STUDIES IN SHIGA TOXIN-PRODUCING *ESCHERCHIA COLI* O157:H7: DETERMINATION OF FACTORS CONTRIBUTING TO THE DISSEMINATION OF *ESCHERICHIA COLI* O157:H7 AMONG DAIRY FARMS

DISSERTATION

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ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 is an important foodborne disease worldwide, and cattle play a central role in the epidemiology of human *E. coli* O157:H7 infection. Cattle feces are considered the primary source from which the food supply and the environment become contaminated with this pathogen. Therefore, reduction in the frequency and magnitude of fecal *E. coli* O157:H7 excretion by cattle is predicted to decrease the incidence of human infection. *Escherichia coli* O157:H7 has been sporadically isolated from other animals, and environmental sources. However, the primary routes of dissemination of *E. coli* O157:H7 within and between farms remains undetermined. The hypothesis that European starlings play a role in the dissemination of *E. coli* O157:H7 between cattle farms was tested by determining if starlings inhabiting Ohio dairy farms are a source for *E. coli* O157:H7 and other foodborne pathogens, and evaluating the extent to which indistinguishable isolates of *E. coli* O157:H7 were shared between dairy farms located in a close geographic proximity. Cultured intestinal contents of starlings captured on Ohio dairy farms showed that starlings seasonally harbor *E. coli* O157:H7 (late summer 20%, winter 0%) and other STEC (62.5%).

Stx-negative O157 isolates could be lysogenized by *stx*$_2$-converting bacteriophage, indicating that these toxin-negative strains may acquire *stx*$_2$.

Pulsed-field gel electrophoresis analysis of *E. coli* O157:H7 isolates recovered during a longitudinal study of 20 dairy farms in Ohio showed *E. coli* O157:H7 subtypes (four indistinguishable subtypes) were disseminated with considerable frequency among farms (7 of 20) in close geographic proximity and non-bovine sources may have contributed to the transmission of this organism between farms.

In summary, these data support a role of wild birds in the dissemination of *E. coli* O157:H7 among dairy farms, but the extent of their role in dissemination of this pathogen is yet to be determined.

Dedicated to

My family

And

My husband, Brian

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PUBLICATIONS

Research Publication

1. Coschigano K.T., Holland A.N., Riders M.E., List E.O., Flyvberg A., Kopchick J.J. Deletion, but not antagonism, of the mouse Growth hormone receptor results in severely decreased body weights, insulin, and insulin-like growth factor I levels and increased life span. *Endocrinology* 2003;144:3799-3810.

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FIELDS OF STUDY

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LITERATURE REVIEW

SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* O157:H7

HISTORY

Escherichia coli O157:H7 was first recognized as a human pathogen in 1982 during an investigation by the Centers for Disease Control and Prevention of two outbreaks of hemorrhagic colitis associated with eating hamburgers from a particular fast-food restaurant chain in Oregon and Michigan (Riley et al., 1983). Subsequently, *E. coli* serotype O157:H7 was shown to belong to a group of *E. coli* that produce toxins similar to Shiga toxin produced by *Shigella dysenteria,* and distinct from previously described *E. coli* heat-stable and heat-labile toxins. However, it was not until 1993, after a large multi-state outbreak of *E. coli* O157:H7 resulting in more than 700 illnesses and four deaths, that this organism was recognized as an important and threatening human pathogen (Bell et al., 1994). This outbreak also resulted in the movement towards the bettering of surveillance of foodborne pathogens and monitoring food production more closely in order to improve the safety of the food supply (McDonald et al., 1993).

