gave a 2.5% occurrence of O111 in comparison to only 0.26% occurrence without. Meanwhile, the use of runoff as a water source showed a significantly higher ($P<0.05$) occurrence of 15.4% *E. coli* O157:H7 (Table 5) in comparison to 4% without.

In both instances, the occurrence of *E. coli* O157:H7 without the use of rivers or streams and runoff was less than 5% (results not shown). However, it was also observed that the occurrence of O45 on these farms was significantly lower ($P<0.05$) at 10% when municipal water was used on the farm in comparison to an occurrence of 29.7% without. Also, for the O121 serogroup, a significantly higher occurrence ($P<0.05$) of 13.3% was observed when well water was used as the water source than without (2.9%).

Water was made accessible to the animals using a combination of methods on 21 of the pastures that were used in this study from Oklahoma. These water access methods were: (1) creeks, (2) ponds or dirt stock tanks, (3) cement or metal stock tacks, and (4) continuous flow tanks (Figure 3). Both creeks and ponds showed a significantly higher ($P<0.05$) occurrence of *E. coli* O157:H7 (17.9% and 16.3%, respectively) (Table 6) in comparison to without the use of these methods of water access. The use of creeks on the farm also indicated that the occurrence of O26 was significantly ($P<0.05$) higher at 13.2% (Table 5) than without (4.2%). Using cement or metal stock tanks also seemed to show a significantly higher ($P<0.05$) occurrence of *E. coli* O157:H7 (Table 6), showing an observed 20.9% occurrence when this method was used, in comparison to a 4.5% occurrence when it was not in use.

The use of continuous flow tanks, however, showed variable results for the various serogroups (Table 5). For *E. coli* O157 a significantly lower ($P<0.05$) occurrence of 0.36% was observed when continuous flow tanks were used in comparison to without (15%). The O26 serogroup also showed a lower occurrence (2.9%) when continuous flow tanks were used than when not in use (11%). This trend was reversed in the case of O45,

however, and a higher occurrence of 25.5% was noted in comparison to the significantly lower ($P<0.05$) occurrence of 7.9%.

Rivers, streams, and runoff are all surface water sources and this water is exposed to many other animals (such as birds and other wildlife like deer) outside the farm that may contaminate the water. The water generated from these sources may then be collected and distributed to the cattle on farms via creeks, ponds, or dirt stock tanks. It was also noted during the fecal collection portion of the study that cattle not only bathed in these creeks, ponds, or dirt stock tanks, but that the majority of fresh fecal pats were easily found close to these water access sites. Hence, should a super-shedder be amongst the herd that drank from these sites, the water could easily be contaminated. Therefore, an increase in the prevalence of *E. coli* O157:H7 and the non-O157 STEC in these sources and the corresponding modes of water accessibility provided for the animals on the farms (creeks, ponds and dirt stock tanks) is to be expected. This can be seen in the results of the study as well, where rivers/streams and runoff water, and creeks and ponds/dirt stock tanks showed a significant ($P<0.05$) increase in the occurrence of the pathogen).

Municipal water is chemically treated and tested for presence of coliforms as well as pathogens before being distributed to the general public. Well water, on the other hand, generates from ground water, and is expected to generally harbor less microbial contamination than surface water. It may be expected, therefore, that water generated from these sources may harbor less pathogenic bacteria. As observed, farms that used well and municipal water also utilized cement/metal stock tanks, or continuous flow tanks. However, while the use of continuous flow tanks showed a very low *E. coli*

O157:H7 and O26 occurrence (0.36% and 2.9% respectively), the use of cement and/or metal stock tanks showed a higher occurrence (20.9%) than without (4.5%). A likely explanation for the difference in results in these two methods of water accessibility may be due to the exposure of the water in the cement and metal stock tanks to rainwater and wildlife in comparison to the continuous flow tanks. Also, cement and metal stocks tanks may be used by more than one animal at a time. In comparison, the continuous flow tanks in Oklahoma were small enough that only one animal may drink from it at a time. Contact with other cattle would therefore be minimized if using automated troughs.

Likewise, it should also be noted that the above explanation does not satisfactorily explain the results obtained for the O45 serogroup, which showed an increased prevalence when continuous flow tanks were in use, or the O121 serogroup that showed an increased prevalence when well water was used. For the discrepancy seen in the trend with the continuous flow tanks, a number of factors should be taken into consideration. Cattle on the pasture may have access to water contained in continuous flow tanks, but also have access to water contained in creeks, ponds, or cement/metal stock tanks. In Oklahoma, six farms that used continuous flow tanks also had at least one other type of water container. Also, in farms that have more than one type of water access method, the more open creeks, ponds, and cement or metal stock tanks may be favored by the animals than an isolated continuous flow tank. The frequency with which these sites are used will also have an impact on the transmission of pathogens among a herd, as well as occurrence within the farm environment. Likewise, while well water may be expected to carry less microbial contamination than surface water, out of the 6 farms that were using well water as a water source, all used these in open cement or metal stock tanks. The

opportunity for water in such an open container to become contaminated in the farm environment is very high.

Table 5. Association between occurrence of STEC and types of water source and water container used in cow/calf operations in Oklahoma

	Positive occurence (%) of STEC serogroups						
	O157	**O26**	**O45**	**O103**	**O111**	**O121**	**O145**
Water source							
River/stream	27.5[A]	14.4[A]	14.4[C]	16.3[C]	2.5[A]	3.1[C]	5.6[C]
Well water	16.7[C]	6.7[C]	21.7[C]	8.3[C]	1.7[C]	13[A]	4.2[C]
Runoff	15.4[A]	8.8[C]	23.5[C]	14.7[C]	0.6[C]	5.3[C]	2.9[C]
Municipal/city water	12.8[C]	5.8[C]	10[B]	7.5[C]	0.4[C]	2[C]	5.4[C]
Water container							
Creeks	17.9[A]	13.2[A]	26.4[C]	16.4[C]	14[C]	3.9[C]	3.9[C]
Dirt stock tank	16.3[A]	9.4[C]	22.5[C]	13.8[C]	0.6[C]	5.6[C]	3.1[C]
Cement/metal stock tank	21[A]	9.1[C]	18[C]	16.8[C]	0.9[C]	6.8[C]	5.9[C]
Continuous flow tank	15[B]	2.9[B]	25.5[A]	11.4[C]	0[C]	1.4[C]	3.6[C]

[1] Average occurrence of STEC serogroups when corresponding water source or water container was present is shown. Significant differences (P<0.05) in occurrence obtained are shown in comparison to when the corresponding water source or container was not present. Average occurrence of STEC serogroups when corresponding category was not present is not shown.

[2] A = significantly higher occurrence in comparison to pastures without corresponding category; B = significantly lower occurrence in comparison to pastures without corresponding category; C = no significant difference between pastures with and without corresponding category

[3] Fecal, water, sediment, and equipment samples analyzed for O157, only fecal samples analyzed for other STEC serogroups

ii. Louisiana

Results from the study show a statistically higher occurrence of 25% for the O26 serogroup (P<0.05) when municipal water was used (Table 6) than when municipal water was not the source of water to the farm (4.2%). On the other hand, using well water showed a significantly lower (P<0.05) occurrence of the same serogroup (5%) (Table 6) when compared to without (23%).

The Louisiana farms used in this study allowed cattle access to water in ponds or dirt stock tanks, cement or metal stocks tanks, or automated troughs. There were no overlapping types of water access. Of the 18 pastures sampled, one used ponds, 9 used automated troughs, and 8 used metal or dirt stock tanks (Figure 3). No statistically significant differences in occurrence were observed when ponds or dirt stock tanks were used (Table 6). Using continuous flow tanks showed a significantly higher ($P<0.05$) occurrence of the non-O157 serogroups O45, O103, and O121 (43.3%, 30%, and 32.7% respectively) (Table 6). Meanwhile, using cement or metal stock tanks showed a statistically lower ($P<0.05$) occurrence of 9.3% for the O121 serogroup (Table 6) in comparison to not using this means of water container (29.5%).

Five of the nine farms that used continuous flow tanks as water containers on their pastures also used municipal water as their water source. While the opposite trend was seen in Oklahoma with continuous flow tanks and municipal water, it is also important to note that the continuous flow tanks in Louisiana were larger than those used in the state of Oklahoma and were not automated, allowing access to more than one animal at a time. Also, unlike in the state of Oklahoma, water access methods in Louisiana did not overlap. Cattle in Louisiana therefore are in constant contact with each other while feeding from continuous flow tanks, and with no other open source of water to drink from, may easily contaminate the water in continuous flow tanks, making re-infection and cross-contamination within the herd easier.

Table 6. Association between occurrence of STEC and types of water source and water container used in cow/calf operations in Louisiana

	Positive occurrence (%) of STEC serogroups						
	O157	**O26**	**O45**	**O103**	**O111**	**O121**	**O145**
Water source							
River/stream	12[C]	0[C]	0[C]	0[C]	0[C]	0[C]	0[C]
Well water	17[C]	5[B]	34[C]	17[C]	1[C]	29[C]	5[C]
Municipal/city water	21[C]	25[A]	37[C]	24[C]	3.8[C]	18.8[C]	8.3[C]
Water container							
Dirt stock tank	12[C]	0[C]	0[C]	0[C]	0[C]	0[C]	0[C]
Cement/metal stock tank	19[C]	13.8[C]	28[C]	13[C]	1.9[C]	9.4[B]	0.6[C]
Continuous flow tank	20[C]	23.9[C]	43[A]	30[A]	3.9[C]	33[C]	13.3[C]

[1] Average occurrence of STEC serogroups when corresponding water source or water container was present is shown. Significant differences ($P<0.05$) in occurrence obtained are shown in comparison to when the corresponding water source or container was not present. Average occurrence of STEC serogroups when corresponding category was not present is not shown.

[2] A = significantly higher occurrence in comparison to pastures without corresponding category; B = significantly lower occurrence in comparison to pastures without corresponding category; C = no significant difference between pastures with and without corresponding category

[3] Fecal, water, sediment, and equipment samples analyzed for O157, only fecal samples analyzed for other STEC serogroups

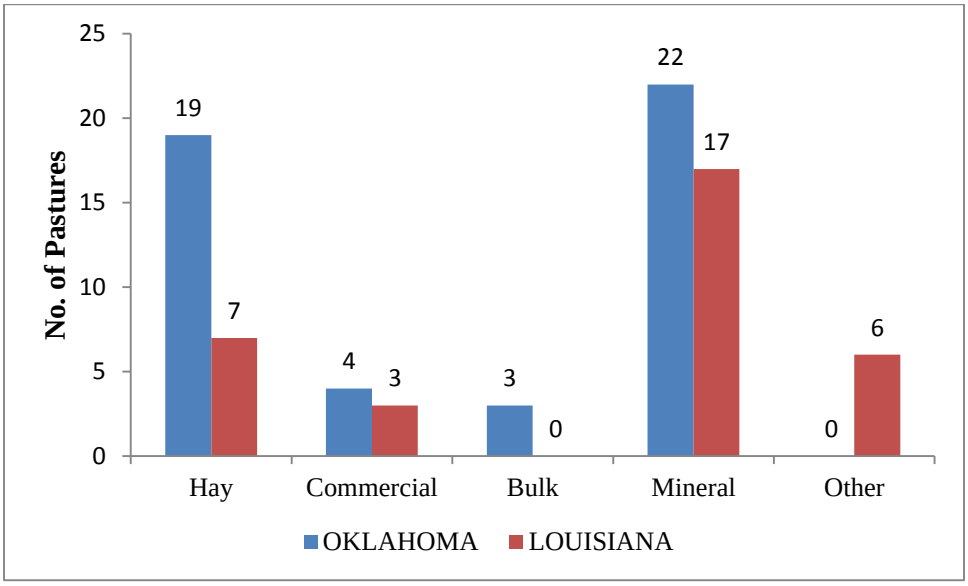
2. Feed

a. Determination of on-farm management practices

A wide variety of feed was provided to cattle on the cow/calf operations sampled during this study. The types of feed, besides pasture grass, provided to animals were categorized as: hay, commercial feed, bulk feed, mineral blocks, and other (mainly protein supplements). In Oklahoma, in addition to pasture grass, cattle were given hay, commercial feed, bulk feed, and mineral blocks (Figure 3), or a combination thereof. A combination of these types of feed was provided to cattle on 21 of the pastures sampled. Apart from pasture grass, Louisiana farms used in this study fed their cattle either hay, commercial feed, provided mineral blocks, or other protein supplements (Figure 3).

Seven of the 18 pastures sampled were using a combination of two or more of the aforementioned sub-categories.

Figure 3. Distribution of types of feed used in Oklahoma and Louisiana cow/calf operations



[1] Other = protein supplements, mineral supplements

b. Identification of contamination sources

i. Oklahoma

Statistically significant ($P<0.05$) differences in occurrence of the pathogens under study were seen only in the 'hay' sub-category, and only with the O157 serogroup (Table 7). Nineteen of the 27 pastures from Oklahoma used in this study provided their cattle with hay in addition to pasture grass. The observed occurrence of O157:H7 was significantly ($P<0.05$) higher (14.5%) when hay was provided to cattle than when it was not (3.4%).

Additionally, it should be noted that the state of Oklahoma experienced drought during the first sampling year. In interviews with the farmers during and after this drought period, it was found that, as a result of crop land and pasture land severely affected by the environmental conditions at the time, cattle were fed predominantly hay during this period. Animals were provided hay even during the summer months when they would usually have foraged on pasture grass. Research results from a study conducted by Diez-Gonzalez et al (1998) that investigated the effect of dietary changes on *E. coli* populations in the ruminant gut demonstrated that an abrupt change from grain-based to hay-based diets significantly reduced the general population of *E. coli.* Since these findings, one of the methods proposed as a means of reducing EHEC in cattle is to abruptly shift the diet from one that is grain-rich to forage based. However, subsequent research in this area has yielded variable results (Hancock et al., 2000, Hovde et al., 1999, Keen et al., 1999). Hoyde et al (1999) found that cattle that were fed hay shed *E. coli* O157:H7 longer than those cattle that were fed a grain-rich diet. However, whether results obtained for Oklahoma were a direct result of feeding hay needs more investigation, since pastures that fed cattle hay also fed them a combination of other types of feed. Additionally, other factor may play a role in affecting the prevalence of STEC.

Table 7. Association between occurrence of STEC and type of feed used in cow/calf operations in Oklahoma

	Positive occurence (%) of STEC serogroups						
Type of feed	**O157**	**O26**	**O45**	**O103**	**O111**	**O121**	**O145**
Hay	14.5^{A}	8.7^{B}	21.3^{B}	15.3^{B}	0.8^{B}	4.7^{B}	2.6^{B}
Commercial feed	8.1^{B}	16.3^{B}	21.2^{B}	11.2^{B}	2.5^{B}	3.7^{B}	5^{B}
Bulk feed	3.3^{B}	5^{B}	30^{B}	28.3^{B}	0^{B}	0^{B}	0B
Mineral blocks	10^{B}	9.3^{B}	19.5^{B}	15.4^{B}	1.1^{B}	4.5^{B}	4.5^{B}

[1] Average occurrence of STEC serogroups when corresponding water source or water container was present is shown. Significant differences ($P<0.05$) in occurrence obtained are shown in comparison to when the corresponding water source or container was not present. Average occurrence of STEC serogroups when corresponding category was not present is not shown.

[2] A = significantly higher occurrence in comparison to pastures without corresponding category; B = no significant difference between pastures with and without corresponding category

[3] Fecal, water, sediment, and equipment samples analyzed for O157, only fecal samples analyzed for other STEC serogroups

ii. Louisiana

Feeding hay showed an increased occurrence (31.4%) of the O26 serogroup (Table 8) in comparison to not feeding hay (9.5%). A similar trend was seen with the O111 serogroup (Table 8) where a statistically higher ($P<0.05$) occurrence of 7.1% was observed when hay was fed to cattle than when not (0%). No statistically significant differences in occurrence of any of the serogroups were obtained when considering the mineral block sub-category (Table 8).

With commercial feed, statistically significant ($P<0.05$) differences in occurrence were observed with the O26, O111, and O145 serogroups (Table 8). In all three cases, the use of commercial feed showed an increased occurrence of the respective serogroup. For the O26 serogroup an occurrence of 36.7% seen when commercial feed was used in comparison to 14.3% when not used. With O111, the occurrence of the serogroup on pastures where commercial feed was provided to cattle was 11.7%. An occurrence of

only 1% was observed among this serotype when this type of feed was not used. For O145, a 30% occurrence was seen in pastures that fed cattle commercial feed, in comparison to a 2.3% occurrence on those pastures that did not.

It should be noted that, out of the seven pastures that provided hay to cattle besides pasture grass, three of these pastures also provided cattle with commercial feed. On the pastures that provided their cattle both hay as well as commercial feed, but did not provide the animals with supplementary protein, the average occurrence of O26 was 36.7%, with occurrence ranging from 0-100% of O26 on these pastures. Likewise, the average occurrence of O111 on these pastures was 11.7% (Table 8) with the occurrence on these farms ranging from 0-40% (results not shown). For the O145 serogroup, an average occurrence of 30% (Table 8) was observed, ranging from 0-100% (results not shown) on these farms. The occurrence for each of the serogroups that seemed to be significantly affected ($P<0.05$) by the provision of hay and commercial feed to the animals were highest in the farms that fed their cattle a combination of these two types of feed. It is also interesting to note that, while the occurrence of *E. coli* O157:H7 was not significantly affected when cattle were fed hay and commercial feed, the highest occurrence of *E. coli* O157:H7 (41%) was seen on a pasture that used this combination of feed. The overall occurrence range for *E. coli* O157:H7 on these pastures was between 0-41% (results not shown), with an average occurrence of 20.5% on each of these pastures. These results therefore indicate that feeding cattle both hay as well as commercial feed in addition to pasture grass may increase the occurrence of STEC in the farm environment.

Three pastures provided cattle with other (generally protein supplements) feed in addition to pasture grass. In this category, significant differences ($P<0.05$) in occurrence

were seen with the O26 and O45 serogroups only (Table 8). In both cases, using this type of feed showed an increased occurrence of the respective serogroup. An occurrence of 36.7% was seen with O26 on pastures that fed their cattle this type of feed, while a occurrence of 14.3% was observed with pastures that did not. Meanwhile, with O45, there was an observed 75% occurrence on pastures where cattle were fed this type of feed.

It is interesting to note that all three pastures that provided cattle with protein supplements in their diet also provided these animals with hay. The occurrence of O26 on these pastures ranged from 10-60% with three of the six farms in this category showing a occurrence of at least 50% O26. Meanwhile the occurrence of O45 ranged from 20-100% on farms that provided their cattle a hay and protein supplement feed combination with two of the three farms in this category showing an occurrence of at least 90% for the O45 serogroup. The highest occurrence of O45 (100%) was seen in pastures that fed hay as well as protein supplements to cattle. Additionally, while the occurrence of *E. coli* O157:H7 did not seem to be significantly affected when this feed combination was used, it should be noted that its occurrence ranged from 14-32% on these pastures. Two of the three pastures in this category showed an occurrence of 32% *E. coli* O157:H7. Although no significant differences in occurrence were observed for the O157:H7 strain, a higher occurrence of the strain was observed in pastures that fed their cattle either a combination of hay and commercial feed, or hay and protein supplements. On average, the occurrence of *E. coli* O157:H7 was 19% for all pastures, while the occurrence for those pastures that fed their cattle either hay and commercial feed or hay and protein supplements, the average occurrence amounted to 24% (results not shown).

Table 8. Association between occurrence of STEC and type of feed used in cow/calf operations in Louisiana

	Positive occurrence (%) of STEC serogroups						
Type of feed	**O157**	**O26**	**O45**	**O103**	**O111**	**O121**	**O145**
Hay	23.8^{B}	31.4^{A}	45^{B}	19.3^{B}	7.1^{B}	13.6^{B}	13.6^{B}
Commercial feed	20.5^{B}	36.7^{A}	30^{B}	10^{B}	11.7^{A}	6.7^{B}	30^{B}
Mineral blocks	19^{B}	19.1^{B}	36^{B}	22^{B}	2.9^{B}	2.2^{B}	7.4^{B}
Other	28.2^{B}	36.7^{A}	75^{A}	35^{B}	3.3^{B}	25^{B}	1.7^{B}

[1] Average occurrence of STEC serogroups when corresponding type of feed was present is shown. Significant differences ($P<0.05$) in occurrence obtained are shown in comparison to when the corresponding type of feed was not present. Average occurrence of STEC serogroups when corresponding category was not present is not shown.

[2] A = significantly higher occurrence in comparison to pastures without corresponding category; B = no significant difference between pastures with and without corresponding category

[3] Fecal, water, sediment, and equipment samples analyzed for O157, only fecal samples analyzed for other STEC serogroups

3. Breed

One of the factors investigated in this study was whether the breed of cattle played a role in influencing the prevalence of STEC found in a farm environment. It has already been established that fecal shedding of *E. coli* O157:H7 and other STEC has also been shown to be seasonal, with peaks in prevalence generally observed during the summer months and dips in prevalence observed in the colder winter season (Chapman et al. 1997; Hancock et al., 1997a; Van Donkersgoed et al., 1999). Additionally, studies have been done on various breeds of cattle in order to determine whether particular breeds of cattle were more susceptible to heat stress than others (Brown-Brandl et al., 2006). Results from the studies conducted by Brown-Brandl et al. (2006) indicate that breed may play a role in determining an animal's ability to tolerate heat. Furthermore, these studies have also shown that the color of the hide and fatness of the animal contribute to the animal's susceptibility towards heat stress. In general, breeds with darker hides and those breeds that quickly accumulated more body fat were at a higher

risk of heat stress (Brown-Brandl et al., 2006). Studies show that heat stress, being one type of physical stress to the host animal (Rostagno, 2009), may have an effect in the shedding patterns of gastrointestinal pathogenic microbes such as *E. coli* O157:H7 and *Salmonella spp*. (Edrington et al., 2004; Rostagno, 2009).

a. Determination of on-farm management practices

Out of the 27 pastures sampled from Oklahoma, 26 pastures had herds containing Angus cattle. One of these pastures also had Charolais cattle in their herd, and another had Simmental in addition to Angus. Seven pastures had various breeds of cattle within a herd, out of which one pasture also had Limosine cattle. One pasture had a herd comprising only Hereford cattle. Meanwhile, of the 18 pastures used in this study from Louisiana, one herd was predominantly Gelbvieh, eight were herds comprising the Brangus breed of cattle, and three herds were made up of predominantly Baldie cattle. Seven of the 18 pastures had cattle from a variety of breeds.

b. Identification of contamination sources

i. Oklahoma

For the results from Oklahoma, only the mixed breeds sub-category showed statistically significant ($P<0.05$) differences in occurrence of the pathogens being investigated (Table 9). Significant ($P<0.05$) results were only seen with *E. coli* O157:H7. The presence of mixed breeds in a herd showed a lower occurrence (0.36%) of O157:H7 (Table 9) than when herds comprised only a single breed (15%).

It is important to note that 96% of the pastures sampled from Oklahoma had herds with predominantly Angus cattle. Cattle belonging to the Angus breed have black hides. According to previous studies done in order to establish a relationship between breed and susceptibility to heat stress, cattle with darker hides are at a higher risk of heat stress than those with lighter hides (Brown-Brandl et al., 2006a, 2006b). Studies done in order to observe patterns of microbial shedding in animals subjected to environmental and physical stress also indicate that heat stress may increase shedding of *E. coli* O157:H7 (Edrington et al., 2004; Rostagno, 2009).

Table 9. Association between occurrence of STEC and breed of cattle in cow/calf operations in Oklahoma

	Positive occurrence (%) of STEC serogroups						
Breed	**O157**	**O26**	**O45**	**O103**	**O111**	**O121**	**O145**
Angus	11.9^{B}	9.4^{B}	19.6^{B}	15^{B}	1^{B}	4.4^{B}	4.4^{B}
Hereford	2.5^{B}	0^{B}	40^{B}	30^{B}	0^{B}	0^{B}	0^{B}
Simmental	5^{B}	10^{B}	15^{B}	20^{B}	0^{B}	0^{B}	0^{B}
Limosine	2.5^{B}	5^{B}	35^{B}	35^{B}	0^{B}	2.1^{B}	0^{B}
Charolais	0^{B}	1.2^{B}	0^{B}	0^{B}	0^{B}	0^{B}	0^{B}
Mixed breeds	0.36^{A}	5^{B}	22.1^{B}	22.1^{B}	0^{B}	0^{B}	3.2^{B}

[1] Average occurrence of STEC serogroups when corresponding cattle breed was present is shown. Significant differences (P<0.05) in occurrence obtained are shown in comparison to when the corresponding cattle breed was not present. Average occurrence of STEC serogroups when corresponding category was not present is not shown.
[2] A = significantly lower occurrence in comparison to pastures without corresponding category; B = no significant difference between pastures with and without corresponding category
[3] Fecal, water, sediment, and equipment samples analyzed for O157, only fecal samples analyzed for other STEC serogroups

ii. Louisiana

Overall, with the exception of Gelbvieh, all other breeds showed significant differences (P<0.05) in the occurrence of particular non-O157 serogroups. With the exception of herds with more than one breed of cattle, significantly higher (P<0.05)

occurrence of non-O157 serogroups were seen when only a single breed of cattle were in a herd. Pastures with the Baldie breed of cattle showed an increased occurrence of O26, O111, and O145 serogroups while the presence of Brangus cattle in a herd showed a significant increase ($P<0.05$) in the occurrence of the O45 serogroup (Table 10). The presence of mixed breeds in a herd, however, showed a significantly ($P<0.05$) lower occurrence of O45 (Table 10). When cattle from mixed breeds were present, the observed occurrence of the O45 serogroup in these pastures was 18.6% in comparison to those pastures where single breeds were present in the herd (44%).

Although no significant differences ($P<0.05$) in occurrence for O157:H7 were observed with the Louisiana pastures, an overall average occurrence of 22% for O157:H7 was seen when mixed breeds were present, and an occurrence of 20% and 14.4% was observed when the herds comprised Brangus and Baldie, respectively. In general the average occurrence of *E. coli* O157:H7 per pasture for Louisiana was 19%. Although Brangus may also have darker hides, they are also known for their resistance to disease and their ability to resist heat and high humidity. However, in general, the average occurrence of *E. coli* O157:H7 in Louisiana with Brangus was higher than in Oklahoma with Angus.

A common trend seen with results from both states is that the presence of cattle from mixed breeds show a significantly ($P<0.05$) decreased prevalence of STEC (O45 serogroup in Louisiana and *E. coli* O157:H7 in Oklahoma). In Oklahoma, these mixed breeds also had predominantly black hides, while those in Lousiana were predominantly red or brown.

Table 10. Association between occurrence of STEC and breed of cattle in cow/calf operations in Louisiana

	Positive occurrence (%) of STEC serogroups						
Breed	**O157**	**O26**	**O45**	**O103**	**O111**	**O121**	**O145**
Gelbvieh	20.5[C]	0[C]	0[C]	0[C]	5[C]	0[C]	0[C]
Brangus	20.1[C]	16.9[C]	49.4[A]	23.7[C]	1.2[C]	27.5[C]	3.7[C]
Baldie	20.1[C]	36.7[A]	30[C]	10[C]	11.7[A]	6.7[C]	30[A]
Mixed breeds	21.8[C]	11.4[C]	18.6[B]	22[C]	0.7[C]	18.6[C]	0.7[C]

[1] Average occurrence of STEC serogroups when corresponding cattle breed was present is shown. Significant differences ($P<0.05$) in occurrence obtained are shown in comparison to when the corresponding cattle breed was not present. Average occurrence of STEC serogroups when corresponding category was not present is not shown.

[2] A = significantly higher occurrence in comparison to pastures without corresponding category; B = significantly lower occurrence in comparison to pastures without corresponding category; C = no significant difference between pastures with and without corresponding category

[3] Fecal, water, sediment, and equipment samples analyzed for O157, only fecal samples analyzed for other STEC serogroups

4. Animal density

Escherichia coli O157:H7 and the other non-O157 Shiga toxin-producing bacteria are enteric pathogens. Following their fecal-oral lifestyle, these bacteria are shed in the feces of animals. This is especially true with cattle when considering the farm environment and their status as the primary reservoirs for *E. coli* O157:H7 and other STEC (Capriola et al., 2005; Elder et al., 2000; Smith et al., 2001). Close proximity of animals would then be expected to increase the likelihood of an animal in coming into contact with the contaminated feces of another animal. The close proximity of these animals in feedlot pens where vertical integration practices are common makes dissemination of STEC throughout a herd becomes easier.

Likewise, super shedders on the farm and in cow-calf operations may contribute to the persistence of these pathogens in the farm environment. More frequent contact with these super shedders or their feces would increase the rate at which these STEC are

disseminated throughout the herd as well as increase persistence on the farm. Therefore, a lower animal density per acre of farmland will be expected to reduce contact between animals and thereby reduce the prevalence of STEC on the pasture. In this study, the correlation between the density of animals on a pasture had with the prevalence of the seven pathogen STEC serogroups was investigated.

Animal density for corresponding cow/calf pastures sampled in the states of Oklahoma and Louisiana are outlined in Table 12, showing an overall higher animal density in cow/calf operations in Louisiana than in Oklahoma. The results from Louisiana showed a negative correlation between animal density and occurrence of O45 (Table 11). For pastures sampled in the state of Oklahoma, results varied with the different serogroups (Table 11). A positive correlation between the animal density and the occurrence of O45 and O103 serogroups was observed, indicating that the occurrence of these *E. coli* serogroups increased as the animal density on the farm increased. At the same time, a negative correlation between animal density and occurrence of the O26 and O121 serogroups was also observed. No significant ($|r|<0.5$) correlation between *E. coli* O157:H7 and animal density was observed from results obtained for either state.

It is difficult, therefore, to conclude with certainty that an increased animal density would result in an observed higher occurrence, in general, of the pathogenic shigatoxigenic *E. coli*, although it should be noted that animal density had varying effects on different serogroups. Also, in general, animal density on cow-calf operations are lower than that in feedlots. Additionally, it is important to note that despite the lower animal density, calves would usually stay very close to their mothers within a herd.

Table 11. Correlation of animal density with occurrence of STEC on cow/calf operations in Oklahoma and Louisiana

	STEC serogroup						
Oklahoma	**O157**	**O26**	**O45**	**O103**	**O111**	**O121**	**O145**
Correlation coefficent*	0.2593	0.1358	0.1264	0.5666	-0.1408	0.4214	-0.0741
|r|**	0.1267	0.4296	0.4627	0.0003	0.4125	0.0105	0.6677
Louisiana	**O157**	**O26**	**O45**	**O103**	**O111**	**O121**	**O145**
Correlation coefficient	-0.1676	-0.0356	0.0230	0.0449	-0.1211	-0.0153	0.2630
|r|	0.2257	0.7984	0.8688	0.7468	0.3830	0.9126	0.0546

* Pearson's correlation coefficient; positive values indicate positive correlation, negative values indicate negative correlation

** $|r| > 0.5$ indicates significant correlation

Table 12. Animal density (cattle/acre) of corresponding cow/calf pastures sampled in Oklahoma and Louisiana

OKLAHOMA				LOUISIANA	
Pasture	**Animal Density**	**Pasture**	**Animal Density**	**Pasture**	**Animal Density**
A	0.4	**S**	0.24	**A**	2.3
B	0.53	**T**	0.23	**B**	3.2
C	0.53	**U**	1.75	**C**	3.5
D	0.44	**V**	1.75	**D**	2
E	0.44	**W**	1.75	**E**	1.3
F	0.31	**X**	1.2	**F**	10.8
G	0.6	**Y**	1.2	**G**	10.8
H	0.6	**Z**	1.2	**H**	13
I	0.33	**A1**	0.18	**I**	2.1
J	0.33			**J**	1.7
K	0.33			**K**	1
L	0.14			**L**	1.3
M	0.14			**M**	2.4
N	0.14			**N**	0.6
O	0.14			**O**	0.6
P	0.23			**P**	0.86
Q	0.11			**Q**	0.86
R	0.42			**R**	0.86

5. Frequency of cleaning common cattle contact areas

Cattle that become infected with *E. coli* O157:H7 and other STEC can cross infect each other on the farm. Besides close proximity to one another, which may increase potential for *E. coli* to spread quickly within a herd, contaminated water sources and equipment may also contribute to the dissemination and persistence of *E. coli* O157:H7 on the farm environment.

Previous studies with *E. coli* O157:H7 have found that this bacterium may be more frequently isolated from the sediments and biofilms of the water troughs used to supply drinking water to cattle (Zottola, 1994). Cattle water troughs can harbor *E. coli* O157:H7 for extended periods of time (Hancock et al., 1998; Murinda et al., 2004; Polifroni et al., 2012; Wetzel and LeJeune, 2006;). Additionally, it has also been shown that approximately 25% of cattle water supply samples contain *E. coli* O157:H7 (Sanderson et al., 2006). These results suggest that water troughs can function as vectors for horizontal transmission of *E. coli* O157:H7 within a group of animals. It has also been suggested that organic material in water troughs may harbor and tend to protect EHEC, and modeling research has been able to show that an increase in water trough cleaning frequency leads to an increase in the death rate of *E. coli* O157:H7 (Ahmadi et al., 2007). The use of chlorinated water supplies to feed cattle water troughs (for example, the use of chlorinated municipal water) has been suggested as a means to reduce *E. coli* O157:H7 populations. However, exposure to sunlight and organic material in the water has a tendency to reduce the effectiveness of chlorine over time, and this has been observed in real world chlorination studies with cattle water troughs (Callaway, 2010).

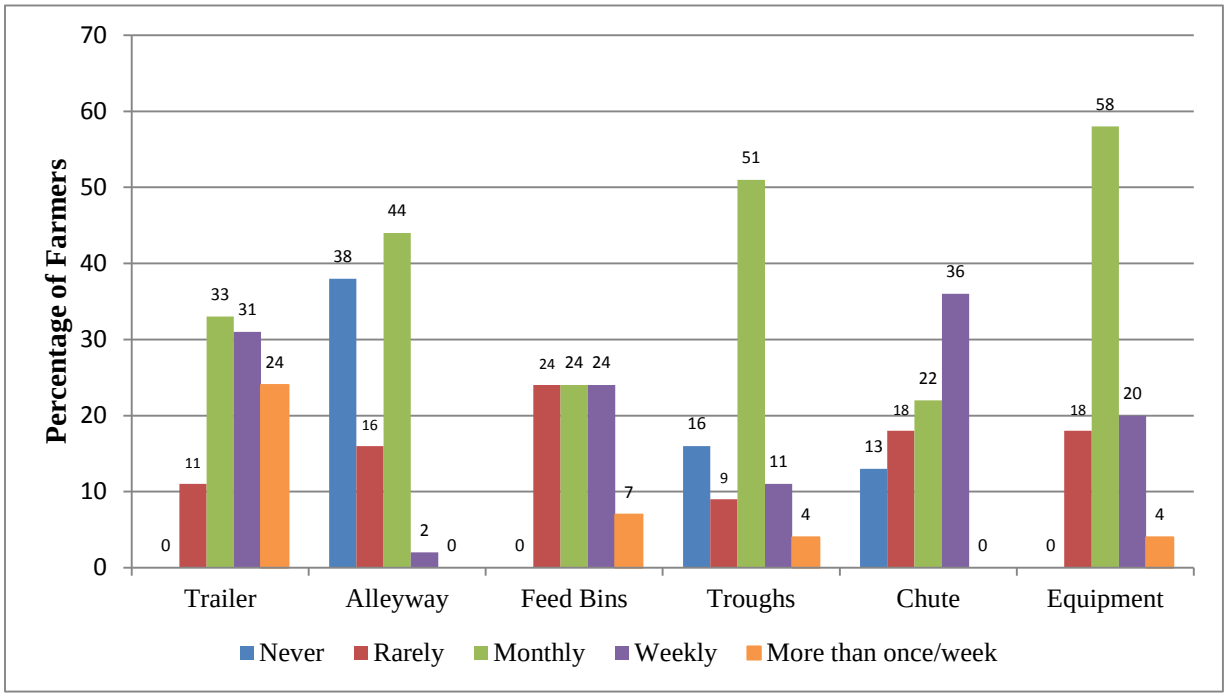
When sampled, cattle trailers have been shown to be frequently positive for *E. coli* O157:H7 (Barham et al., 2002; Reicks et al, 2007) and may present as important vectors of *E. coli* O157:H7 to uninfected cattle. Cattle from cow/calf operations may be transported to feedlots in these trailers, or new batches of cattle may be brought into the farm, transported on trailers. Transportation may add stress to an animal. It has been shown that stress may have an effect on the gut microbiota, resulting in an increase in pathogenic bacterial population within the animal (Carroll et al, 2007; Kelley, 1980; Salak-Johnson and McGlone, 2007).There is an increased risk of transmission of these bacteria to farms and feedlots through cattle on these trailers (Alonso et al., 2007). Although washing of cattle trailers has been shown to be effective only against *Salmonella* contamination in swine (Rajkowski, 1999), it is a logically intuitive solution to prevent some degree of cross-contamination of cattle during a stressful period like transportation.

Other potential sources of cross-contamination among cattle in a herd include alleyways, chutes, and farm equipment. Feed bins were also included in this category because cattle are at very close proximity to each other when feeding at these structures.

a. Determination of on-farm management practices

The percentage of farmers (Oklahoma and Louisiana combined) cleaning common cattle contact areas is shown in Figure 4. The majority of farmers in both states cleaned trailers, alleyways, water troughs and heavy farm equipment at least once a month. The percentage of farmers cleaning these areas of common cattle contact more than once a week was generally low.

Figure 4. Cleaning frequency of common areas of cattle contact on cow/calf operations in Oklahoma and Louisiana



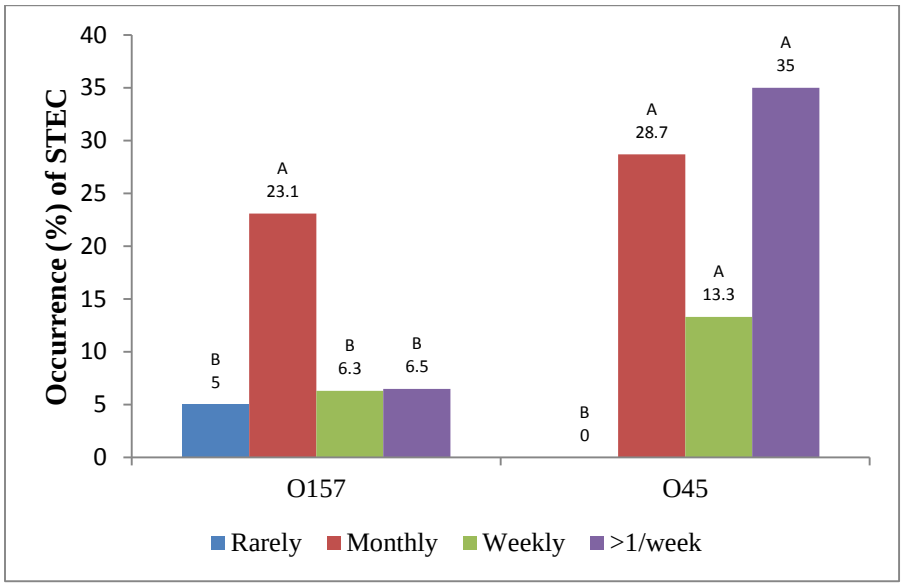
b. Identification on contamination sources

i. Oklahoma

Significant differences in occurrence ($P<0.05$) were observed only for the 'trailer', 'feed bins', and 'chutes' categories in Oklahoma (Figures 5-7). A significantly lower ($P<0.05$) occurrence of *E. coli* O157:H7 was observed on pastures that cleaned trailers either weekly or more than once a week in comparison to those that cleaned their trailers only monthly (Figure 5). With no significant difference ($P>0.05$) observed between levels 4 and 5 (weekly cleaning and cleaning more than once a week), the results indicate that cleaning trailers at least once a week may help significantly reduce the prevalence of *E. coli* O157:H7 on the farm. However, for the O45 serogroup the opposite

trend was seen (Figure 5), indicating that the frequency of cleaning trailers may affect the various serogroups differently. Pastures that cleaned trailers more than once a week showed a significantly higher (P<0.05) occurrence of the O45 serogroup than those pastures that cleaned their trailers only rarely. Only two pastures of the 27 that were sampled from Oklahoma responded as cleaning their trailers at a frequency of more than once a week, and of these two pastures, O45 was detected in only one pasture.