Escherichia coli O157:H7 became a nationally notifiable infection in 1994, and by 2000, mandatory reporting was implemented in 48 states (Rangel et al., 2005). It is estimated that more than 70,000 illnesses due to *E. coli* O157:H7 infection occur annually in the US, leading to an estimated 2,168 hospitalizations and 61 deaths each year (Mead et al., 1999). *Escherichia coli* O157:H7 is currently the most frequently isolated serotype in North America (Griffin et al., 1998), and outbreaks have occurred with the highest incidences in Scotland, Canada, Japan, and the United States (Parry et al., 2000). Other serotypes, such as O111, O103, O26, and O145 are emerging human pathogens predominantly in Europe, Australia, and South America (Bettlheim, 1996; Bettleheim, 2000). To date, there are over 700 serotypes of *E. coli* identified, and more than 200 of those are different Shiga toxin-producing *E. coli* (STEC) serotypes that have been isolated from humans, animals, food, and other sources (LeBlanc, 2003; World Health Org. 1999). Although not all STEC serotypes have been shown to cause human illness, *E. coli* O157:H7 have caused a series of outbreaks of HC and hemolytic uremic syndrome (HUS) worldwide (Doyle et al., 1997; Griffin et al., 1995).

GENERAL BIOLOGY

Classification. *Escherichia coli*, belonging to the family Enterobacteriacea, are part of the normal gastrointestinal tract of humans and other warm-blooded animals and are acquired by infants within a very few days of birth (Bell, 2002). *Escherichia coli* strains are described serologically by identifying the surface components O antigen of lipopolysaccharide (LPS) and the flagella H-antigen.

Most *E. coli* do not cause disease, however the acquisition of mobile virulence genes located on pathogenicity islands, integrated bacteriophages, or on plasmids has led to the emergence of hundreds of pathogenic *E. coli* strains causing different types of pathogenicity and a wide spectrum of pathophysiological symptoms (Bell, 2002). Such strains are grouped into five virotypes based on their virulence factors, mechanisms of pathogenicity, clinical symptoms, and serology; enterotoxigenic (ETEC), enteroaggregative (EAEC), enteroinvasive (EIEC), enteropathogenic (EPEC), and enterohemorrhagic (EHEC). Four of these recognized virotypes cause gastroenteritis in humans: ETEC, EIEC, EPEC and EHEC, with the first three of these leading to illness with diarrhea as a hallmark symptom, and EHEC being the cause of bloody diarrhea and hemolytic uremic syndrome (HUS). ETEC strains are characterized by their ability to produce enterotoxins (heat-stable and heat labile) and adhesive fimbriae. ETEC strains are frequently associated with traveler's diarrhea and diarrhea in young piglets, calves, and lambs. ETEC is not regarded as a zoonotic pathogen due to the species-specific binding of the fimbrial adhesions. The role of EAEC as an enteropathogen is still unknown, but EAEC strains have been associated with chronic diarrhea in children, and *in vivo* have been demonstrated to produce an aggregative adherence pattern on HEp2 cells. Some EAEC strains can produce a heat-stable enterotoxin. EIEC strains are known to cause disease in humans similar to that caused by *Shigella* spp., and differs from the other *E. coli* virotypes in their ability to invade and multiply within colonic epithelial cells (Wasteson 2001).

EPEC and EHEC are distinct from the other pathogenic *E. coli* strains in that they produce unique histopathological lesions on intestinal epithelial cells called attaching and effacing (A/E) lesions mediated by a pathogenicity island called LEE for Locus of Enterocyte Effacement (McDaniel et al., 1995; Alfredo et al., 2005). However, the differences between EPEC and EHEC are the ability of EHEC to produce Shiga toxins (Stx), also called by many researchers Verocytotoxins (VT), that cause bloody diarrhea, and serious secondary complications such as HUS and thrombotic thrombocytopenic purpura (TTP). The term Shiga toxin-producing *E. coli* (STEC), or Verocytotoxin-producing *E. coli* (VTEC), refers to *E. coli* serotypes capable of producing Shiga toxins (or verotoxins) associated with bloody diarrhea and hemolytic uremic syndrome (HUS) in humans. STEC is broadly used to describe *E. coli* strains that express Stx, whereas the term EHEC is used to describe a subset of STEC strains that also contain LEE and a large plasmid (pO157). The majority of STEC strains associated with human disease possess LEE and the large plasmid pO157, therefore most EHEC are also considered STEC (O'Brien et al., 1998; Alfredo et al., 2005). *Escherichia coli* serotype O157:H7 is therefore classified as STEC (or VTEC) that belongs to the subgroup EHEC (Caprioli et al., 2005). STEC/EHEC strains are considered zoonotic pathogens that are of great interest due to their ability to cause severe disease in humans when transmitted through the food chain and the environment from their animal reservoirs.

The toxins produced by *Escherichia coli* O157:H7 are either termed Verocytotoxins (VT), because of their inhibitory activity on Vero cells (African green monkey kidney cells) in tissue culture, or Shiga toxins (Stx), because of their similarity with the toxin produced by *Shigella dysenteriae* type 1, with the latter being the preferred nomenclature used by investigators now (Melton-Celsa et al., 1998).

There are *E. coli* O157 strains that do not express a functional H, flagellar or motility, antigen designated as *E. coli* O157:H- or O157:NM. They can also produce Shiga toxin, and cause the same pattern of human disease (Griffin et al., 1995). In addition to being nonmotile, *E. coli* O157:H- strains can be atypical in the characteristic to ferment sorbitol after overnight incubation, and such strains have emerged as important causes of human disease in continental Europe during the past decade. However, the epidemiology of sorbitol fermenting (SF) *E. coli* O157:H^- is poorly understood, and limited data suggest that SF *E. coli* O157:H^- human infections occur predominantly during the cold months and in children less than 3 years of age, and only a single SF STEC O157: H^- strain has been isolated to date from a cow, although only a small fraction of bovine feces have been tested (less than 1,300) (Bielaszewska et al., 2000). Except for a single isolation of SF *E. coli* O157:H^- from a pony (Robert Koch-Institute, 1999), SF *E. coli* O157:H^- has not been isolated from other domestic or wild animals. It has been suggested that SF *E. coli* O157:H^- strains might be adapted to the human intestine and that humans may be the major reservoir of this strain of *E. coli* O157 (as reviewed in Karch and Bielaszewska, 2001). Often these sorbitol-fermenting *E. coli* O157:H- strains are missed by diagnostic procedures recommended for the detection of non-sorbitol fermenting *E. coli* O157:H7.

The bacterium. *Escherichia coli* O157:H7 is a Gram-negative flagellated rod-shaped, facultative anaerobe. Phenotypic traits include; oxidase negative, indole positive, sorbitol negative, and 4-methylumbelliferyl-β-D-glucuronide (MUG) negative (produce a nonfunctional form of the enzyme β-glucuronidase that uses the MUG subtrate). The major virulence factors include; Stx production, formation of the adhesion factor intimin, and the presence of EHEC hemolysin. Generally nonpathogenic *E. coli* do not grow well under the refrigeration temperature conditions commonly used in the food industry (i.e. 2-5 ºC), however *E. coli* O157:H7 has been demonstrated to survive in ground beef stored at -20 ºC for over 9 months (Doyle et al., 1984). In addition to meat, like other *E. coli, E. coli* O157:H7 can grow well in a variety of other foods; pasteurized milk (Heuvelink et al., 1998), cantaloupe and watermelon stored at 25 ºC (del Rosario et al., 1995), in cheddar cheese during manufacturing for more than 60 days (Reitsma et al., 1996), and can survive at pH levels as low as 2.5 and persist for up to several weeks when inoculated into apple cider (Conner et al., 1995; Miller et al., 1994) or mayonnaise (Weagant et al., 1994; Raghubeer et al., 1995). *Escherichia coli* O157:H7 can survive in food products of relatively low water activity (~ 0.9); in river water (Wang et al., 1996); grow in bean sprout seeds and on prepared pre-packed fresh vegetables; and can survive meat fermentation processes (Glass et al., 1992). Studies showing increased survival of *E. coli* O157:H7 in mildly acidic foods (less than pH 2.5) indicates that this organism is more acid tolerant than other *E. coli* (Leyer et al., 1995; Gorden et al., 1993).

Escherichia coli O157:H7 is not unusually heat resistant, and adequate heating or cooking procedures will kill the pathogen.

The regulations set forth by the USDA Food Safety Inspection Service (FSIS) recommend an internal temperature of 160 ºF (71.2 ºC) for 15 s for ground beef (Meng et al., 1998).