Figure 5. Effect of cleaning frequency of trailers on occurrence of STEC in Oklahoma cow/calf operations

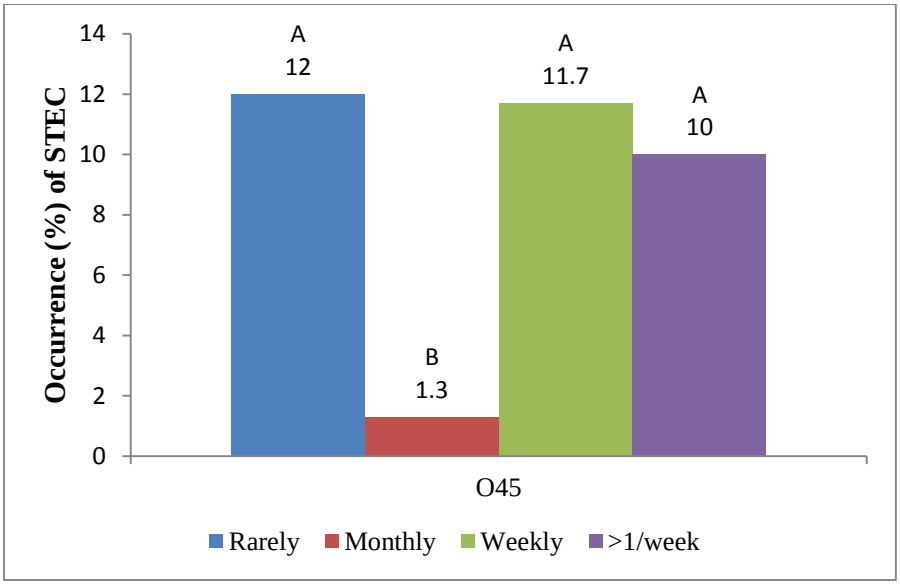


[1] Statistical significance (P<0.05) between mean values is identified by letters A, B. Data bars with different letters represent a significant difference (P<0.05). Statistical significance is calculated between cleaning frequencies for each serogroup.
[2]Fecal, water, sediment, and equipment swab samples used in analysis for O157; only fecal samples used in analysis for non-O157 serogroups

For the 'feed bins' category, significant (P<0.05) results were seen only with the O45 serogroup (Figure 6). More frequent cleaning of feed bins seemed to show an increased occurrence of this serogroup. Pastures that cleaned feed bins on a weekly basis showed higher occurrence of this serogroup than those that cleaned monthly or rarely. Of

the six pastures that cleaned feed bins on a weekly basis, a 0 to 100% occurrence range for this serogroup was seen. No significant difference (P>0.05) in occurrence was observed between pastures that rarely cleaned their feed bins and those that cleaned them on a monthly basis.

Figure 6. Effect of cleaning frequency of feed bins on occurrence of STEC in Oklahoma cow/calf operations

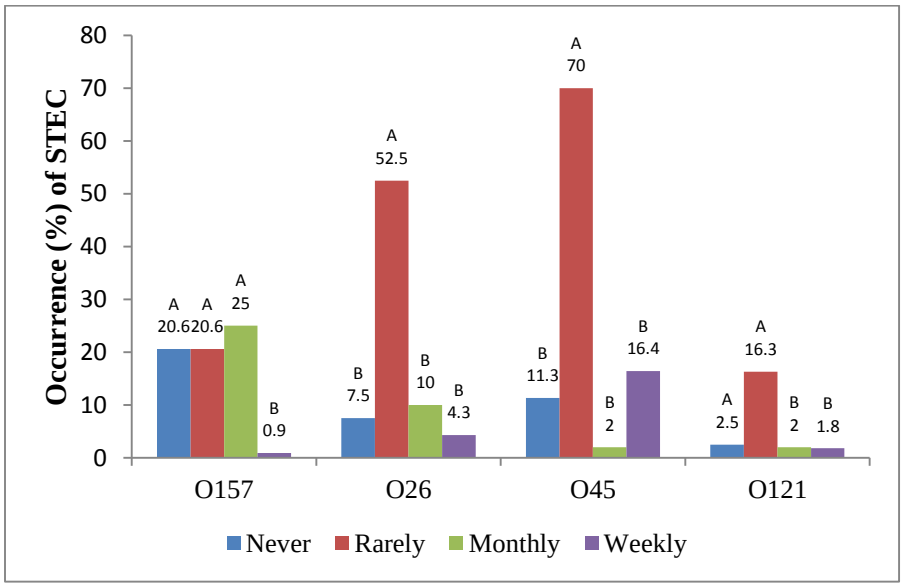


[1] Statistical significance (P<0.05) between mean values is identified by letters A, B. Data bars with different letters represent a significant difference (P<0.05). Statistical significance is calculated between cleaning frequencies for each serogroup.
[2]Only fecal samples used in analysis for non-O157 serogroups

Responses for cleaning chutes ranged from 1 to 4 (never cleaned to cleaning on a weekly basis). For both *E. coli* O157:H7 and *E. coli* O121, more frequent cleaning of chutes showed a lower occurrence of the respective pathogen on the farm. Pastures that cleaned chutes on a weekly basis showed a significantly lower (P<0.05) occurrence of *E. coli* O157:H7 than those pastures that cleaned either rarely, on a monthly basis, or never cleaned their trailers at all (Figure 7). Likewise, for *E. coli* O121, a significantly lower

(P<0.05) occurrence of O121 was observed on pastures that cleaned chutes either monthly or weekly in comparison to those that cleaned chutes only rarely (Figure 7). No significant difference (P>0.05) in occurrence was seen between pastures that cleaned chutes monthly or weekly. Similar results were observed with the O26 serogroup as well. These results indicate that cleaning chutes at least once a week may help reduce the occurrence of *E. coli* O121 and *E. coli* O157:H7 in the farm environment. However, as with the trailer category, variable results were seen with the O45 serogroup for 'chutes' as well, once again indicating that the serogroups may behave differently to different management practices, or that other environmental factors may play a more significant role in determining prevalence.

Figure 7. Effect of cleaning frequency of chutes on occurrence of STEC in Oklahoma cow/calf operations



[1] Statistical significance (P<0.05) between mean values is identified by letters A, B. Data bars with different letters represent a significant difference (P<0.05). Statistical significance is calculated between cleaning frequencies for each serogroup.
[2]Fecal, water, sediment, and equipment swab samples used in analysis for O157; only fecal samples used in analysis for non-O157 serogroups

ii. Louisiana

Significant differences in occurrence ($P<0.05$) were observed for all six on-farm cleanliness categories for the Louisiana samples (Figures 8-13). A significant difference ($P<0.05$) in occurrence of *E. coli* in response to the frequency of cleaning cattle trailers was seen for the O26, O103, O111, and O145 serogroups (Figure 8). Responses for this category ranged from 2 (cleaned rarely) to 5 (cleaned more than once a week). For the O103 serogroup, cleaning trailers more often seemed to have a significant impact ($P<0.05$) on the average occurrence in the farm environment. Pastures that cleaned trailers only rarely showed a significantly higher ($P<0.05$) occurrence of the O103 serogroup than pastures that cleaned trailers either monthly, weekly, or more than once a week. No significant differences in occurrence were observed between the cleaning levels 3, 4, and 5, suggesting that cleaning trailers at least once every month may help to significantly reduce the occurrence of *E. coli* O103 on the farm.

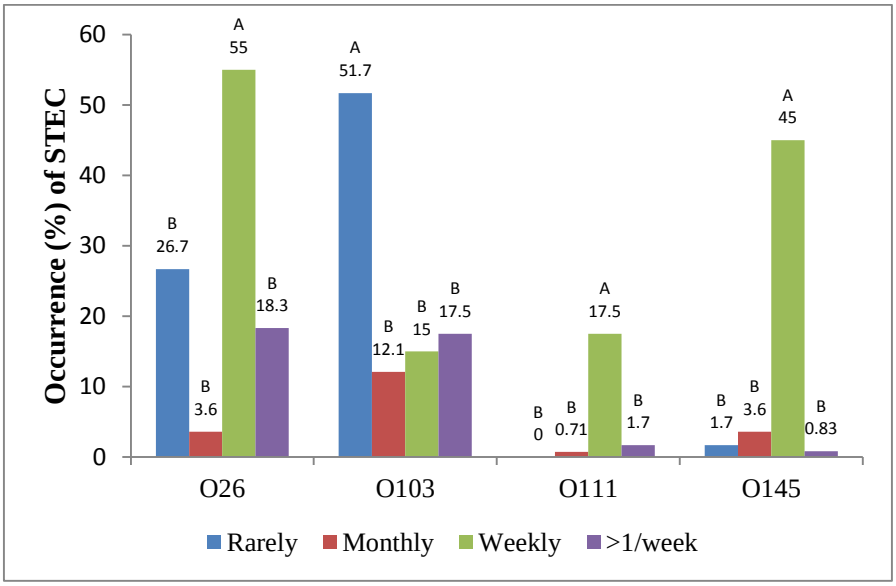
However, variable results were obtained for the O26, O111, and O145 serogroups. For the O26 serogroup, a significant difference ($P<0.05$) in occurrence was seen between pastures that cleaned their trailers at least once a week and those that cleaned their trailers more than once a week, with pastures that cleaned more frequently exhibiting a lower occurrence of the serogroup (Figure 8). However, the occurrence of this serogroup was significantly lower ($P<0.05$) in pastures that cleaned their trailers on an average of once every month in comparison to those that cleaned their trailers once every week. The occurrence of O26 on pastures that cleaned their trailers once a month ranged from 0 to 30%, while pastures that cleaned their trailers once a week and more than once a week ranged from 0 to 50% and 0 to 60% respectively. Only two pastures

responded as cleaning their trailers once a week. Additionally, while not statistically significant ($P>0.05$), the occurrence of O26 on pastures that cleaned their trailers rarely was also lower than that of pastures that cleaned their trailers more than once a week. It is possible, therefore, that the frequency with which trailers are cleaned may not have a direct effect on the occurrence of O26. Results were also contradictory for the O111 serogroup. A significantly lower ($P<0.05$) average occurrence was observed in pastures that cleaned trailers rarely and monthly in comparison to those that cleaned them once every week (Figure 8). On the other hand, a significantly lower ($P<0.05$) occurrence of *E. coli* O111 was seen in pastures that cleaned troughs more than once a week than those that cleaned weekly. However, it is important to note that only two of the 18 pastures sampled tested positive for presence of O111. For the O145 serogroup, a significantly higher ($P<0.05$) occurrence of this serogroup was seen on pastures that cleaned trailers on a weekly basis in comparison to those that cleaned more than once a week (Figure 8). However, a significantly higher ($P<0.05$) average occurrence was also seen when comparing pastures that cleaned trailers weekly to those that cleaned them rarely or monthly (Figure 8). Only seven pastures tested positive for *E. coli* O145 and only four pastures cleaned trailers weekly, out of which two pastures showed 80 to 100% occurrence.

From the results, therefore, it is difficult to confirm if trailer cleaning frequency has a direct impact on the occurrence of STEC serogroups O26, O111, and O145 even though significant differences ($P<0.05$) in occurrence between cleaning levels were seen. No significant results were obtained for *E. coli* O157:H7, or the O45 and O121 serogroups. For the O103 serogroup, however, the results suggest that cleaning trailers at

least on a monthly basis may reduce populations of this serogroup in the farm environment.

Figure 8. Effect of cleaning frequency of trailers on occurrence of STEC in Louisiana cow/calf operations

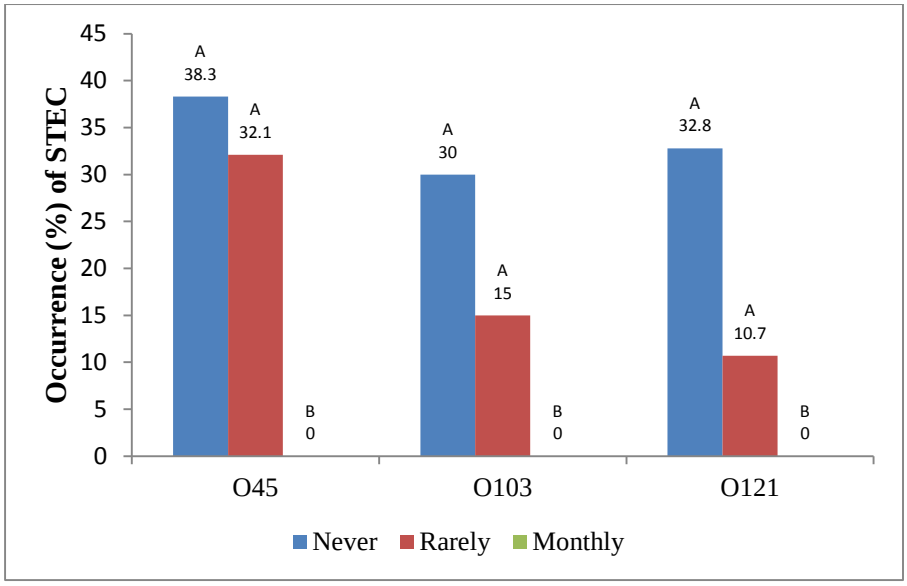


[1] Statistical significance (P<0.05) between mean values is identified by letters A, B. Data bars with different letters represent a significant difference (P<0.05). Statistical significance is calculated between cleaning frequencies for each serogroup.
[2]Only fecal samples used in analysis for non-O157 serogroups

The cleaning frequency of alleyways showed significant differences (P<0.05) in bacterial occurrence with regards to the O45, O103, and O121 serogroups (Figure 9). Responses to cleaning alleyways ranged from 1 (never) to 3 (monthly). In this category, the occurrence of O45 was significantly lower (P<0.05) when alleyways on pastures were cleaned monthly in comparison to never being cleaned. No significant difference (P>0.05) between cleaning once every few months and not cleaning alleyways at all was observed, suggesting that that cleaning alleyways at least once a month may significantly reduce the occurrence of O45 on the farm. Likewise, for the O103 serogroup, a significant difference (P<0.05) in average occurrence was observed when comparing

pastures that never cleaned alleyways to those that cleaned alleyways monthly. The results indicate that cleaning alleyways at least once a month may help reduce *E. coli* O103 occurrence in the farm environment. Similar trends were observed with the O121 serogroup as well. The occurrence of O121 was significantly lower ($P<0.05$) in pastures that cleaned alleyways more frequently. Pastures that did not clean alleyways showed a higher occurrence of *E. coli* O121 than those pastures that cleaned them either rarely or monthly. No significant difference was seen when comparing occurrence of O121 between pastures that cleaned rarely and those that cleaned on a monthly basis ($P>0.05$), indicating that cleaning alleyways at least once every few months would significantly reduce the occurrence of this serogroup in the farm environment. In all cases where significant differences ($P<0.05$) in occurrence were seen, increasing the frequency of cleaning alleyways seemed to show a decreased average occurrence of bacteria. Therefore, farmers that clean alleyways more frequently may expect to see a reduced pathogen load with regards to O45, O103, and O121 serogroups.

Figure 9. Effect of cleaning frequency of alleyways on occurrence of STEC in Louisiana cow/calf operations



[1] Statistical significance (P<0.05) between mean values is identified by letters A, B. Data bars with different letters represent a significant difference (P<0.05). Statistical significance is calculated between cleaning frequencies for each serogroup.
[2]Only fecal samples used in analysis for non-O157 serogroups

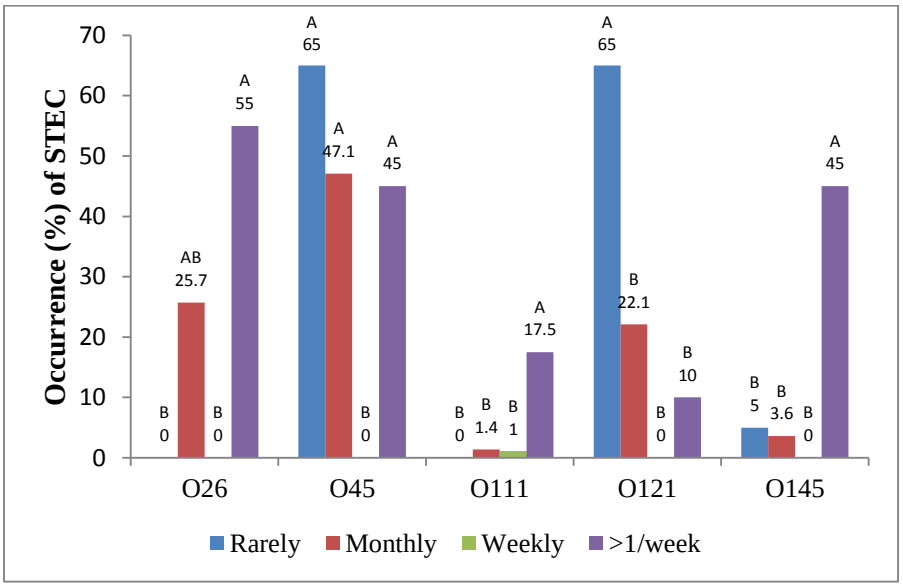
In the 'feed bins' category, responses ranged from 2 (cleaned rarely) to 5 (cleaned more than once a week). Out of the 18 pastures sampled, three did not use feed bins and responded as 'not applicable'. With the exception of *E. coli* O157:H7 and *E. coli* O103, significant differences (P<0.05) in prevalence was seen in the other serogroups (Figure 10). Significant differences (P<0.05) in occurrence of O45 were observed when comparing pastures that had their feed bins cleaned monthly and those that cleaned feed bins weekly (response levels 3 and 4), as well as between pastures that cleaned feed bins only rarely and those that cleaned them weekly (response levels 2 and 4) (Figure 10). In both cases, the occurrence of O45 was significantly lower (P<0.05) when feed bins were cleaned weekly. No significant difference was observed between levels 4 and 5 (weekly

cleaning and cleaning more than once a week), suggesting that a minimum cleaning frequency of at least once a week may help significantly reduce occurrence of this *E. coli* serogroup. Likewise, a significant difference ($P<0.05$) in average O121 occurrence was observed when comparing pastures that cleaned feed bins weekly with those that cleaned them only once every few months (rarely). A higher occurrence of this serogroup was observed in pastures that cleaned feed bins on a weekly basis than those that cleaned feed bins only rarely. No significant difference ($P>0.05$) was observed between levels 4 and 5 (weekly cleaning and cleaning more than once a week), suggesting that the occurrence of *E. coli* O121 may be reduced if feed bins were cleaned at least once a week.

However, variable results in the 'feed bins' category were obtained with the O26, O111, and O145 serogroups. The occurrence of O26 was significantly higher ($P<0.05$) when feed bins were cleaned very often (at least once a week) in comparison to those pastures that cleaned their feed bins weekly as well as those that cleaned their trailers monthly (Figure 10). Out of the 18 pastures sampled, only one responded as cleaning feed bins rarely (with a 0% O26 occurrence), seven responded as cleaning their feed bins monthly (occurrence range from 0 to 60%), five responded as cleaning their feed bins weekly (no O26 serogroup detected), and only two responded as cleaning their feed bins more than once a week (0 to 100% occurrence). These results indicate that increasing frequency of cleaning feed bins may not have a direct effect on the occurrence of O26 on the farm; other environmental factors may most likely also play a more significant role. Likewise, for the O111 serogroup, cleaning more than once a week showed an increased average occurrence than cleaning monthly or weekly (Figure 10). This serogroup was detected in only two pastures, and one of these pastures that reported as cleaning feed

bins more than once a week showed the highest occurrence for the serogroup (40%). From these results, it is difficult to say if the cleaning regimen of feed bins has a direct effect on the occurrence of *E. coli* O111. Similar results were seen with the O145 serogroup. Overall, cleaning feed bins at least once a week may help farmers to significantly reduce the occurrence of O121 on the farm.