EMERGENCE

In terms of distribution and public recognition, *E. coli* O157:H7 is considered a new emerging pathogen as a result of an increase in the number of outbreaks and outbreak-related cases of *E. coli* O157:H7 reported to the CDC since the first outbreaks were recognized in 1982; however, it is not clear at the molecular level whether this is a new pathogen, nor the factors underlying the emergence of *E. coli* O157:H7. At the molecular level *E. coli* O157:H7 shows signs of clonal origin. Theories of the molecular origin of *E. coli* O157:H7 are based on data obtained from analysis of multilocus enzyme electrophoresis studies of O157:H7 and other EHEC and EPEC strains. Evolutionary analysis has shown that *E. coli* O157:H7 are genetically most closely related to EPEC O55:H7 strains. Based on a model by Feng et al. (1998) (Figure 1.1), *E. coli* O55:H7 and *E. coli* O157:H7 descended from a common EPEC-like ancestorial strain, that contained the Locus of Enteroctye Effacement pathogenicity island (LEE), ability to ferment sobitol (SOR+) and express β-glucuronidase (GUD+), and could presumably elicit diarrhea via an attachment and effacing mechanism. The first step in the evolution of *E. coli* O157:H7 began with the acquisition of the stx_2 gene, resulting in a Stx2-positive O55:H7.

In the next step, the large virulence plasmid (pO157) was gained and the somatic antigen switched from O55 to O157 (through acquisitoion of the *rfb*O157 gene), and from this stage two separate lines evolved. One branch lost the H7 motility antigen by mutation in the flagellar operon, resulting in a SOR+ or sorbitol fermenting (SF) *E. coli* O157:H$^-$ clone, first discovered in HUS human cases in Germany in 1988. The second branch is split into two lineages (termed EHEC lineage I and II); both lineages arose from the loss of the ablitiy to ferment sorbitol (SOR-), and the acquisition of *stx*$_1$ gene, resulting in a GUD+ O157:H7 strain (EHEC lineage II). Subsequently the GUD+ O157:H7 evolved further into a SOR-, GUD-, phenotype typical of *E. coli* O157:H7 (EHEC lineage I) as a result of mutational inactivation of the *uidA* gene (Wick et al., 2005; Feng et al., 1998). From population studies looking at human clinical and bovine *E. coli* O157:H7 isolates from the US, both human and bovine isolates were shown to be nonrandomly distributed between the two lineages; lineage I strains were mostly human derived, whereas lineage II strains were mostly derived from cattle (Kim et al., 1999). Due to this biased distribution of human clinical and bovine strains, a model has been suggested in which the lineages are not equally capable of causing disease in humans or are not equally capable of being transmitted to humans from bovine sources (Kim et al., 1999). However, these findings are not absolute because examination of human clinical and bovine isolates from Australia showed that several human isolates were detected in lineage II, suggesting that either the relatively small sample sizes used in the studies were biased, or that the Australian lineage II strains are distinct from those in the US (Kim et al., 1999).

What is known, is that *Escherichia coli* O157:H7 (EHEC linage I) have spread geographically and now account for the majority of human disease caused by EHEC. Unfortunately, from the available data, it is not possible to determine exactly when *E. coli* O157:H7 acquired the ability to cause hemolytic uremic syndrome (acute kidney failure, anemia, and low platelet count), nor when it emerged in animal populations (as reviewed in Armstong et al., 1996).

Many questions regarding the determinants responsible for the emergence of *E. coli* O157:H7 in human populations remain unanswered. Although, Armstong et al. (1996) suggests that changes in the livestock industry management, and/or changes in slaughter and meat processing practices in such a way as to promote contamination of meat with this organism may have contributed to the emergence of *E. coli* O157:H7 in human populations.

In the time period between the 1970's and the 90's in the US, several structural changes in the livestock industry occurred. Cattle were often passed through more different geographic locations before going to slaughter (usually calves are produced or raised on a different farm, and many cattle go to auctions/sale barns prior to slaughter), giving them more chances to come in contact with other infected cattle. The size of a cattle herd also changed in both the beef and dairy cattle industries, from many small operations to a smaller number of farms with a large number of cattle per farm. The number of feedlots decreased from 121,000 in 1970 to 43,000 in 1998, and the number of feedlots with over 1,000 head of cattle increased.