Figure 10. Effect of cleaning frequency of feed bins on occurrence of STEC in Louisiana cow/calf operations



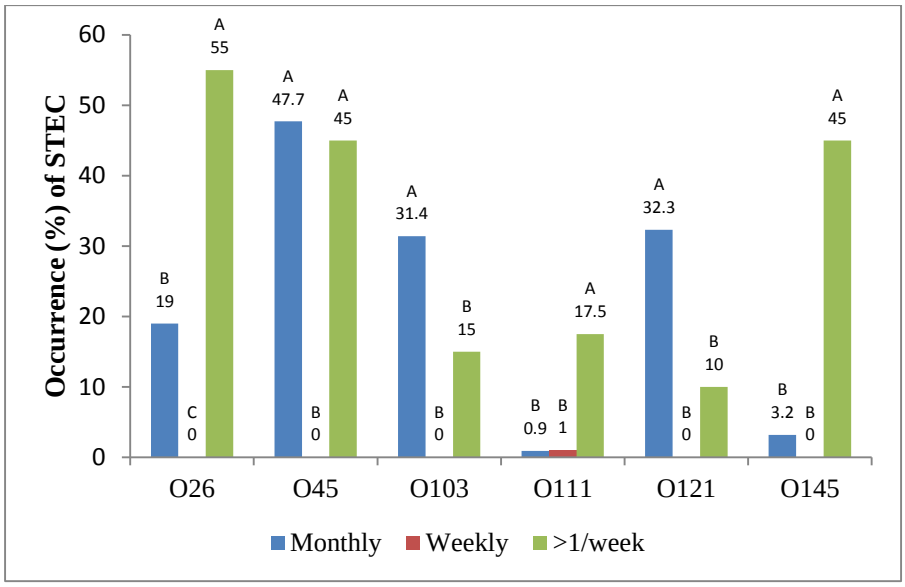
[1] Statistical significance (P<0.05) between mean values is identified by letters A, B. Data bars with different letters represent a significant difference (P<0.05). Data bars that share the same letter are not significantly different (P>0.05). Statistical significance is calculated between cleaning frequencies for each serogroup.
[2]Only fecal samples used in analysis for non-O157 serogroups

The responses from farmers to the 'trough' category ranged from 3 (cleaning monthly) to 5 (cleaning weekly). Cleaning troughs weekly showed a significantly lower (P<0.05) occurrence of the O103 serogroup in comparison to cleaning troughs once every month (Figure 11). No significant difference was observed when comparing occurrence of O103 in pastures that cleaned troughs weekly to those that cleaned troughs more than

once a week indicating that cleaning troughs at least once a week may help significantly reduce the occurrence of O103 on the farm. Likewise, similar results were obtained for the *E. coli* O121 serogroup (Figure 11). These results indicate that cleaning troughs at least once a week may also significantly reduce the occurrence of *E. coli* O121 in the farm environment.

Cleaning frequency of troughs and occurrence of *E. coli* serogroups O45, O111, and O145, however, gave variable results (Figure 11). For the O45 serogroup, a significantly higher ($P<0.05$) occurrence was seen in pastures that cleaned their water troughs monthly in comparison to those that cleaned them weekly. However, a significantly higher ($P<0.05$) average occurrence of the O45 serogroup was also seen when troughs were cleaned more than once a week, in comparison to cleaning weekly. The O45 serogroup was not detected on any of the pastures that cleaned their troughs weekly. Only two pastures cleaned water troughs more than once a week, but had a occurrence of O45 ranging from 30 to 70%. Meanwhile, 11 pastures out of the 18 that were sampled responded as cleaning their water troughs monthly, and O45 occurrence detected on these pastures ranged from 0 to 90%. The results suggest that cleaning water troughs may not have a direct effect on the occurrence of O45 on the farm. With the O111 and O145 serogroups, pastures that employed more frequent cleaning showed significantly higher ($P<0.05$) occurrence.

Figure 11. Effect of cleaning frequency of troughs on occurrence of STEC in Louisiana cow/calf operations



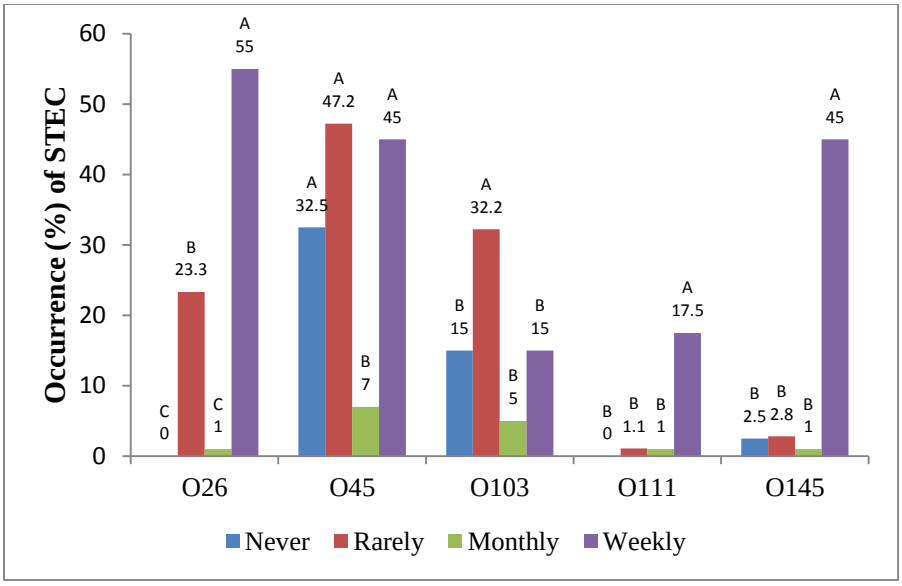
[1] Statistical significance (P<0.05) between mean values is identified by letters A, B, C. Data bars with different letters represent a significant difference (P<0.05). Statistical significance is calculated between cleaning frequencies for each serogroup.
[2]Only fecal samples used in analysis for non-O157 serogroups

Responses for the 'chutes' category ranged from 1 (never cleaned) to 4 (cleaned on a weekly basis). When comparing occurrence of O45 in response to cleaning frequency of chutes, a significant difference was observed between cleaning chutes rarely and cleaning them monthly (Figure 12). Cleaning chutes rarely resulted in a higher occurrence of O45 than cleaning monthly. However, a higher occurrence was seen in pastures that cleaned chutes weekly. For the O103 serogroup, a significant difference (P<0.05) in occurrence was seen when comparing pastures that cleaned chutes monthly to those that only rarely cleaned chutes, with a higher occurrence observed in those pastures that only rarely cleaned chutes than those that cleaned chutes on a monthly basis. The

results indicate that, for *E. coli* O103, cleaning chutes at least once a month may help to significantly reduce the occurrence of the bacteria in the farm environment.

Results with the O26, O111, and O145 serogroups were variable (Figure 12). With the O26 serogroup, a significantly higher occurrence ($P<0.05$) was seen when cleaning chutes were more frequent than not. Similar results were obtained for the O111 and O145 serogroup, where pastures that employed more frequent cleaning showed significantly higher ($P<0.05$) occurrence of *E. coli* O111. Therefore, it may be concluded that, while more frequent cleaning of chutes may significantly decrease the occurrence of the O103 serogroup, this trend might not be achieved with the O26, O45, O111, and O145 serogroups. No significant results were obtained for *E. coli* O157:H7 or *E. coli* O121.

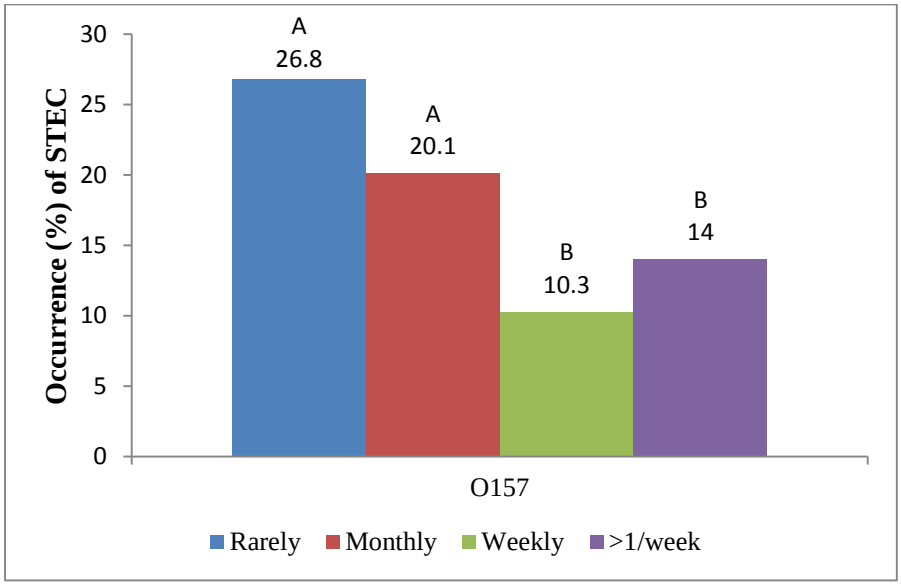
Figure 12. Effect of cleaning frequency of chutes on occurrence of STEC in Louisiana cow/calf operations



[1] Statistical significance ($P<0.05$) between mean values is identified by letters A, B. Data bars with different letters represent a significant difference ($P<0.05$). Statistical significance is calculated between cleaning frequencies for each serogroup.
[2]Only fecal samples used in analysis for non-O157 serogroups

Significant differences (P<0.05) were observed only for *E. coli* O157:H7 with respect to cleaning farm equipment (Figure 13). Responses for this category ranged from 2 (rarely cleaning farm equipment) to 5 (cleaning equipment more than once a week). For this serogroup, cleaning farm equipment at least once a week resulted in a significantly lower (P<0.05) *E. coli* O157:H7 population, with no significant difference (P>0.05) in average occurrence when comparing *E. coli* O157:H7 populations on farms that cleaned their equipment weekly to those that cleaned their equipment more than once a week. These results indicate that, for *E. coli* O157:H7, cleaning equipment at least once a week may help significantly reduce its occurrence in the farm environment.

Figure 13. Effect of cleaning frequency of farm equipment on occurrence of STEC in Louisiana cow/calf operations



[1] Statistical significance (P<0.05) between mean values is identified by letters A, B. Data bars with different letters represent a significant difference (P<0.05). Statistical significance is calculated between cleaning frequencies for each serogroup.
[2]Fecal, water, sediment, and equipment swab samples used in analysis for O157

6. Conclusions

Results from this study indicate that the total positive occurrence of *E. coli* O157:H7 is higher in the state on Louisiana than Oklahoma. However, the total occurrence of non-O157 STEC is higher in both states than *E. coli* O157:H7, although the proportion of pathogenic strains at the farm level is much lower than the reported number for each serogroup. Results from this study also suggest that the occurrence of STEC may be affected by farm management practices. However, certain STEC serogroups may respond differently to particular farm management practices. In general, more frequent cleaning of common cattle contact areas may help reduce the occurrence of certain STEC serogroups. Farm management practices also differ from state to state and may depend on the availability of resources. Breed and animal density also had an effect on the occurrence of particular types of STEC. Having established that farm management practices may have an effect on the occurrence of STEC, control measures such as well-defined cleaning regimens for areas of cattle contact and minimum acreage per herd of 100 cattle need to be assessed. These control measures could thereafter be implemented on cow/calf operations as a means of reducing the occurrence of STEC at the farm and animal levels, and thereby reducing the pathogen load entering the food processing chain.

REFERENCES

Ahmadi, B. V., Frankena, K., Turner, J., Velthuis, A. G., Hogeveen, H. and Huirne, R. B. 2007. Effectiveness of simulated interventions in reducing the estimated prevalence of *E. coli* O157:H7 in lactating cows in dairy herds. *Veterinary Research*, 38: 755–771

Alonso, G., Vilchez, G. and Vidal, R. L. 2000. How bacteria protect themselves against channel-forming colicins. *International Microbiology*, 3: 81-88

Ayscue, P., Lanzas, C., Ivanek, R. and Gröhn, Y. T. 2009. Modeling on-farmO157:H7 population dynamics. *Foodborne Pathogens and Disease,* 6: 461-70.

Bai, J., Paddock, Z. D., Shi, X., Li, S., An, B. and Nagaraja, T. G. 2012. Applicability of a multiplex PCR to detect the seven major shiga toxin-producing *Escherichia coli* based on genes that code for serogroup-specific O-antigens and major virulence factors in cattle feces. *Foodborne Pathogens and Disease,* 9: 541-548

Bai, J., Shi, X. and Nagaraja, T. G. 2010. A multiplex PCR procedure for the detection of six major virulence genes in *Escherichia coli* O157:H7. *Journal of Microbiological Methods,* 82: 85-89

Barham, A. R., Barham, B. L., Clound, C. E., Hays, A. F., Johnson, A. K., Allen, D. M., Blanton, J. R. and Miller, M. F. 2002. Effect of shipping stress in beef cattle on prevalence levels of *Escherichia coli* O157 and *Salmonella* spp. from the feedyard to the packing plant. *Journal of Food Protection,* 65: 280-283

Barrett, T. J., Kaper, J. B., Jerse, A. E. and Wachsmuth, I. K. 1992. Virulence factors in shiga-like toxin-producing *Escherichia coli* isolated from humans and cattle. *Journal of Infectious Diseases,* 165: 979-80.

Bentley, R. and Meganathan, R. 1982. Biosynthesis of Vitamin K (Menaquinone) in Bacteria. *Microbiol Rev,* 46 (3):241-280

Bernier, C., Gounon, P. and Le Bouguenec, C. 2002. Identification of an adhesion fimbria (AAF) type III-encoding operon in enteroaggregative *Escherichia coli* as a sensitive probe for detecting the AAF-encoding operon family. *Infection and Immunity,* 70: 4302-4311

Beutin, L., Geier, D., Zimmermann, S. and Karch, H. 1995. Virulence markers of shiga-like toxin-producing *Escherichia coli* strains originating from healthy domestic animals of different species. *Journal of Clinical Microbiology,* 33: 631-35.

Blanco, J., Blanco, M., Blanco, J. E., Mora, A., Alonso, M. P., Gonzalez, E. A. and Bernardez, M. I. 2001. Epidemiology of verocytotoxigenic *Escherichia coli* (VTEC) in ruminants. Food and Nutrition Press, Trumbull, Conn, pp 113-148

Blanco, M., Blanco, J. E. , Blanco, J. , Gonzalez, E. A. , Mora, A. , Prado, C. , Fernandez, L., Rio, M. , Ramos, J. and Alonso, M. P. 1996. Prevalence and characteristics of *Escherichia coli* serotype O157:H7 and other verotoxin-producing *E. coli* in healthy cattle. *Epidemiology and Infection,* 117: 251.

Boerlin, P., McEwen, S.A., Boerlin-Petzold, F., Wilson, J.B., Johnson R.P., and Gyles, C.L. 1999. Association between virulence factors of Shiga toxin-producing *Escherichia coli* and disease in humans. *Journal of Clinical Microbiology,* 37: 497-503.

Brooks, J. T., Sowers, E. G., Wells, J. G., Greene, K. D., Griffin, P. M., Hoekstra, R. M. and Strockbine, N. A. 2005. Non-O157 shiga toxin-producing *Escherichia coli* infections in the United States, 1983-2002. *Journal of Infectious Disease,* 192: 1422-1429

Brown-Brandl, T. M., Nienaber, J. A., Eigenberg, R. A., Mader, T. L., Morrow, J. L. and Dailey, J. W. 2006. Comparison of feedlot heifers of different breeds. *Livestock Science, 105,* 19-26

Buchko, S. J., Holley, R. A. , Olsen, W. O. , Gannon, V. P. J. and Veira, D. M. 2000. The effect of different grain diets on fecal shedding of *Escherichia coli* O157:H7 by steers. *Journal of Food Protection,* 11: 1467-474.

Burland, V., Shao, Y., Perna, N.T., Plunkett, G., Sofia, H.J. and Blattner, F.R. 1998. The complete DNA sequence analysis of the large virulence plasmid of *Escherichia coli* O157:H7. *Nucleic Acids Research* 26: 4196-4204.

Callaway, T. R. 2010. Pre-harvest management controls and intervention option for reducing *Escherichia coli* O157:H7 shedding in cattle. In: *National Cattlemen's Beef Association; Research and Knowledge Management,* pp 1-24

Caprioli, A., Morabito, S., Brugere, H. and Oswald, E.. 2005. Enterohaemorrhagic *Escherichia coli*: emerging issues on virulence and modes of transmission. *Veterinary Research*, 36: 289–311.

Caprioli, A., Luzzi, I., Rosmini, F., Resti, C., Edefonti, A., Perfumo, F., Farina, C., Goglio, A., Gianviti, A. and Rizzoni, G. 1994. Communitywide outbreak of hemolytic-uremic syndrome associated with non-O157 verocytotoxin-producing *Escherichia coli*. *Journal of Infectious Diseases*, 1994; 169:208–11.

Carroll, J. A. and Forsberg, N. E. 2007. Influence of stress and nutrition on cattle immunity. Pp 105-149. In Olson, K. C. and Hollis, L. C (ed.), Veterinary clinics of North America: food animal practice Elsevier Saunders, Amsterdam, The Netherlands

Centers for Disease Control and Prevention. 2012a. Multistate Outbreak of Shiga Toxin-producing Escherichia Coli O145 Infections (Final Update). http://www.cdc.gov/ecoli/2012/O145-06-12/index.html?s_cid=ccu061812_019

Centers for Disease Control and Prevention. 2012b. Multistate Outbreak of Shiga Toxin-producing Escherichia Coli O26 Infections Linked to Raw Clover Sprouts at Jimmy John's Restaurants (Final Update). http://www.cdc.gov/ecoli/2012/O26-02-12/index.html?s_cid=fb1445

Centers for Disease Control and Prevention. 2014. Reports of Selected E. Coli Outbreak Investigations. http://www.cdc.gov/ecoli/outbreaks.html

Chapman, P. A., Siddons, C. A., Cerdan Malo, A. T. and Harkin, M. A. 1997. A 1-year study of *Escherichia coli* O157 in cattle, sheep, pigs and poultry. *Epidemiology and Infection*, 119: 245–250.

Chase-Topping, M. E., Mckendrick, I. J. , Pearce, M. C. , Macdonald, P., Matthews, L., Halliday, J., Allison, L., Fenlon, D., Low, J. C. , Gunn, G. and Woolhouse, M. E. J. 2007. Risk factors for the presence of high-level shedders of *Escherichia coli* O157 on Scottish farms. *Journal of Clinical Microbiology,* 45: 1594-603.

Chase-Topping, M. E., Rosser, T. , Allison, L. J. , Courcier, E. , Evans, J., McKendrick, I. J. , Pearce, M. C. , Handel, I., Caprioli, A. , Karch, H. , Hanson, M. F. , Pollock, K. G. J. , Locking, M. E. , Woolhouse, M. E. J., Matthews, L., Low, J. C. and Gally, D. L. 2012. Pathogenic potential to humans of bovine *Escherichia coli* O26, Scotland. *Emerging Infectious Disease,* 18: 439-448

Chase-Topping, M., Gally, D., Low, C., Matthews, L. and Woolhouse, M. 2008. Super-shedding and the link between human infection and livestock carriage of *Escherichia coli* O157. *Nature Reviews Microbiology,* 6: 904-12.

Cobbold, R. N., Hancock, D. D., Rice, D. H., Berg, J., Stilborn, R., Hovde, C. J. and Besser, T. E. 2007. Rectoanal junction colonization of feedlot cattle by *Escherichia coli* O157:H7

and its association with supershedders and excretion dynamics. *Applied and Environmental Microbiology,* 73: 1563-568.

Comstock, N., Towle, M. , Warner, A., Reynolds, S., Durso, L., Campbell, C. and Kiefer, M. 2012. Epidemiologic and occupational investigation of an *Escherichia coli* O111 outbreak associated with a correctional facility dairy-Colorado. *Morbidity and Mortality Weekly Reports,* 61: 149-52.