The number of commercial dairy farms also decreased from 600,000 in 1955 to 160,000 in 1989, and the number of farms with milk cows decreased from 2,800,000 to 205,000 (reviewed in Armstrong et al., 1996). However, more recent studies looking at the relationship of herd size and *E. coli* O157:H7 prevalence in cattle, have shown that herd size has no effect on the prevalence of *E. coli* O157:H7 (Hancock et al., 1994; Herriot et al., 1998; Dodson and LeJeune, 2005). Changes in cattle management practices on individual farms, such as the introduction of ionophores (carboxylin polyether ionophore antibiotic) in the 1970's to increase feed efficiency in cattle, or the increased use of manure slurries to fertilize pastures, may have played a role in the emergence of *E. coli* O157:H7. Ionophores work by inhibiting the gram-positive bacteria of the rumen and thus promoting the growth of Gram-negative bacteria. Speculation arose that the use of ionophores brought about physiological changes in the rumen, making conditions selective for *E. coli* O157:H7; however, this has not been supported by any experimental data. One case-control study comparing 64 farms, found no association between ionophore use and the presence of *E. coli* O157:H7-positive cattle (Garber et al., 1995). It was also suggested that intensification of farming, i.e. increased herd sizes, and the number of housed animals have resulted in increased quantitities of livestock waste that must be disposed of more often. Prior to agricultural intensification in 1970's, livestock wastes were managed as farmyard manure and the process of aerobic composting (reaches 70ºC temperatures) was capable of killing the majority of pathogens. As a consequence of intensification of farming, slurry (a mixture of manure, urine, feed debris, and water), stored in settling tanks create anaerobic conditions which are more condusive for pathogen survival (Mawdsley et al., 1995). *Escherichia coli* O157:H7 can proliferate

and survive in manure slurries for at least several weeks. The emergence of *E. coli* O157:H7 in the farm environment may have occurred as a result of farmers applying untreated slurry on land more often (even during periods of good weather in the winter) and in larger quantities, due to pressures on storage space. Although, studies looking at the association between the use of manure slurries and the presence of *E. coli* O157:H7 in cattle, failed to find any significant association (Hancock et al., 1994).

It is also possible that with the findings of other non-bovine animal reservoirs for *E. coli* O157:H7, such as deer, the organism could have been maintained in non-bovine populations prior to introduction into a cattle reservoir. The increase in the number of deer could have contributed to more co-mingling with cattle and their environments, thus either increasing the risk of transmission of *E. coli* O157:H7 between deer and cattle, or maintaining the organism in the cattle population.

Changes in food processing and the food industry to produce massive amounts of ground beef could have also been a contributing factor to the emergence of *E. coli* O157:H7 in humans. Large commercial meat packers purchase raw carcass meat from different sources (both domestic and foreign), of which can be ground using the same grinder (machines can grind 4,000 to 12,000 pounds per h continuously for 20 h each day), thus increasing the chances of contamination a large amounts of ground beef with *E. coli* O157:H7. On a hypothetical level, it has been estimated that an evenly contaminated carcass with approximately 700 CFU/g of bacteria (the number of organisms that caused the 1993 multistate *E. coli* O157:H7 outbreak in humans due to undercooked hamburger) would be sufficient enough to contaminate 8 tons of ground beef.

There was also an increase in the number of meat producers during this time, due to the consumer demand for ground beef, that in turn has led to an increase in the size of feedlot operations and potentially increasing the volume of contaminated hamburger that could have led to human outbreaks.

Although experimental data does not support many of the above mentioned factors in the emergence of *E. coli* O157:H7, its possible that these changes, alone or together, may have created a setting conducive for *E. coli* O157:H7 to survive and spread more readily through and into animal and human populations. On the other hand, it is possible that this organism would have emerged as a major pathogen regardless of these mentioned farm management and processing changes, based on its ability to survive in other ecological niches (water, feed, other animal species, and insects) in cattle operations, and its virulence and low infectious dose in humans. The factor(s) responsible for the emergence of *E. coli* O157:H7 in the early 1980's, still remain undetermined.

MOLECULAR BIOLOGY AND MECHANISMS OF DISEASE

Clinical Presentation

In humans clinical symptoms associated with *E. coli* O157:H7 infection, typically appear within 3-5 days following ingestion of the bacteria through contaminated food, water, or by animal-to-human or person-to-person transmission (Griffin et al., 1998). The gastrointestinal manifestations of the illness begin with watery diarrhea, abdominal pain and occasionally nausea and vomiting.

Fever is not a prominent feature of infection, distinguishing this illness from inflammatory colitis associated with infection of organisms such as *Salmonella* (Thorpe 2004). The watery diarrhea may or may not progress within 1-2 days to bloody diarrhea, known as hemorrhagic colitis (HC) (Griffin, et al., 1990). In most cases, symptoms resolve within a week without obvious squeal; however further life-threatening complications could arise such as hemolytic uremic syndrome (HUS), thrombotic thrombocytopenic purpura (TTP), acute renal failure, and even death (Nataro et al., 1998; Paton et al., 1998; Phillips et al., 2000). Some individuals infected with STEC may be asymptomatic carriers, in spite of the presence of large numbers of organisms and free toxin detected in their feces (Paton et al., 1998; Brian et al., 1992). Asymptomatic carriers of *E. coli* O157:H7 are usually found periodically, as a result follow-up to an outbreak case. Because there has never been a large scale surveillance of healthy individuals or individuals at a higher risk for infection with *E. coli* O157:H7, very little is known of the true incidence of asymptomatic carriers (Paton and Paton 1998). The median duration of fecal excretion (interval from onset of diarrhea to the first of two negative stool cultures) reported by Shah et al. (1996) was 29 days among 12 culture–positive children in a day-care-associated outbreak. Other studies have reported less than 7 days and up to 17 days for the median duration of fecal excretion of *E. coli* O157:H7 after the onset of clinical symptoms (Tarr et al., 1990; Belongia et al., 1993). However, the duration of fecal excretion of *E. coli* O157:H7 varies inversely with age, with younger children reported to excrete O157:H7 for longer periods after resolution of symptoms than older children and adults (Belongia et al., 1993; Oai et al., 1988).

Studies have not found a correlation between the amount of toxin in the feces of humans and the severity of illness, or the amount of toxin in the feces and the duration of toxin excretion (Scotland et al., 1988).

Human illness associated with *E. coli* O157:H7 and other STEC infections are more likely to be severe in infants, young children (less than 5 years of age), and the elderly. Those infections that progress to HUS, characterized by renal failure, low platelet count and, hemolytic anemia, tend to occur within 2-14 days after the onset of diarrhea (Thorpe 2004). Progression of STEC infection to HUS is estimated to be 5%-8% (Ochoa et al., 2003). *Escherichia coli* O157:H7 is the leading cause of HUS and the most common cause of acute kidney failure in children in the United States (Altekruse et al., 1997). STEC infection can also result in a variant form of HUS, referred to as thrombotic thrombocytopenic purpura (TTP) which is more common in adults than in children. The clinical symptoms of TTP resemble HUS but also include fever and marked neurological abnormalities (Altekruse et al, 1997; Tan et al., 2001).

Pathogenesis

Shiga toxin production is essential for many of the pathological features as well as the development of life-threatening sequelae of STEC infection. Pathogenesis is a multi-step process, involving a complex interaction between bacterial and host factors. The infectious dose of *E. coli* O157:H7 is very low, approximately 10^2 organisms or less have been associated with foodborne disease outbreaks (Peacock et al., 2001).

STEC orally ingested, often with low initial doses, must initially survive the harsh environment of the stomach, followed by competition with normal gut flora to establish intestinal colonization. Stx, produced by STEC in the lumen of the gut, must first be absorbed by the intestinal epithelium and then be translocated to the bloodstream in order to allow for delivery to receptors located on target cell surfaces.