Coombes, B. K., Wickham, M. E., Mascarenhas, M., Gruenheid, S., Finlay, B. B. and Karmali, M. A. 2008. Molecular analysis as an aid to assess the public health risk of non-O157 shiga toxin-producing *Escherichia coli* strains. *Applied and Environmental Microbiology,* 74: 2153-2160

Cray, W. C., Jr., and H. W. Moon. 1995. Experimental infection of calves and adult cattle with *Escherichia coli* O157:H7. *Applied Environmental Microbiology,* 61**:** 1586-1590

Croxen, M. A. and Finlay, B. B. 2010. Molecular mechanisms of *Escherichia coli* pathogenicity. *Nature Reviews. Microbiology,* 8: 26-38

Davies, M., Engel, J. , Griffin, D., Ginzl, D., Hopkins, R., Blackmore, C., Nathan, L., Levy, C., Briggs, G., Kioski, C., Kreis, S., Keen, J. and Durso, L. 2005. Outbreaks of *Escherichia coli* associated with petting zoos-North Carolina, Florida, and Arizona, 2004 and 2005. *Morbidity Mortality Weekly Report,* 54: 1277-1280

Deng, W., Li, Y., Vallance, B. A. and Finlay, B. B. 2001. Locus of enterocyte effacement from *Citrobacter rodentium*: sequence analysis and evidence for horizontal transfer among attaching and effacing pathogens. *Infection and Immunity,* 69: 6323-6335

Diez-Gonzalez, F., Callaway, T. R., Kizoulis, M. G. and Russell, J. B. 1998. Grain feeding and the dissemination of acid-resistant *Escherichia coli* from cattle. *Science,* 281: 1666–1668.

Dodd, C. C., Sanderson, M. W. , Sargeant, J. M. , Nagaraja, T. G. , Oberst, R. D. , Smith, R. A. and Griffin, D. D. 2003. Prevalence of *Escherichia coli* O157 in cattle feeds in Midwestern feedlots. *Applied and Environmental Microbiology,* 69: 5243-247.

Donnenberg, M. S. and Whittam, T. S. 2001. Pathogenesis and evolution of virulence in enteropathogenic and enterohaemorrhagic *Escherichia coli. The Journal of Clinical Investigation,* 107: 539-548

Döpfer, D., Geue, L., Schares, S., Mintel, B., Hoffmann, B. and Fischer, E. A. J. 2012. Dynamics of shiga-toxin producing *Escherichia coli* (STEC) and their virulence factors in cattle. *Preventive Veterinary Medicine,* 103: 22-30.

Drasar, B.S. and Barrow, P.A. 1985. Intestinal Microbiology. In: (eds), Amer. Soc. Microbiol. Press, Washington, DC pp. 19-40.

DuPont, H. L., Formal, S. B., Hornick, R. B., Snyder, M J., Labonati, J. P., Sheanon, D. G, La Brec, E. H. and Kalas, J. P. 1971. Pathogenesis of *Escherichia coli* diarrhea. *New England Journal of Medicine,* 285: 1-9

Durso, L.M., and Keen, J. E. 2007. Shiga-toxigenic *Escherichia coli* O157 and non-shiga-toxigenic *E. Coli* O157 respond differently to culture and isolation from naturally contaminated bovine faeces. *Journal of Applied Microbiology,* 103: 2457-464.

Durso, L. M., Reynolds, K., Bauer, N. and Keen, J. E. 2005. Shiga-toxigenic O157:H7 infections among livestock exhibitors and visitors at a Texas county fair. *Vector-Borne and Zoonotic Diseases* 5: 193-201.

Edrington, T. S., Schultz, C. L., Genovese, K. J., Callaway, T. R. , Looper, M. L. , Bischoff, K. M. , McReynolds, J. L. , Anderson, R. C. and Nisbet, D. J. 2004. Examination of heat stress and stage of lactation (early versus late) on fecal shedding of *E. coli* O157:H7 and *Salmonella* in dairy cattle. *Foodborne Pathogens and Disease,* 1: 114-119

Edrington, T. S., Callaway, T. R., Ives, S. E., Engler, M. J., Looper, M. L., Anderson, R. C. and Nisbet, D. J. 2006. Seasonal shedding of O157:H7 in ruminants: a new hypothesis. *Foodborne Pathogens and Disease,* 3: 413-21.

Eklund, M., Scheutz, F. and Siitonen, A. 2001. Clinical isolates of nonO157 shiga toxin-producing *Escherichia coli*: serotypes, virulence characteristics, and molecular profiles of strains of the same serotype. *Journal of Clinical Microbiology,* 39: 2829–2834

Elder, R. O., Keen, J. E., Siragusa, G. R. , Barkocy-Gallagher, G. A., Koohmaraie, M. and Laegreid, W. W. 2000. Correlation of enterohemorrhagic *Escherichia coli* O157 prevalence in feces, hides, and carcasses of beef cattle during processing. *Proceedings of the National Academy of Sciences,* 97: 2999-3003.

Elliot, S.J., Wainwright L.A., McDaniel T.K., Jarvis K.G., Deng, Y., Lai, L., McNamara, B. P., Donnenberg, M. S. and Kaper, J. B. 1998. The complete sequence of the locus of enterocyte effacement (LEE) from enteropathogenic *Escherichia coli* E2348/69. *Molecular Microbiology,* 28: 1-4

Escherich, T. 1989. The intestinal bacteria of the neonate and breast fed infant. 1885. *Rev Infect Dis* 11: 352-356

Frank, C., Kapfhammer, S., Werber, D., Stark, K. and Held, L.. 2008. Cattle density and shiga toxin-producing Escherichia coli infection in Germany: Increased risk for most but not all serogroups. *Vector-Borne Zoonotic Diseases,* 8: 635-643.

Frankel, G., Phillips, A. D., Rosenshine, I., Dougan, G., Kaper, J. B. and Knutton, S. 1998. Enteropathogenic and enterohemorrhaegic *Escherichia coli*: more subversive elements. *Molecular Microbiology,* 44: 361-379

Gaastra, W. and Svennerholm, A.-M. 1996. Colonization factors of human enterotoxigenic *Escherichia coli* (ETEC). *Trends in Microbiology,* 4: 444-452

Gansheroff, L. J. and O'Brien, A. D. 2000. *Escherichia coli* O157:H7 in beef cattle presented for slaughter in the U.S.: higher prevalence rates that previously estimated. *Proceedings of the National Academy of Science (USA),* 97: 2959–2961

Gautam, R., Kulow, M., Döpfer, D., Kaspar, C., Gonzales, T., Pertzborn, K. M., Carroll, R. J., Grant, W. and Ivanek, R. 2012. The strain-specific dynamics of O157:H7 faecal shedding in cattle post inoculation. *Journal of Biological Dynamics,* 6: 1052-066.

Gillespie, J., Seon-Ae, K., and Paudel, K. 2007. Why don't producers adopt best management practices? An analysis of the beef cattle industry. *Agricultural Economics,* 36: 89-102.

Goldwater, P. N. and Bettelheim, K. A. 1994. The role of enterohaemorrhagic *E. coli* serotypes other than O157:H7 as causes of disease. In: Karmali MA, Goglio AG , editors. Recent advances in verocytotoxin-producing *Escherichia coli* infections. *Amsterdam: Elsevier Science*, pp 57-60.

Goode, B., O'reilly, C., Dunn, J., Fullerton, K., Smith, S., Ghneim, G., Keen, J., Durso, L., Davies, M. and Montgomery, S. 2009. Outbreak of *Escherichia coli* O157: H7 infections after petting zoo visits, North Carolina state fair, October-November 2004. *Archives of Pediatrics and Adolescent Medicine,* 163: 42-48.

Gould, H. L., Mody, R. K., Ong, K. L., Clogher, P., Cronquist, A. B., Garman, K. N., Lathrop, S., Medus, C., Spina, N. L., Webb, T. H., White, P. L., Wymore, K., Gierke, R. E., Mahon, B. E. and Griffin, P. M. 2013. Increased recognition of non-O157 shiga toxin-producing *Escherichia coli* infections in the United States during 2000-2010: epidemiologic features and comparison with *E. coli* O157 infections. *Foodborne Pathogens and Disease,* 10: 453-460

Griffin, P.M. and Tauxe, R.V. 1991. The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohaemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. *Epidemiologic Reviews,* 13: 60-98

Gyles, C., Johnson, R., Gao, A., Ziebell, K., Pierard, D., Aleksic, S. and Boerlin, P. 1998. Association of enterohaemorrhagic *Escherichia coli* hemolysin with serotypes of shiga-like toxin producing *Escherichia coli* of human and bovine origins. *Applied and Environmental Microbiology,* 64: 4134-4141

Hancock, D. D., Besser, T. E., Gill, C. and Hovde-Bohach, C. 1999. Cattle, hay, and *E. coli*. *Science, 284*, 51-52

Hancock, D. D., Besser, T. E., Rice, D. H., Herriott, D. E. and Tarr, P. I. 1997. A longitudinal study of *Escherichia coli* O157 in fourteen cattle herds. *Epidemiology and Infection,* 118: 193-95.

Hancock, D. D., Besser, T. E., Rice, D. H., Ebel, E. D., Herriott, D. E. and Carpenter, L. V. 1998. Multiple sources of *Escherichia coli* O157 in feedlots and dairy farms in the Northwestern USA. *Preventive Veterinary Medicine* 35: 11-19.

Hedican, B. E., Medus, C., Besser, J. M., Juni, B. A., Koziol, B., Taylor, C. and Smith, K. E. 2009. Characteristics of O157 versus Non-O157 shiga toxin–producing *Escherichia coli* infections in Minnesota, 2000–2006. *Clinical Infectious Diseases,* 49: 358-364

Heijnen, L., and Medema, G. 2006. Quantitative detection of *E. Coli*, *E. Coli* O157 and other shiga toxin producing *E. Coli* in water samples using a culture method combined with real-time PCR. *Journal of Water and Health,* 4: 487-98.

Hovde, C. J., Austin, P. R., Cloud, K. A., Williams, C. J. and Hunt, C. W. 1999. Effect of cattle diet on *Escherichia coli* O157:H7 acid resistance. *Applied and Environmental Microbiology, 65,* 3233-3235

Hussein, S., and Bollinger, L. M. 2008. Influence of selective media on successful detection of shiga toxin–Producing *Escherichia coli* in food, fecal, and environmental samples. *Foodborne Pathogens and Disease,* 5: 227-44.

Ibekwe, A.M. and Grieve, C. M. 2003. Detection and quantification of *Escherichia coli* O157:H7 in environmental samples by real-time PCR. *Journal of Applied Microbiology,* 94: 421-31.

Jacob, M. E., Shi, X., An, B., Nagaraja, T. G. and Bai, J. 2012. Evaluation of a multiplex real-time polymerase chain reaction for the quantification of *Escherichia coli* O157 in cattle feces. *Foodborne Pathogens and Disease,* 9: 79-85.

Jarvis, K. G., Giron, J. A., Jerse, A. E., McDaniel, T. K., Donnenberg, M. S. and Kaper, J. B. 1995. Enteropathogenic *Escherichia coli* contains a putative type III secretion system necessary for the export of proteins involved in attaching and effacing lesion formation. *Proceedings of the National Academy of Sciences (USA),* 92: 7994-8000

Johnson, K. E., Thorpe, C. M., and Sears, C. L. 2006. The emerging clinical importance of Non-O157 shiga toxin-producing *Escherichia coli*. *Clinical Infectious Diseases, 43*, 1587-1595

Johnson, R.P., Clarke, R.C., Wilson, J.B., Read, S.C., Rahn, K., and Renwick, S.A. 1996. Growing concerns and recent outbreaks involving non-O157:H7 serotypes of verotoxigenic *Escherichia coli. Journal of Food Protection* 59: 1112-1122

Kalchayanand, N., T. M. Arthur, J. M. Bosilevac, J. E. Wells, and T. L. Wheeler. 2013. Chromogenic agar medium for detection and isolation of *Escherichia coli* serogroups O26, O45, O103, O111, O121, and O145 from fresh beef and cattle feces. *Journal of Food Protection* 76: 192-99.

Kaper, J.B., Elliott, S., Sperandio, V., Perna, N.T. and Matthew G.F. 1998a. Attaching-and-effacing intestinal histopathology and the locus of enterocyte effacement: in *Escherichia coli* O157:H7 and other shiga-toxin-producing *E. coli* Strains ed. Kaber, J.B. and O'Brien, A.D. pp 163-182 Washington D.C: American Society for Microbiology

Kaper, J.B., Gansheroff, L.J., Wachtel, M.R. and O'Brien, A.D. 1998b. Intimin-mediated adherence of shiga-toxin-producing *Escherichia coli* and attaching and effacing pathogens" In *Escherichia coli O157:H7 and Other Shiga-Toxin-Producing E. coli Strains Washington D.C: American Society for Microbiology* ed. Kaber, J.B. and O'Brien, A.D pp 148-156 Washington D.C: American Society for Microbiology

Kappeli, U., Hachler, H., Giezendanner, N., Cheasty, T. and Stephan, R. 2011. Shiga toxin-producing *Escherichia coli* O157 associated with human infections in Switzerland, 2000-2009. *Epidemiology and Infection,* 139: 1097-1104

Kelley, K. W. 1980. Stress and immune function: A bibliographic review. *Annales de Recherches Veterinaires,* 11: 445-461

Kenny, B. 2002. Enteropathogenic *Escherichia coli* (EPEC) – a crafty subversive little bug. *Microbiology,* 148: 1967-1978

Knutton, S., Baldwin, T., Williams, P. H. and McNeish, A. S. 1989. Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis for a new diagnostic test for enteropathogenic and enterohaemorrhagic *Escherichia coli. Infection and Immunity,* 57: 1290-1298

Laegreid, W. W., R. O. Elder, and J. E. Keen. 1999. Prevalence of *Escherichia coli* O157:H7 in range beef calves at weaning. *Epidemiology and Infection,* 123: 291-98.

LeJeune, J. T., Besser, T. E. and Hancock, D. D. 2001. Cattle water troughs as reservoirs of Escherichia coli O157. *Applied Environmental Microbiology*, 67: 3053–3057

Levine, M. M., Bergquist, E. J., Nalin, D. R., Waterman, D. H., Hornick, R. B., Young, C. R., Sotman, S. and Rowe, B. 1987. *Escherichia coli* strains that cause diarrhea but do not produce heat-labile or heat-stable enterotoxins and are non-invasive. *Lancet* 1: 1119-1122

Lin, J., M. P. Smith, K. C. Chapin, H. S. Baik, G. N. Bennett, and J. W. Foster. 1996. Mechanisms of acid resistance in enterohemorrhagic *Escherichia coli*. *Applied and Environmental Microbiology.* 63: 3094-100.

Louise, C. B., and T. G. Obrig. 1995. Specific interaction of *Escherichia coli* O157:H7-derived shiga-like toxin II with human renal endothelial cells. *Journal of Infectious Diseases,* 172: 1397-401.

Lowe, R. M. S., Munns, K., Selinger, L. B., Kremenik, L., Baines, D., Mcallister, T. A and Sharma, R. 2010. Factors influencing the persistence of *E. coli* O157:H7 lineages in feces from cattle fed grain versus grass hay diets. *Canadian Journal of Microbiology*, 56: 667-75.

Luzzi, I., Tozzi, A. E., Rizzoni, G., Niccolini, A., Benedetti, I., Minelli, F. and Caprioli, A. 1995. Detection of serum antibodies to the lipopolysaccharide of *Escherichia coli* O103 in patients with Hemolytic-uremic syndrome. *Journal of Infectious Diseases,* 171: 514-515

Madic, J., de Garam, C. P., Brugere, H., Loukiadis, E., Fach, P., Jamet, E. and Auvray, F. 2011. Duplex real-time PCR detection of type III effector *tccP* and *tccP2* genes in pathogenic

Escherichia coli and prevalence in raw milk cheeses. *Letters in Applied Microbiology,* 52: 538-545

Matthews, L., J. C. Low, D. L. Gally, M. C. Pearce, D. J. Mellor, J. A. Heesterbeek, M. Chase-Topping, M. Naylor, S. W. Shaw, S. W. Reid, G. J. Gunn, and M. E. Woolhouse. 2006. Heterogeneous Shedding of *Escherichia coli* O157 in Cattle and Its Implications for Control. *Proceedings of the National Academy of Sciences* 103: 547-52.

Matthews, L., R. Reeve, M. E. J. Woolhouse, M. Chase-Topping, D.J. Mellor, M.C. Pearce, L.J. Allison, G.J. Gunn, J.C. Low, and S.W.J. Reid. 2009. Exploiting Strain Diversity to Expose Transmission Heterogeneities and Predict the Impact of Targeting Supershedding. *Epidemics* 1: 221-29.

McDaniel, T.K. and Kaper, J.B. 1997. A cloned pathogenecity island from enteropathogenic *Escherichia coli* confers the attaching and effacing phenotype on E. coli K-12. *Molecular Microbiology,* 23: 399-407

Menrath, A., Wieler, L.H., Heidemanns, K., Semmler, T., Fruth, A. and Kemper, N. 2010. Shiga Toxin Producing *Escherichia Coli*: Identification of Non-O157:H7-Super-Shedding Cows and Related Risk Factors. *Gut Pathogens,* 2: 7.

Mol, O. and Oudega, B. 1996. Molecular and structural aspects of fimbriae biosynthesis and assembly in *Escherichia coli*. *FEMS Microbiology Reviews,* 19: 25-52

Moon, H. W., Whipp, S. C., Argenzio, R. A., Levine, M. M. and Gianella, R. A. 1983. Attaching and effacing activities of rabbit and human enteropathogenic *Escherichia coli* in pig and rabbit intestines. *Infection and Immunity,* 53: 1340-1351

Mull, B., and V. R. Hill. 2009. Recovery and Detection of *Escherichia coli* O157:H7 in Surface Water, Using Ultrafiltration and Real-Time PCR. *Applied and Environmental Microbiology,* 75: 3593-597.

Murinda, S. E., L. T. Nguyen, H. M. Nam, R. A. Almeida, S. J. Headrick, and S. P. Oliver. 2004. Detection of Sorbitol-Negative and Sorbitol-Positive Shiga Toxin-Producing *Escherichia*

coli, *Listeria monocytogenes*, *Campylobacter jejuni*, and *Salmonella* spp. in Dairy Farm Environmental Samples. *Foodborne Pathogens and Disease*, 1: 97-104.

Nataro, J. P. and Kaper, J. B. 1998. Diarrhaegenic *Escherichia coli. Clinical Microbiology Reviews*, 11: 142-201

Ogura, Y., Ooka, T., Iguchi, A, Toh, H., Asadulghani, M., Oshima, K., Kodama, T., Abe, H., Nakayama, K., Kurokawa, K., Tobe, T., Hattori, M. and Hayashi, T. 2009. Comparative genomics reveal the mechanism of the parallel evolution of O157 and non-O157 enterohemorrhagic *Escherichia coli*. *Proceedings of the National Academy of Sciences (USA)*, 106: 17939-17944

Okeke, I. N. and Nataro, J. P. 2001. Enteroaggregative *Escherichia coli*. *Lancet Infectious Diseases*, 1: 304-313

Paddock, Z. D., Bai, J., Shi, X., Renter, D. G. and Nagaraja, T. G. 2013. Detection of *Escherichia coli* O104 in the feces of feedlot cattle by a multiplex PCR assay designed to target major genetic traits of the virulent hybrid strain responsible for the 2011 German outbreak. *Applied Environmental Microbiology*, 79: 3522-3525

Perna, N. T., G. F. Mayhew, G. Posfai, S. Elliott, M. S. Donnenberg, and J. B. Kaper. 1998. Molecular Evolution of a Pathogenecity Island from Enterohemorrhagic *Escherichia coli* O157:H7. *Infection and Immunity*, 66: 3810-817.

Phillips, A. D. and Frankel, G. 2000. Intimin-mediated tissue specificity in enteropathogenic *Escherichia coli* interaction with human intestinal organ cultures. *Journal of Infectious Diseases*, 181: 1496-1500

Polifroni, Rosana, Analía I. Etcheverría, Marcelo E. Sanz, Rosana E. Cepeda, Alejandra Krüger, Paula M. A. Lucchesi, Daniel Fernández, Alberto E. Parma, and Nora L. Padola. 2012. Molecular Characterization of Shiga Toxin-Producing *Escherichia coli* Isolated from the Environment of a Dairy Farm. *Current Microbiology*, 65: 337-43.

Possè, B., De Zutter, L., Heyndrickx, M. and Herman, L. 2008. Novel differential and confirmation plating media for shiga toxin-producing *Escherichia coli* serotypes O26,

O103, O111, O145 and sorbitol-positive and-negative O157. *FEMS Microbiology Letter,* 282: 124-131

Rajkowski, K. T., Eblen, s. and Laubauch, C. 1998. Efficacy of washing and sanitizing trailers used for swine transport in reduction of *Salmonella* and *Escherichia coli. Journal of food protection,* 61: 31-35

Rangel, J. M., Sparling, P. H., Crowe, C., Griffin, P. M. and Swerdlow, D. L. 2005. Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982-2002. *Emerging Infectious Diseases*, 11: 603-609

Rasmussen, M. A., and Casey, T. A. 2001. Environmental and Food Safety Aspects Of *E coli* O157:H7 Infections in Cattle. *Critical Reviews in Microbiology*, 27: 57-73.

Reicks, A. L., Brashears, M. M., Adams, K. D., Brooks, J. C., Blanton, J. R. and Miller, M. F. 2007. Impact of transportation of feedlot cattle to the harvest facility on the prevalence of *Escherichia coli* O157:H7, *Salmonella*, and total aerobic microorganisms on hides. *Journal of Food Protection,* 70: 17-21

Reid, C.A., A. Small, S.M. Avery and S. Buncic. 2002. Presence of food-borne pathogens on cattle hides. *Food Control*. 13: 411-415.

Rice, D. H., K. M. McMenamin, L. C. Pritchett, D. D. Hancock, and T. E. Besser. 1999. Genetic Subtyping of *Escherichia coli* O157 Isolates from 41 Pacific Northwest USA Cattle Farms. *Epidemiology and Infection,* 122: 479-84.

Salak-Johnson, J. L. and McGlone, J. J. 2007. Making sense of apparently conflicting data: stress and immunity in swine and cattle. *Journal of Animal Science,* 85: E81-88

Sanderson, M. W., J. M. Sargeant, X. Shi, T. G. Nagaraja, L. Zurek, and M. J. Alam. 2006. Longitudinal Emergence and Distribution of *Escherichia coli* O157 Genotypes in a Beef Feedlot. *Applied and Environmental Microbiology*, 72: 7614-619.

Sargeant, J.M, M.W Sanderson, R.A Smith, and D.D Griffin. 2003. *Escherichia coli* O157 in Feedlot Cattle Feces and Water in Four Major Feeder-cattle States in the USA. *Preventive Veterinary Medicine,* 61: 127-35

Sargeant, J. M., Sanderson, M. W., Griffin, D. D. and Smith, R. A. 2004. Factors Associated with the Presence of *Escherichia coli* O157 in Feedlot–cattle Water and Feed in the Midwestern USA. *Preventive Veterinary Medicine,* 66: 207-37.

Scaletsky, I. C. A., Silva, M. L. M. and Trabulsi, L. R. 1984. Distinctive patterns of adherence of enteropathogenic *Escherichia coli* to HeLa cells. *Infection and Immunity,* 45: 534-536

Schmidt, H., Geitz, C., Tarr, P. I., Frosch, M. and Karch, H. 1999. Non-O157:H7 pathogenic shiga toxi-producing *Escherichia coli*: phenotypic and genetic profiling of virulence traits and evidence for clonality. *Journal of Infectious Diseases,* 179: 115-123

Scotland, S.M., Smith, H.R., Willshaw, G.A. and Rowe, B. 1990. Properties of strains of *Escherichia coli* O26:H11 in relation to their enteropathogenic or enterohaemorrhagic classification. *Journal of Infectious Diseases,* 162: 1069-1074

Scotland, S.M., Smith, H.R., Willshaw, G.A. and Rowe, B. 1983. Verocytotoxin production in strains of *Escherichia coli* is determined by genes carried on bacteriophage. *Lancet,* ii: 216

Sears, C. L., and Kaper, J. B. 1996. Enteric bacterial toxins: mechanisms of action and linkage to intestinal secretion. *Microbiology Reviews,* 60: 167-215

Sen, Keya, James L. Sinclair, Laura Boczek, and Eugene W. Rice. 2011. Development of a Sensitive Detection Method for Stressed *Escherichia coli* O157:H7 in Source and Finished Drinking Water by Culture-qPCR. *Environmental Science & Technology,* 45: 2250-256.

Shelton, Daniel R., James A. Higgins, Jo Ann S Van Kessel, Yakov A. Pachepsky, Kenneth Belt, and Jeffrey S. Karns. 2004. Estimation of Viable *Escherichia coli* O157 in Surface Waters Using Enrichment in Conjunction with Immunological Detection. *Journal of Microbiological Methods* 58: 223-31.

Sjogren, R., R. Neill, D. Rachmilewitz, D. Fritz, J. Newland, and D. Sharpnack. 1994. Role of Shiga-like Toxin I in Bacterial Enteritis: Comparison between Isogenic *Escherichia coli* Strains Induced in Rabbits. *Gastroenterology,* 106: 306-17.

Smith, D., M. Blackford, S. Younts, R. Moxley, J. Gray, L. Hungerford, T. Milton, and T. Klopfenstein. 2001. Ecological Relationships between the Prevalence of Cattle Shedding *Escherichia coli* O157:H7 and Characteristics of the Cattle or Conditions of the Feedlot Pen. *Journal of Food Protection,* 12: 1891-2110.

Smith, H.R., Cheasty, T., Roberts, D., Thomas, A., Rowe, B. 1991. Examination of retail chickens and sausages in Britain for verocytotoxin-producing *Escherichia coli*. *Applied and Environmental Microbiology,* 57: 2091-2093.

Steiner, T. S., Lima, A. A., Nataro, J. P. and Guerranto, R. I. 1998. Enteroaggregative *Escherichia coli* produce intestinal inflammation and growth impairment and cause interleukin-8 release from intestinal epithelial cells. *Journal of Infectious Diseases,* 177: 88-96

Tarr, P. I., and M. A. Neill. 1996. Perspective: The Problem of Non-O157:H7 Shiga Toxin (Verocytotoxin)--Producing *Escherichia Coli*. *Journal of Infectious Diseases* 174: 1136-139.

Tarr, P., Gordon, C. and Chandler, W. 2005. Shiga toxin-producing *Escherichia coli* and the haemolytic uraemic syndrome. *Lancet,* 365:1073–86

Tauschek, M., Gorrell, R. J., Strugnell, R. A. and Robins-Browne, R. M. 2002. Identification of a protein secretory pathway for the secretion of heat-labile enterotoxin by an enterotoxigenic strain of *Escherichia coli*. *Proceedings of the National Academy of Sciences (USA),* 99: 7066-7071

Tesh, V. L. 2004. Foodborne enterohaemorrhagic *Escherichia coli* infections. Preharvest and postharvest food safety; contemporary issues and future directions. Chapter 3, pp 27-42

Tillman, G. E., J. L. Wasilenko, M. Simmons, T. A. Lauze, J. Minicozzi, B. B. Oakley, N. Narang, P. Fratamico, and W. C. Cray, Jr. 2012. Isolation of Shiga Toxin-producing *Escherichia coli* Serogroups O26, O45, O103, O111, O121, and O145 from Ground Beef Using Modified Rainbow Agar and Post-immunomagnetic Separation Acid Treatment. *Journal of Food Protection,* 75: 1548-554.

Tkalcic, Suzana, Cathy A. Brown, Barry G. Harmon, Anant V. Jain, Eric P.O. Mueller, Andrew Parks, Karen L. Jacobsen, Scott A. Martin, Tong Zhao, and Michael P. Doyle. 2000. Effects of Diet on Rumen Proliferation and Fecal Shedding of *Escherichia coli* O157:H7 in Calves. *Journal of Food Protection*, 12: 1628-760

Tzipori, S., Montanaro, J., Robins-Browne, R. M., Vial, P., Gibson, R. and Levine, M. M. 1992. Studies with enteroaggregative *Escherichia coli* in the gnotobiotic piglet gastroenteritis model. *Infection and Immunity*, 60: 5302-5306

United States Department of Agriculture – Food Safety and Inspection Service. 2011. Federal Register, 76 (182). http://www.gpo.gov/fdsys/pkg/FR-2011-09-20/pdf/2011-24043.pdf

United States Department of Agriculture –Animal and Plant Health Inspection Service. 1997. An update: Escherichia coli O157:H7 in humans and cattle

Van Donkersgoed, J., Berg, J., Potter, A., Hancock, D., Besser, T., Rice, D., LeJeune, J. and Klashinsky, S. 2001. Environmental sources and transmission of *Escherichia coli* O157 in feedlot cattle. *The Canadian Veterinary Journal*, 42: 714–720.

Vidovic, S., and D. R. Korber. 2006. Prevalence of *Escherichia coli* O157 in Saskatchewan cattle: Characterization of isolates by using random amplified polymorphic DNA PCR, antibiotic resistance profiles, and pathogenicity determinants. *Applied Environmental Microbiology*, 72: 4347-4355.

Vimont, A., M.-L. Delignette-Muller, and C. Vernozy-Rozand. 2007. Supplementation of Enrichment Broths by Novobiocin for Detecting Shiga Toxin-producing *Escherichia coli* from Food: A Controversial Use. *Letters in Applied Microbiology*, 44: 326-31.

Vugia, D., A. Cronquist, J. Hadler, M. Tobin-D'Angelo, D. Blythe, K. Smith, S. Lathrop, D. Morse, P. Cieslak, T. Jones, K.G. Holt, J.J. Guzewich, O.L. Henao, E. Scallan, F.J. Angulo, P.M. Griffin and R.V. Tauxe. 2007. Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food --- 10 states, 2006. *Morbidity and Mortality Weekly Reports*, 56: 336-339

Waghela, D. S. 2004. Pathogenic *Escherichia coli* in: Preharvest and postharvest food safety; Contemporary issues and future directions. Chapter 2, pp 13-25

Waterman, S.R. and Small, P.L. 1996. Characterization of the acid resistance phenotype and *rpoS* alleles of Shiga-like toxin-producing *Escherichia coli*. *Infection and Immunity,* 64: 2808-2811

Wells, J. G., L. D. Shipman, K. D. Greene, E. G. Sowers, J. H. Green, D. N. Cameron, F. P. Downes, M. L. Martin, P. M. Griffin, and S. M. Ostroff. 1991. Isolation of *Escherichia coli* Serotype O157:H7 and Other Shiga-like-toxin-producing *E. Coli* from Dairy Cattle. *Clinical Microbiology,* 29: 985-89.

Wetzel, A. N., and J. T. Lejeune. 2006. Clonal Dissemination of *Escherichia coli* O157:H7 Subtypes among Dairy Farms in Northeast Ohio. *Applied and Environmental Microbiology,* 72: 2621-626.

Wickham, M. E., Lupp, C., Mascarenhas, M., Vazquez, A., Coombes, B. K., Brown, N. F., Coburn, B. A., Deng, W., Puente, J. L., Karmali, M. M. and Finlay, B. B. 2006. Bacterial genetic determinants of non-O157 STEC outbreaks and hemolytic-uremic syndrome after infection. *Journal of Infectious Diseases* , 194: 819–27.

Willshaw, G.A., Scotland, S.M., Smith, H.R. and Rowe, B. 1992. Properties of Vero cytotoxin-producing *Escherichia coli* of human origin of O serogroups other than O157. *Journal of Infectious Diseases,* 166: 797-802

Winfield, M. D. and E. A. Groisman. 2003. Role of nonhost environments in the lifestyles of *Salmonella* and *Escherichia coli*. *Applied and Environmental Microbiology*, 69: 3687–3694.

Wylie, J. L., P. Van Caeseele, M. W. Gilmour, D. Sitter, C. Guttek, and S. Giercke. 2013. Evaluation of a New Chromogenic Agar Medium for Detection of Shiga Toxin-Producing *Escherichia coli* (STEC) and Relative Prevalences of O157 and Non-O157 STEC in Manitoba, Canada. *Journal of Clinical Microbiology,* 51: 466-71.

Yoshitomi, K.J., R. Zapata, K.C. Jinneman, S.D. Weagant, and W. Fedio. 2012. Recovery of *E. coli* O157 Strains after Exposure to Acidification at pH 2. *Letters in Applied Microbiology*, 54: 499-503.

Zhang, W-L., Bielaszewska, M., Liesegang, A., Tschape, H., Schmidt, H., Bitzan, M. and Karch, H. 2000. Molecular characteristics and epidemiological significance of shiga toxin-producing *Escherichia coli* O26 strains. *Journal of Clinical Microbiology*, 38: 2134-2140

Zhao, Tong, Michael P. Doyle, Jack Shere, and Lindsey Garber. 1995. Prevalence of Enterohemorrhagic *Escherichia coli* O157:H7 in a Survey of Dairy Herds. *Applied and Environmental Microbiology* 61: 1290-293.

Zottola, E. A. 1994. Microbial attachment and biofilm formation: a new problem in the food industry? *Food Technology*, 48: 107-114

APPENDICES

Apppendix A: Production practices survey

Questionnaire page 1

Cow/Calf Operations Production Practices Survey

Farm:

1. Cattle in the sample have access to water that is contained in….

	Yes	**No**
Free-flowing creeks	☐	☐
Streams or Rivers	☐	☐
Dirt stock tanks	☐	☐
Cement/metal stock tanks	☐	☐
Small capacity/continuous flow stock tank	☐	☐

2. Water available to cattle in the sample comes from...

	Yes	No
River/stream	☐	☐
Well/windmill	☐	☐
Runoff Capture	☐	☐
Municipal/City Water	☐	☐

3. The cattle in the sample are currently being fed....

	Yes	No
Hay (list below)	☐	☐
Commercial product (list below)	☐	☐
Bulk single commodity (list below)	☐	☐
_____		
Silage (list below)	☐	☐
_____		
Distillers Grain	☐	☐
Mineral Blocks	☐	☐
Other additives (list below)	☐	☐

4. How many cattle are in this sample?

5. How many total cattle are in the operation?

6. How many acres are in this sample?

7. How many acres are in this operation?

8. How many days have the cattle in the sample been in the current location?

9. The cattle in the sample are...

☐ Pure bred only (Indicate breed) _____

☐ Cross bred only _____

☐ A mixture of pure and cross breeds _____

10. What is the primary hide color of the sample?

☐ Black

☐ White

☐ Red

☐ Gray

☐ Mixed

11. How often do you clean the following:

	Never	Rarely	Sometimes	Quite Often	Very Often
Trailer	☐	☐	☐	☐	☐
Alleyways	☐	☐	☐	☐	☐
Feed bins	☐	☐	☐	☐	☐
Water Troughs	☐	☐	☐	☐	☐
Chutes	☐	☐	☐	☐	☐
Heavy equipment- tractors, skid loaders, etc.	☐	☐	☐	☐	☐

Please answer the following questions about yourself or the Farm Manager/Owner

12. Gender (Check One): ☐ Male ☐ Female

13. In what year were you born?

14. Please indicate the highest level of education you have completed.

☐ Some Formal Education

☐ High School Diploma

☐ Associate's Degree

☐ Bachelor's Degree

☐ Graduate Degree

15. What is your zip code?

16. Which of the following industry associations are you a member of? (Select All that Apply)

☐ National Cattlemen's Beef Association

☐ Independent Cattlemen's Association of Texas

☐ Texas and Southwestern Cattle Raisers Association

☐ Louisiana Cattlemen's Association

☐ Breed Association (please specify)

☐ Other Industry Association (please specify)

Appendix B: Anti-*E. coli* Dynabeads® manual

Anti-*E. coli* O157:H7 Dynabeads® manual page 1

applied biosystems
by Life technologies

Dynabeads® anti-E. coli O157

Catalog nos. 71003, 71004 **Store at 2°C to 8°C**

Rev. Date: August 2012 (Rev. 011)

Product Contents

Cat. no.	Volume
71003	1 mL
71004	5 × 1 mL

Product capacity
Capacity for 71003: 50 tests
Capacity for 71004: 250 tests

Dynabeads® anti-E. coli O157 are supplied in a suspension of phosphate buffered saline (PBS) pH 7.4 with 0.1% bovine serum albumin (BSA) and 0.02% sodium azide.
Caution: Sodium azide may react with lead and copper plumbing to form highly explosive metal azides.

Product Description

Intended Use

Dynabeads® anti-E. coli O157 is designed for rapid selective separation of *E. coli* O157:H7 from food, water, or environmental samples. This process can be automated using a BeadRetriever™ benchtop instrument or performed using a manual method. Dynabeads® anti-E. coli O157 are designed for rapid, selective concentration of *E. coli* O157 directly from a pre-enriched sample aliquot using immunomagnetic separation (IMS). Dynabeads® anti-E. coli O157 reacts with all *E. coli* O157 strains including pathogenic and non-pathogenic, sorbitol fermenting and non-sorbitol fermenting isolates. Dynabeads® anti-E. coli O157 are simply incubated with an aliquot of the pre-enriched sample, and the antibodies coated onto the beads will specifically bind the target bacteria. The bead-bacteria complexes are subsequently separated by applying a magnetic field. The whole IMS process can be automated using a BeadRetriever™ instrument or performed manually.

Intended User

Any user who is skilled in using conventional microbiological techniques, equipped, and/or certified to do pathogen testing on food, feed, and environmental samples, may use Dynabeads® anti-E. coli O157. The user must be skilled in using conventional microbiological techniques and in interpreting results.

Sample Matrix

Any food, water, feed, or environmental sample that has been pre-enriched for 6–18 hours in Buffered Peptone Water (BPW), Tryptone Soya Broth (TSB), or Brilliant-Green Bile Broth (BGBB) is suitable for IMS with Dynabeads® anti-E. coli O157.

Interpretation Criteria

The test is based on plating the concentrated bead-bacteria complexes onto internationally accepted *E. coli* O157 culture media, such as Cefixime Tellurite Sorbitol MacConkey agar (CT-SMAC) and CHROMagar® O157. Interpretation of presumptive results depends on the skill of the user to correctly identify and differentiate the isolated colonies based on typical *E. coli* O157 morphology. Suspect colonies must be confirmed by standard biochemical and serological test methods.

Required Materials

For performing automated IMS:
- BeadRetriever™ instrument.
- BeadRetriever™ tubes and tips.

For performing manual IMS:
- Magnets: MPC™-6, MPC™-1, MPC™-S.
- Mixer allowing tilting and rotation of tubes (e.g. MX 1, Sample Mixer).

Other materials:
- CT-supplement.
- CHROMagar® O157.
- Micropipette (10–100 µL).
- 1-mL dispenser pipette.
- Pre-enrichment broths such as BPW, TSB, BGBB, or other pre-enrichment broths.
- Stomacher apparatus and stomacher bag with filter.
- Test tubes, glassware, loops, swabs.
- Washing buffer (PBS Tween®): 0.15 M NaCl, 0.01 M Sodium-Phosphate buffer, pH 7.4, with 0.05% Tween®-20. (Autoclave at 121°C for 15 min, store at 2°C to 8°C.)
- Sorbitol MacConkey agar.

Note: Use analytical grade reagents.

General Guidelines

- Read the instrument operating instructions of the BeadRetriever™ before use.
- To avoid cross-contamination and for safety reasons, perform IMS using the BeadRetriever™. In the absence of the BeadRetriever™, strict adherence to good laboratory practice and the following instructions are a prerequisite to obtaining valid results.

For testing of Food and Environmental samples only.

Protocol

The following protocol applies to all samples. Place all of the discarded material in appropriate microbiological containers and autoclave.

Prepare Sample

Food samples
1. Weigh 25 g of food sample and place into a filter homogenizer bag.
2. Add 225 mL of the enrichment medium (e.g. BPW, TSB, or BGBB) and homogenize.
3. Incubate for 6–18 hours at 37°C or 41.5°C.
4. Mix the pre-enriched sample thoroughly by homogenizing once more.

Human stools, bovine feces, and environmental swab samples
1. For human and animal stool samples, prepare a 10% suspension in physiological saline and transfer 1 mL into 10 mL of a suitable enrichment broth.
2. Human rectal swab and environmental swabs samples should be transferred into 10 mL of a suitable enrichment broth.
3. Food samples should also be incubated as described in the preceding guidelines.

Water samples
1. Filter 1 L of water according to standard local procedures.
2. Use flat-ended forceps to remove the filter and transfer directly into a wide-mouthed bottle.
3. Add 90 mL of BPW or TSB to the contents of the bottle and shake vigorously to dislodge bacteria from the surface of the membrane.
4. Incubate for 6–24 hours at 37°C or 41.5°C.
5. The use of a filter aid is recommended for samples that are too turbid for membrane filtration.

Automated Immunomagnetic Separation

Place one disposable sample tube strip into a BeadRetriever™ sample rack for each sample to be processed and, using aseptic technique, dispense reagents into each tube. The tab on the tube strip may be used for labelling samples.
1. Load one BeadRetriever™ sample tube strip for each sample into a sample rack.
2. Resuspend Dynabeads® anti-E. coli O157 until the pellet in the bottom disappears by vortexing.
3. Aseptically add 10 µL of properly mixed Dynabeads® anti-E. coli O157 into the two sample tubes 1 and 2.
4. Aseptically add 500 µL of wash buffer to sample tubes 1 and 2.
5. Aseptically add 1 mL of wash buffer to tubes 3 and 4 within the strip.
6. Aseptically add 100 µL of wash buffer to the 5th tube.
7. Remove the desired tube from the sample rack and place it in a second sample rack one meter away. Add 500 µL of a test sample to tubes 1 and 2 and transfer the inoculated tube back to the first sample rack. Repeat for the remaining samples.
8. Aseptically insert the sterile protective tip combs into the instrument.
9. Insert the rack with filled tubes into the instrument to lock it in place.
10. Check that everything is properly aligned and close the instrument door.
11. Select the EPEC/VTEC program sequence by scrolling with the arrow key and press the START button.
12. While the instrument is in operation, the door must be kept closed. Each processing step and the total time remaining can be followed on the LC display.
13. At the end of the program run, remove the tube's rack from the instrument and plate one half of the bead-bacteria complexes from the 5th tube onto each of the appropriate plating media as recommended in the "Detection & Confirmation of *E. coli* O157" section.
14. Remove the tip combs and discard into a biohazard waste container together with the tube strips.

Manual Immunomagnetic Separation

1. Remove the magnetic plate and load the necessary number of 1.5-mL microcentrifuge tubes into the MPC™-S .
2. Resuspend Dynabeads® anti-E. coli O157 until the pellet in the bottom disappears by vortexing. Pipet 20 µL of Dynabeads® anti-E. coli O157 and dispense into each tube.
3. Add 1 mL of the pre-enriched sample aliquot and close the tube. Change to a new pipette or pipette tip for each new sample.
4. Invert the MPC™-S rack several times. Incubate at room temperature for 10 min with gentle continuous agitation to prevent the beads from settling (e.g. in a MX4 sample mixer).
5. Insert the magnetic plate into the MPC™-S. Invert the rack several times to concentrate the beads into a pellet on the side of the tube. Allow the tube to stand for 3 min for maximum recovery of Dynabeads® anti-E. coli O157.
6. Open the tube cap using the tube opener provided and carefully aspirate and discard the sample supernatant as well as any remaining liquid in the tube cap. (See "Factors Affecting Product Performance".)
7. Remove the magnetic plate from the MPC™-S.

8. Add 1 mL of wash buffer using a different disposable pipette or tip for each sample to prevent cross-contamination between samples as well as the wash buffer. Close the cap and invert the MPC™-S several times to resuspend the beads.
9. Repeat steps 5–8 once.
10. Repeat steps 5–7 once.
11. Resuspend the Dynabeads®-bacteria complex in 100 µL of wash buffer using a different disposable pipette or tip for each sample. Mix briefly by vortexing and proceed to "Detection & Confirmation of *E. coli* O157".

Confirmation

After IMS, transfer the resuspended beads onto each internationally accepted *E. coli* O157 culture media plate. Use two different culture media to increase the chances of detecting suspect colonies that have distinct differential features on each media. We recommend Sorbitol MacConkey (SMAC) media supplemented with CT-supplement and CHROMagar® O157.

1. Spread the bead-bacteria complexes over one half of the plate with a sterile swab to ensure break-up of the bead-bacteria complexes.
2. Dilute further by streaking with a loop. Always carry the loop back into the previously streaked quadrant several times to ensure that the beads reach a fresh, unstreaked quadrant.
3. Incubate the plates at 35°C to 37°C for 18–24 hours. Read the plates for suspect E. coli O157 colorless colonies on CT-SMAC and pink-mauve colored colonies on CHROMagar® O157.

The choice of plating media has been based on some distinct characteristics of *E. coli* 0157:H7. It is the only *E. coli* among clinical isolates which does not ferment sorbitol within 24 hours and which is glucuronidase–negative. The organisms are resistant to potassium tellurite and cefixime. Presumptive *E. coli* O157 colonies must be confirmed by standard biochemical and serological testing.

Specificity And Sensitivity

The protocol for use with Dynabeads® anti-E. coli O157 will determine the presence or absence of one viable *E. coli* O157 in the sample size described if this one cell is able to replicate and is not obstructed by resident background flora. Dynabeads® anti-E. coli O157 will bind both motile and non-motile strains of *E. coli* O157. The binding is independent of the ability to produce either Shiga toxins 1 or 2, or both. Antigenically similar organisms, (e.g. *Escherichia hermannii*, *Salmonella O group N*, or *Proteus spp.*), can crossreact and bind to a limited extent. In addition, extremely "sticky" organisms like *Pseudomonas spp.* or *Serratia* liquefaciens could bind non-specifically. However, the presence of high numbers of competitive background flora in the sample will not affect the binding of *E. coli* O157 to the beads. In naturally contaminated samples the Dynabeads® anti-E. coli O157 protocol, in combination with CT-SMAC agar, can detect *E. coli* O157 from pre-enriched sample aliquots containing as low as 100 *E. coli* O157 cells against high numbers of background flora of 10^8 organisms or more per mL.

False Negatives/Positive Rates

A false negative rate ranging between 2–10% may be expected using the Dynabeads® anti-E. coli O157 protocol depending on the inoculum level, background flora, and sample matrix. However, in identical samples tested without IMS, this false negative rate is significantly increased and is often more than 25%. Therefore, use of the Dynabeads® anti-E. coli O157 protocol will consistently decrease the sample false negative rate by more than 15%. False positives do not occur since all presumptive colonies must always be verified by suitable identification methods. However, the methods depends on the user following good laboratory practices and avoiding cross-contamination of samples. The accuracy of the method is not measurable since IMS is a qualitative, not a quantitative technique. Several bacteria may be bound to the Dynabeads®, but only give rise to one colony-forming unit on the culture media. The precision is dependent on the extent to which particles are recovered from different sample matrices.

Factors Affecting Product Performance

- Perform the IMS procedure on a benchtop at room temperature between 15°C to 25°C using room temperature reagents.
- Ensure that the Dynabeads® are fully dispersed by vortexing >10 sec before use.
- Use filtered pipette tips to transfer samples into the test tubes for manual and automated IMS.
- When performing manual IMS, do not aspirate and discard the isolated bead-bacteria complexes. If the bead-bacteria complexes are aspirated from the sample tube, immediately dispense back into the tube and dilute with wash buffer, then repeat step 5 in the "Manual Immunomagnetic Separation" section before aspirating again.
- In extremely fatty, viscous, or particulate samples, a two-fold sample dilution using the wash buffer must be made prior to IMS to ensure maximum particle recovery.
- During bead-bacteria complex magnetic capture, it is essential with continued gentle rocking of the MPC™-S to prevent binding of magnetic or magnetizable low-mass debris.
- To avoid cross-contamination of the prepared tubes during automated IMS, perform the sample transfer into the tubes in a designated area at least one meter from the prepared tubes.

Description of Materials

Dynabeads® anti-E. coli O157 are uniform, superparamagnetic, polystyrene 2.8 µm beads with adsorbed and affinity-purified antibodies against *E. coli* O157 covalently bound to the surface.

Related Products

Product	Cat. no.
MPC™-1	12001D
MPC™-6	12002D
MPC™-S	A13346
MX1	15907
Sample Mixer	94701
BeadRetriever™	15950
BeadRetriever™ Tubes and Tips	15951

REF on labels is the symbol for catalog number.

References

- Japan - Official Method of the Japanese Health Ministry
- Canada - Health Canada Compendium Official Method – MFLP 90 http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume3/mflp90-01-eng.php

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SPEC-06020

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applied biosystems
by Life technologies

Dynabeads® EPEC/VTEC O26

Catalog no. 71013 **Store at 2˚C to 8˚C**

Rev. Date: August 2012 (Rev. 003)

Product Contents

Product contents	Volume
Dynabeads® EPEC/VTEC 026	2 mL

Dynabeads® EPEC/VTEC O26 contains a suspension of paramagnetic Dynabeads® specific for the O serogroup O26 of *E. coli*. The beads are suspended in phosphate buffered saline (PBS) pH 7.4, with 0.1% bovine serum albumin (BSA) and 0.02% sodium azide.
Caution: Sodium azide may react with lead and copper plumbing to form highly explosive metal azides.

Product Description

Introduction

Vero cytotoxin-producing *E. coli* serotypes other than O157 VTEC are important human pathogens, and their disease causing abilities as enteropathogenic *E. coli* (EPEC) in animals have been recognized long ago. Non-O157 VTEC infections may be associated with consumption of animal products, although knowledge of their incidence in foods throughout the entire food chain is limited. Some strains of *E. coli* O26 exhibit increased susceptibility to cefixime and tellurite in CT-SMAC and do not seem to grow on this medium. However, Immunomagnetic Separation (IMS) using Dynabeads® EPEC/VTEC O26 represents a physically selective concentration procedure needed to improve the isolation and detection of the organisms from diverse sample matrices. The performance of this product is improved significantly by using the BeadRetriever™, the automated IMS instrument that removes all the major problems associated with manual IMS and assures the safety of test performers.

Intended Use

Dynabeads® EPEC/VTEC O26 is designed for rapid selective concentration of *E. coli* serotype O26 directly from a pre-enriched sample aliquot using the BeadRetriever™. Dynabeads®, wash buffers, and samples are loaded into the tube-strips provided. All incubations and washing steps are carried out automatically in the instrument. During the incubation process the antibodies coated onto the beads specifically bind the target bacteria. Washing of the beads is achieved by moving the bead-bacteria complexes from tube-to-tube until a final resuspension into the 5th tube for further processing to detect and/or isolate the target organisms.

Intended User

Any user who is skilled in using conventional microbiological techniques, equipped, and/or certified to do pathogen testing on food, feed, and environmental samples may use Dynabeads® EPEC/VTEC O26. The user must be skilled in using conventional microbiological techniques and in interpreting results.

Sample Matrix

Any food, water, feed, and environmental samples that has been pre-enriched for 24 hours in Buffered Peptone Water (BPW) at 42°C can be used for automated IMS with Dynabeads® EPEC/VTEC O26. Environmental samples include swab streaks of surfaces and containers and fecal material of animal or human origin. A water sample is defined as any source water for potable supply, food is defined as material intended for use in human consumption, and feed is defined as material used for animal consumption.

Interpretation Criteria

Since strains of *E. coli* O26 possess no distinguishing diagnostic feature like sorbitol negativity of *E. coli* O157, no plating medium is particularly recommended except that the medium must be rich enough to allow profuse growth. Modified sorbitol MacConkey agar (CT-SMAC) used with *E. coli* O157 must never be used since some strains of *E. coli* O26 seem to be inhibited on this medium.

Recommended agar/plating media:

- Washed sheep blood agar, or bovine/equine based blood agar with sodium citrate.
- CHROMagar®, MacConkey agar, modified Haemorharragic colitis agar (mHC), Eosin Methylene Blue (EMB), Chromocult® Coliform Agar (Merck).

Follow the swab-streak technique when plating the bead-bacteria complexes as this will result in better isolated colony formation on the culture media. Colonies of presumptive *E. coli* O26 would show the same morphology as any generic *E. coli* on blood agar or any of the previously mentioned plating media. However, these colonies should be serologically confirmed with the agglutination sera recommended for use with the kit and/or by performing other standard differential biochemical tests if necessary.

Required Materials

- 1-mL dispenser pipette.
- BPW (available from most media manufacturers).
- Stomacher and stomacher bag with filter.
- Test tubes, glassware, loops, swabs, pipettes.
- Washing buffer (PBS-Tween®): 0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.4, with 0.05% Tween®-20. (Autoclave at 121°C for 15 min, store at 2°C to 8°C.)
- CHROMagar® O157.
- Modified Haemorrhagic Colitis medium (for isolating haemorrhagic colitis strains of *E. coli*) may be prepared from (g/L), tryptone-20; bile salts #3 (1.12), sodium chloride (5), sorbitol (20), bromocresol purple (0.015), distilled water, and Bacto agar (15).
- EPEC/VTEC O26 Antiserum, purchased as Colony Verification Kit from Statens Serum Institut, Denmark.
- All reagents should be of analytical grade.

General Guidelines

- To avoid cross-contamination of the prepared tubes, transfer of sample into the tubes in a designated area at least one meter away from the prepared tubes (see "Automated Immunomagnetic Separation"). Tube-strips for the BeadRetriever™ are designed to fit into the rack in one direction only. Insert tip combs and tube tray as instructed until a click sound is heard. After processing a sample, remove the sample tray first before removing the tip combs. Remove the tip combs at least 10 min after the assay is complete to allow for air-drying before removal.
- For manual IMS, be careful not to aspirate the beads from the sample tube when discarding the supernatant as this results in lack of recovery of *E. coli* O26. If aspiration becomes difficult, leave some of the supernatant in the tube and dilute with wash buffer as this will break the fat content which causes the beads to slide down the tube wall.
- In extremely fatty, viscous, or particulate samples, prepare a two-fold dilution of the sample using the described wash prior to IMS to ensure maximum recovery of particles. Use filtered pipette tips to transfer samples into the test tubes for manual and automated IMS.
- Wear standard laboratory protective clothing.
- Avoid pipetting by mouth.

Protocol

The following protocol applies to all samples. Place all of the discarded material in appropriate microbiological containers and autoclave.

Prepare Sample

Food Samples

1. Weigh 25 g of food sample and place into a filter homogenizer 1 bag.
2. Add 225 mL of Buffered Peptone Water (BPW).
3. Incubate at 42°C for 24 hours.
4. Mix the pre-enriched sample thoroughly by homogenizing once more.
5. Using a sterile pipette, transfer a 2 × 0.5 mL or 1-mL aliquot of the filtered suspension to be tested to the assay tubes using immunomagnetic separation (see "Automated Immunomagnetic Separation" and "Manual Immunomagnetic Separation".)

Human Stools, Bovine Faeces, and Environmental Swab Samples

Refrigerate whole stool specimens as soon as possible after collection and examine within 1–2 hours of collection. If they cannot be examined within 1–2 hours, place whole stools or a swab of the stool or rectal swabs in a transport medium (e.g. Stuart's, Cary Blair, etc.) and refrigerate until examination within 2–3 days. If a sample will be held longer than 3 days before examination, freeze at -70°C. Specimens in transport medium should not be left at ambient temperature.

- Transfer 1 mL of human liquid stool sample into 10 mL of BPW.
- For solid human stool samples and bovine faeces, prepare a 10% suspension and transfer 1 mL into 10 mL BPW.
- Human rectal and environmental swab samples should be transferred into 10 mL of BPW.

Human stool, bovine feces and environmental samples must be pre-enriched for 24 hours at 42°C.

Water Samples

1. Filter 1 L of water according to standard local procedures.
2. Use flat-ended forceps to remove the filter and transfer directly into a wide-mouthed bottle.
3. Add 90 mL of BPW to the contents of the bottle and shake vigorously to dislodge bacteria from the membrane surface.
4. Incubate at 42°C for 24 hours.
5. The use of a filter aid is recommended for samples that are too turbid for membrane filtration.

Automated Immunomagnetic Separation

All reagents and samples must be aseptically dispensed sequentially into the strips of tubes, after they are fitted into the rack. Users must read the user instructions provided with Dynabeads® EPEC/VTEC O26 before use as follows:

1. Resuspend beads until the pellet in the bottom disappears by using a vortex machine and aseptically add 10 µL into sample tubes 1 and 2.
2. Aseptically add 500 µL of wash buffer to sample tubes 1 and 2.
3. Aseptically add 1 mL of wash buffer to tubes 3 and 4 within the strip.
4. Aseptically add 150 µL of wash buffer to the tube 5.

For testing of Food and Environmental samples only.

5. Remove the desired tube from rack A and place in rack B (one meter away). Add 500 µL of a test sample to tubes 1 and 2 and transfer the inoculated tube to rack A. Repeat for the remaining samples.
6. Aseptically insert the sterile protective tip combs into the instrument.
7. Insert the rack containing filled tubes into the instrument, locking it in place.
8. Check that all components are properly aligned and close the instrument door.
9. Select the EPEC/VTEC program sequence by scrolling with the arrow key and press the START button.
10. While the instrument is in operation, the door must be kept closed. Each processing step and the total time remaining can be followed on the LC display.
11. At the end of the program run, remove the tube rack from the instrument and plate the bead-bacteria complexes from the 5th tube onto the appropriate plating media as recommended in "Culture *E. coli* O26".
12. Remove the tip combs and discard into a biohazard waste container together with the tube strips.

Manual Immunomagnetic Separation

To avoid cross-contamination and for safety reasons, perform immunomagnetic separation using the BeadRetriever™. In the absence of the BeadRetriever™, strict adherence to good laboratory practice and the following instructions are a pre-requisite to obtaining valid results.

1. Remove the magnetic plate and load the necessary number of 1.5-mL microcentrifuge tubes into the MPC®-S magnet.
2. Resuspend Dynabeads® EPEC-VTEC O26 until the pellet in the bottom disappears by using a vortex machine. Pipet 20 µL of Dynabeads® EPEC-VTEC O26 and dispense into each tube.
3. Add 1 mL of the pre-enriched sample aliquot from "Prepare Samples" and close the tube. Change to a new pipette tip for each new sample.
4. Invert the MPC®-S rack a few times. Incubate at room temperature for 10 min with gentle continuous agitation to prevent the beads from settling (e.g. in a MX1 sample mixer).
5. Insert the magnetic plate into the MPC®-S. Invert the rack several times to concentrate the beads into a pellet on the side of the tube. Allow 3 min for proper recovery.
6. Open the tube cap using the tube opener provided and carefully aspirate and discard the sample supernatant as well as the remaining liquid in the tube's cap.
7. Remove the magnetic plate from the MPC®-S.
8. Add 1 mL of wash buffer (PBS-Tween®). Do not touch the tube with the pipette tip since this can cross-contaminate the samples as well as the wash buffer. Close the cap and invert the MPC®-S a few times to resuspend the beads.
9. Repeat steps 5–8 once.
10. Repeat steps 5–7 once.
11. Resuspend the Dynabeads®-bacteria complex in 100 µL of wash buffer (PBS-Tween®). Mix briefly using a vortex mixer. Proceed to "Culture *E. coli* O26".

Culture *E. Coli* O26

After manual or automated IMS, transfer all the resuspended bead-bacteria complex onto blood agar. Alternatively, transfer one half of the bead-bacteria complex onto blood agar and the remaining half onto any one of the following plating media: CHROMagar® O157, MacConkey agar, modified Haemorharragic Colitis agar (mHC), Eosin Methylene Blue (EMB), Chromocult Coliform Agar (Merck).

1. Spread the bead-bacteria complexes over one half of the plate with a sterile swab. This is to ensure the break-up of the bead-bacteria complexes. Dilute further by streaking with a loop. Always carry the loop back into the previously streaked quadrant several times to ensure that the beads are applied to a fresh, unstreaked quadrant.
2. Incubate the plates at 35°C to 37°C for 18–24 hours.
3. Proceed to "Presumptive Identification and Confirmation".

Presumptive Identification and Confirmation

1. Add 10 µL of physiological saline onto a glass slide placed on a dark background. Two or three tests may be performed on one slide.
2. Transfer a sweep of mixed growth from the first half of the blood agar plate onto the slide and make a smooth, milky suspension.
3. Observe for auto-agglutination.
4. In the absence of any auto-agglutination, add 10 µL of the OK O26 antiserum provided in the Colony Verification Kit to the suspension and mix well. Observe for agglutination by tilting the slide for 10–30 sec.
5. If auto-agglutination occurs, test 1–5 distinct individual colonies as described in step 6. A visible agglutination reaction within 30 sec is a strong indication of a presumptive positive sample.
6. When testing the sweep of mixed growth, confirm the initial presumptive result by testing 1–5 distinct individual colonies from the blood agar plate in a similar manner using the OK O26 antiserum. If no distinct colonies could be picked, plate further for purity from the other plating media onto blood agar and proceed as described in this procedure.
7. The reaction is read with the naked eye by holding the slide in front of a light source against a black background (indirect illumination). A positive reaction is seen as a visible agglutination. A negative reaction is persistence of the homogenous milky turbidity.
8. Plate the agglutination positive colonies further for purity and confirm them by standard biochemical, serological, and DNA tests (e.g. PCR).

Specificity and Sensitivity

Following the described protocol for use with Dynabeads® EPEC/VTEC O26 will determine the presence or absence of one viable *E. coli* O26 in the sample sizes described if this one cell is able to replicate and is not obstructed by resident background flora. Dynabeads® EPEC/VTEC O26 will bind both motile and non-motile strains of *E. coli* O26. Antigenically similar (e.g. Escherichia hermannii, Salmonella O group N, or Proteus spp.) can cross-react and bind to a limited extent. In addition, extremely "sticky" organisms like Pseudomonas spp. or Serratia liquefaciens could bind non-specifically. However, the presence of high numbers of competitive background flora in the sample will not affect the specific binding of target organisms to the beads. Routinely, immunomagnetically selected and concentrated *E. coli* O26 are detectable on any enteric plating media from pre-enriched sample aliquots containing as little as 100 target cells against high numbers of background flora of 10^9 organisms or more per mL. The accuracy of the method is not measurable since IMS is a qualitative, not a quantitative technique. Several bacteria may be bound to the beads, but only give rise to one colony-forming unit on the culture media. The precision depends on the extent to which particles are recovered from different sample matrices.

False/Negative Rates

Dynabeads® EPEC/VTEC O26 might record a false negative rate ranging between 2–10% depending on the inoculum level, background flora, and sample matrix. In the same sample without IMS, this false negative rate is significantly increased and is often more than 25%. Hence Dynabeads® EPEC/VTEC O26 will consistently decrease the false negative rate by more than 15%. False positives do not occur since the possibility to verify presumptive colonies is always applicable. However the efficacy, of the methods employed depends on the users aptitude in following good laboratory practices and avoiding cross-contamination of samples.

Description of Materials

Dynabeads® EPEC/VTEC O26 are uniform, superparamagnetic, polystyrene microscopic beads with purified antibodies against *E. coli* O26 covalently bound to the surface. Dynabeads® EPEC/VTEC O26 specifically reacts with all strains of *E. coli* O26 serotypes of both human and animal origin.

Related Products

Product	Cat. no.
MPC®-S	A13346
MPC®-1	12001D
MPC®-6	12002D
MX1	15907
BeadRetriever™	15950
CHROMagar® O157	74002

REF on labels is the symbol for catalog number.

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SPEC-06023

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NB: Same protocol as Anti-*E. coli* O26 Dynabeads® was followed for O103, O111, and O145 serogroups

invitrogen
by Life technologies

Dynabeads® MAX EPEC/VTEC O45 Kit

Catalog nos. A14631, A14683 **Store at 2˚C to 8˚C**

Publication Part no. MAN0007701 **Rev.** 1.0

Kit contents

Product	Cat. no.	Volume
Dynabeads® MAX EPEC/VTEC O45 Kit	A14631	2 x 1 mL
	A14683	4 x 1 mL

The Dynabeads® MAX EPEC/VTEC O45 Kit contains a paramagnetic bead suspension specific for the O serogroup O45 of *E. coli*. The beads are suspended in phosphate buffered saline (PBS) pH 7.4, with 0.1% bovine serum albumin (BSA) and 0.02% sodium azide.
Caution: Sodium azide may react with lead and copper plumbing to form highly explosive metal azides.

Description

Introduction

Vero cytotoxin-producing *E. coli* serotypes other than O157 VTEC are important human pathogens, and their disease causing abilities as enteropathogenic *E. coli* (EPEC) in animals have been recognized long ago. Non-O157 VTEC infections may be associated with consumption of animal products, although knowledge of their incidence in foods throughout the entire food chain is limited. Some strains of *E. coli* O45 exhibit increased susceptibility to cefixime and tellurite in CT-SMAC and do not seem to grow on this medium. However, Immunomagnetic Separation (IMS) using the Dynabeads® MAX EPEC/VTEC O45 Kit represents a physically selective concentration procedure needed to improve the isolation and detection of the organisms from diverse sample matrices. The performance of this product is improved significantly by using the BeadRetriever™ System, the automated IMS instrument that removes major problems associated with manual IMS, providing increased safety for test performers.

Procedure overview

The Dynabeads® MAX EPEC/VTEC O45 Kit is designed for rapid, selective concentration of the *E. coli* serotype O45 directly from a pre-enriched sample aliquot using the BeadRetriever™ System. The bead suspension, wash buffers, and samples are loaded into the tube-strips provided. All incubations and washing steps are carried out automatically in the instrument. During the incubation process the antibodies coated onto the beads specifically bind the target bacteria. The BeadRetriever™ System washes the beads by moving the bead-bacteria complexes from tube-to-tube until a final resuspension into the 5th tube for further processing to detect and/or isolate the target organisms.

Any user who is skilled in using conventional microbiological techniques, equipped, and/or certified to do pathogen testing on food, feed, and environmental samples may use the Dynabeads® MAX EPEC/VTEC O45 Kit. The user must be skilled in using conventional microbiological techniques and in interpreting results.

Sample matrix

Any food, water, feed, and environmental sample that has been pre-enriched for 24 hours in Buffered Peptone Water (BPW) at 42°C can be used for automated IMS with the Dynabeads® MAX EPEC/VTEC O45 Kit. Environmental samples include swab streaks of surfaces and containers and animal litter. A water sample is defined as any source water for potable supply, food is defined as material intended for use in human consumption, and feed is defined as material used for animal consumption.

Interpretation criteria

Since strains of *E. coli* O45 possess no distinguishing diagnostic feature like sorbitol negativity of *E. coli* O157, no plating medium is particularly recommended except that the medium must be rich enough to allow profuse growth. Modified sorbitol MacConkey agar (CT-SMAC) used with *E. coli* O157 must never be used since some strains of *E. coli* O45 seem to be inhibited on this medium.

Recommended agar/plating media:

- Washed sheep blood agar, or bovine/equine based blood agar with sodium citrate.
- MacConkey agar, modified Hemorrhagic colitis agar (mHC), Eosin Methylene Blue (EMB), Chromocult® Coliform Agar (Merck).

Follow the swab-streak technique when plating the bead-bacteria complexes since this will result in better isolated colony formation on the culture media. Colonies of presumptive *E. coli* O45 would show the same morphology as any generic *E. coli* on blood agar or any of the previously mentioned plating media. However, these colonies should be serologically confirmed with the agglutination sera recommended for use with the kit and/or by performing other standard differential biochemical tests if necessary.

Required materials

- 1-mL dispenser pipette.
- Buffered Peptone Water (BPW, available from most media manufacturers).
- Stomacher and stomacher bag with filter.
- Test tubes, glassware, loops, swabs, pipettes.
- Washing buffer (PBS-Tween®): 0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.4, with 0.05% Tween®-20 Solution.
  (Autoclave at 121°C for 15 minutes, store at 2°C to 8°C.)
- Appropriate plating media for *E. coli* O45: Blood agar, and MacConkey agar, modified Hemorrhagic Colitis agar (mHC), Eosin Methylene Blue (EMB), or Chromocult Coliform Agar (Merck).
- Modified Hemorrhagic Colitis medium (for isolating hemorrhagic colitis strains of *E. coli*) may be prepared from (g/L), tryptone-20; bile salts #3 (1.12), sodium chloride (5), sorbitol (20), bromocresol purple (0.015), distilled water, and Bacto agar (15).
- EPEC/VTEC O45 Antiserum, purchased as Colony Verification Kit from Statens Serum Institut, Denmark.

Note: Use only analytical grade reagents.

Guidelines

- To avoid cross-contamination of the prepared tubes, transfer samples into the tubes in a designated area at least one meter away from the prepared tubes (see **Automated immunomagnetic separation**). Tube-strips for the BeadRetriever™ System are designed to fit into the rack in one direction only. Insert tip combs and the tube tray as instructed until you hear a click sound. After processing a sample, remove the sample tray first before removing the tip combs. Remove the tip combs at least 10 minutes after the assay is complete to allow for air-drying before removal.
- For manual IMS, be careful not to aspirate the beads from the sample tube when discarding the supernatant because this results in lack of recovery of *E. coli* O45. If aspiration becomes difficult, leave some of the supernatant in the tube and dilute with wash buffer, which will break the fat content and cause the beads to slide down the tube wall.
- In extremely fatty, viscous, or particulate samples, prepare a two-fold dilution of the sample using the described wash prior to IMS to ensure maximum recovery of particles. Use filtered pipette tips to transfer samples into the test tubes for manual and automated IMS.
- Wear standard laboratory protective clothing.
- Avoid pipetting by mouth.

Protocol

The following protocol applies to all samples. Place all of the discarded material in appropriate microbiological containers and autoclave.

Prepare samples

Food and environmental samples

1. Collect and enrich food and environmental samples according to your standard laboratory procedure.
2. Using a sterile pipette, transfer two 0.5-mL aliquots or one 1-mL aliquot of the sample to the assay tubes that will be used for immunomagnetic separation (see **Automated immunomagnetic separation** and **Manual immunomagnetic separation**).

Water samples

1. Filter 1 L of water according to standard local procedures.
2. Use flat-ended forceps to remove the filter and transfer directly into a wide-mouthed bottle.
3. Add 90 mL of BPW to the contents of the bottle and shake vigorously to dislodge bacteria from the membrane surface.
4. Incubate at 42°C for 24 hours.
5. We recommend using a filter aid for samples that are too turbid for membrane filtration.

Automated immunomagnetic separation

Aseptically dispense all reagents and samples sequentially into the strips of tubes, after they are fitted into the rack. Read and use the following user instructions before use:

1. Resuspend beads until the pellet in the bottom disappears by using a vortex machine and aseptically add 10 µL into sample tubes 1 and 2.
2. Aseptically add 500 µL of wash buffer to sample tubes 1 and 2.
3. Aseptically add 1 mL of wash buffer to tubes 3 and 4 within the strip.
4. Aseptically add 150 µL of wash buffer to tube 5.
5. Remove the desired tube from rack A and place in rack B (one meter away). Add 500 µL of a test sample to tubes 1 and 2 and transfer the inoculated tube to rack A. Repeat for the remaining samples.
6. Aseptically insert the sterile protective tip combs into the instrument.

For testing of Food and Environmental samples only.

7. Insert the rack containing filled tubes into the instrument, locking it in place.
8. Check that all components are properly aligned and close the instrument door.
9. Select the **EPEC/VTEC** program sequence by scrolling with the arrow key and press the **START** button.
10. Keep the door closed while the instrument is in operation. You may follow each processing step and the total time remaining on the LC display.
11. At the end of the program run, remove the tube rack from the instrument and plate the bead-bacteria complexes from the 5th tube onto the appropriate plating media and proceed to **Culture *E. coli* O45**.
12. Remove the tip combs and discard into a biohazard waste container together with the tube strips.

Manual immunomagnetic separation

To avoid cross-contamination and for safety reasons, perform immunomagnetic separation using the BeadRetriever™ System. In the absence of the BeadRetriever™ System, strict adherence to good laboratory practice and the following instructions are a pre-requisite to obtaining valid results.

1. Remove the magnetic plate and load the necessary number of 1.5-mL microcentrifuge tubes into the MPC®-S magnet.
2. Resuspend Dynabeads® MAX EPEC/VTEC O45 until the pellet in the bottom disappears by using a vortex machine. Pipet 20 µL of Dynabeads® MAX EPEC/VTEC O45 and dispense into each tube.
3. Add 1 mL of the pre-enriched sample aliquot from **Prepare samples** and close the tube. Change to a new pipette tip for each new sample.
4. Invert the MPC®-S rack a few times. Incubate at room temperature for 10 minutes with gentle continuous agitation to prevent the beads from settling (e.g. in a MX1 sample mixer).
5. Insert the magnetic plate into the MPC®-S concentrator. Invert the rack several times to concentrate the beads into a pellet on the side of the tube. Allow 3 minutes for proper recovery.
6. Open the tube cap using the tube opener provided and carefully aspirate and discard the sample supernatant as well as the remaining liquid in the tube cap.
7. Remove the magnetic plate from the MPC®-S concentrator.
8. Add 1 mL of wash buffer (PBS-Tween®). Do not touch the tube with the pipette tip since this can cross-contaminate the samples as well as the wash buffer. Close the cap and invert the MPC®-S concentrator a few times to resuspend the beads.
9. Repeat steps 5–8 once.
10. Repeat steps 5–7 once.
11. Resuspend the bead-bacteria complex in 100 µL of wash buffer (PBS-Tween® solution). Mix briefly using a vortex mixer and proceed to **Culture E. coli O45.**

Culture *E. coli* O45

After manual or automated IMS, transfer all of the resuspended bead-bacteria complex onto blood agar. Alternatively, transfer one half of the bead-bacteria complex onto blood agar and the remaining half onto any one of the following plating media: MacConkey agar, modified Hemorrhagic Colitis agar (mHC), Eosin Methylene Blue (EMB), Chromocult Coliform Agar (Merck).

1. Spread the bead-bacteria complexes over one half of the plate with a sterile swab to ensure the break-up of the bead-bacteria complexes. Dilute further by streaking with a loop. Always carry the loop back into the previously streaked quadrant several times to ensure that the beads are applied to a fresh, unstreaked quadrant.
2. Incubate the plates at 35°C to 37°C for 18–24 hours.
3. Proceed to **Presumptive identification and confirmation**.

Presumptive identification and confirmation

1. Add 10 µL of physiological saline onto a glass slide placed on a dark background. You may perform 2 or 3 tests on one slide.
2. Transfer a sweep of mixed growth from the first half of the blood agar plate onto the slide and make a smooth, milky suspension.
3. Observe for auto-agglutination.
4. In the absence of any auto-agglutination, add 10 µL of the OK O45 antiserum provided in the Colony Verification Kit to the suspension and mix well. Observe for agglutination by tilting the slide for 10–30 seconds.
5. If auto-agglutination occurs, test 1–5 distinct individual colonies as described in step 6.
   Note: A visible agglutination reaction within 30 seconds is a strong indication of a presumptive positive sample.
6. When testing the sweep of mixed growth, confirm the initial presumptive result by testing 1–5 distinct individual colonies from the blood agar plate in a similar manner using the OK O45 antiserum. If you cannot pick any distinct colonies, plate further for purity from the other plating media onto blood agar and proceed as described in this procedure.
7. Read the reaction with the naked eye by holding the slide in front of a light source against a black background (indirect illumination). A positive reaction is seen as a visible agglutination. A negative reaction is persistence of the homogenous milky turbidity.
8. Plate the agglutination positive colonies further for purity and confirm them by standard biochemical, serological, and DNA tests (e.g. PCR).

Performance considerations

Following the described protocol for use with Dynabeads® MAX EPEC/VTEC O45 will determine the presence or absence of one viable *E. coli* O45 in the sample sizes described if this one cell is able to replicate and is not obstructed by resident background flora. Dynabeads® MAX EPEC/VTEC O45 will bind both motile and non-motile strains of *E. coli* O45. Antigenically similar (e.g. *Escherichia hermannii*, *Salmonella* O group N, or *Proteus* spp.) can cross-react and bind to a limited extent. In addition, extremely "sticky" organisms like *Pseudomonas* spp. or *Serratia liquefaciens* could bind non-specifically. However, the presence of high numbers of competitive background flora in the sample will not affect the specific binding of target organisms to the beads. Routinely, immunomagnetically selected and concentrated *E. coli* O45 are detectable on any enteric plating media from pre-enriched sample aliquots containing as little as 100 target cells against high numbers of background flora of 10^9 organisms or more per mL. The accuracy of the method is not measurable since IMS is a qualitative and not a quantitative technique. Several bacteria may be bound to the beads, but only give rise to one colony-forming unit on the culture media. The precision depends on the extent to which particles are recovered from different sample matrices.

The Dynabeads® MAX EPEC/VTEC O45 Kit might record a false negative rate ranging between 2–10% depending on the inoculum level, background flora, and sample matrix. In the same sample without IMS, this false negative rate is significantly increased and is often more than 25%. Hence the Dynabeads® MAX EPEC/VTEC O45 Kit will consistently decrease the false negative rate by more than 15%. However, the efficacy of the methods employed depends on the users aptitude in following good laboratory practices and avoiding cross-contamination of samples.

Description of materials

Dynabeads® MAX EPEC/VTEC O45 are uniform, superparamagnetic, polystyrene microscopic beads with purified antibodies against *E. coli* O45 covalently bound to the surface. Dynabeads® MAX EPEC/VTEC O45 specifically react with all strains of *E. coli* O45 serotypes of both human and animal origin.

Related products

Product	Cat. no.
Dynabeads® MPC®-S	A13346
Dynabeads® MPC®-1	12001D
Dynabeads® MPC®-6	12002D
Dynabeads® MX1	15907
BeadRetriever™ System	15950

REF on labels is the symbol for catalog number.

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13 June 2013

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NB: Anti-*E. coli* O45 Dynabeads® protocol was followed for O121 isolation as well.

Appendix C: BAX® System RT-PCR protocol for *E. coli*

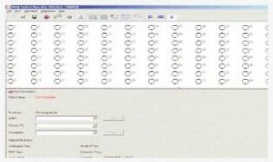
DuPont™ BAX® System

Ready Reference for Real-Time PCR Assays

DUPONT The miracles of science™

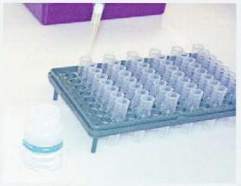
Enrich samples (See *User Guide*)

1. Create rack file with data on each sample.



2. Add 150 uL protease to 12 mL lysis buffer.

3. Add 200 uL lysis reagent to lysis tubes.



4. Transfer 5 uL* sample to lysis tubes.

* *For E. coli O157:H7* and *STEC, use 20 uL*

5a. Heat lysis tubes - First Stage*

37°C for 20 minutes:
Campylobacter
E. coli O157:H7
E. coli - STEC suite
Salmonella
Shigella
Vibrio

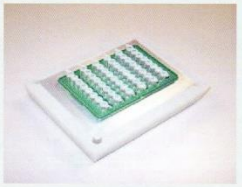
55°C for 60 minutes:
Staphylococcus aureus

* Steps 5 and 6 can also be performed using the DuPont™ Thermal Block. See the Thermal Block User Guide for details and instructions.

5b. Heat lysis tubes - Second Stage

95°C for 10 minutes:
All targets

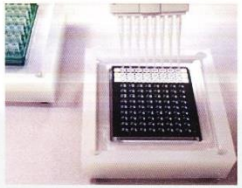
6. Cool lysis tubes 5 minutes in cooling block.



7. Warm up cycler/detector.

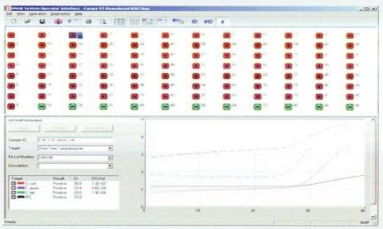
8. Arrange PCR tubes in PCR cooling block.

9. Transfer 30 uL lysate to PCR tubes.



10. Place PCR tubes in cycler and run program.

11. Review results on screen. See User Guide for details.



VITA

Buddhini Piyumika Kumari Jayasundera

Candidate for the Degree of

Master of Science

Thesis: IDENTIFICATION OF CONTAMINATIN SOURCES AND PREVALENCE OF SHIGATOXIGENIC *E. COLI* ON SMALL-SCALE COW/CALF OPERATIONS IN OKLAHOMA AND LOUISIANA

Major Field: Animal Science

Biographical:

Education:

Completed the requirements for the Bachelor of Science in Biochemistry at Oklahoma State University, Stillwater, Oklahoma, USA in 2008.