The pathogen and host factors that contribute to *E. coli* O157:H7 infection and the pathogenesis process are still not fully understood. However, there are certain key determinant virulence factors involved in the pathogenesis if this organism; acid resistance mediated by *rpoS,* genes implicated in the attaching and effacing phenotype (A/E) located on a pathogenicity island, genes for hemolysin and other potential virulent factors located on a large plasmid, and *stx* genes encoding Shiga toxins (Stx) located on temperate bacteriophages. Most genes associated with *E. coli* O157:H7 pathogenesis seem to have been acquired from multiple recombination events with foreign bacterial DNA through horizontal transmission (Boerlin, 1999; Boerlin et al., 1999; Feng et al., 1998; Kim et al., 2001) (Figure 1.1).

Acid resistance: An important initial feature of STEC strains for colonizing the gut is resistance to the acidity of the stomach, mediated by *rpoS*, which encodes a stationary-phase sigma factor (Leyer et al., 1995; Gorden et al., 1993).

Locus of enterocyte effacement (LEE): STEC must establish colonization of the lower gastrointestinal tract (GI) by adhering to intestinal epithelial cells. The adhesion of the bacteria to intestinal epithelial cells (attachment) and destruction of the brush-border of microvilli (effacement) process is called the attaching and effacing histopathology (A/E) (Nataro et al., 1998; Torres et al., 2002).

Beneath the adherent bacteria, marked cytoskeletal changes occur, including the accumulation of polymerized actin, that result in the formation of pedestals; this is recognizable by electron microscopy and flurorescene microscopy (Knutton et al., 1989). Tzipori et al, first reported this A/E histopathology in gnotobiotic piglets infected with STEC *E. coli* O157:H7; and since then, A/E lesions have been found to occur in other animal models infected with STEC *E. coli* O157:H7 (infant rabbits, colostrum- deprived neonatal calves, 1-day-old gnotobiotic pigs, 1-day-old chickens, and macaque monkeys), as well as in cultured epithelial cells infected with *E. coli* O157:H7 (Moxley et al., 1998). The A/E lesion is routinely not detected in clinical specimens from patients infected with STEC, not because they do not occur, but because colonic biopsy specimens are usually collected late in the disease and A/E lesions are present only early in the disease before the cytotoxic effects of Stx occur (Kaper et al, 1998).

This A/E process is mediated by genes located on a 35 kb chromosomal pathogenicity island called the Locus for Enterocyte Effacement (LEE). This region called LEE is not present in normal flora *E. coli,* or enterotoxigenic *E. coli* strains, but is found in EPEC, EHEC and some STEC strains. *Escherichia coli* O157:H7 LEE locus is composed of 41 different open reading frames (ORF) organized into five major operons (LEE 1-5) (Devinney, et al., 1999; Elliot et al., 1998). The LEE locus is organized into three functioning regions: (1) the middle region contains *eae,* encoding intimin, and the *tir* gene, encoding a translocated receptor for intimin, (2) downstream of *eae* are the *esp* genes, which encode the secreted proteins required for inducing the epithelial cell signal transduction events leading to the A/E lesion, (3) upstream of the *eae* and *tir* are numerous genes (*esc* and *sep)* that encode a type III secretion system (TTSS) that

facilitate the transport of effector molecules to their active sites (Kaper et al., 1998). There are three other secretion systems, I, II, and IV, described in other gram-negative bacteria. These four pathways of protein secretion all actively transport proteins from the bacterial cytoplasm across the inner and outermembranes into the bacterial supernatant or onto the surface of the bacterial cell and inject pathogenicity proteins into the cytosol of eukaryotic host cells. However, many of the protein components and mechanisms for secretion of proteins across the outermembrane differ between each of the four protein secretion systems (as reviewed in Hueck 1998).

There are numerous intimin (*eae*) subtypes produced by EHEC and EPEC serotypes and a particular intimin subtype produced by a given A/E *E. coli* serotype influences tissue tropism in the intestine (Oswald et al., 2000; Zhang et al., 2002). Through intimate attachment, intimin is thought to enhance intestinal colonization, increase bacterial counts in the gastrointestinal tract and feces, and prolong fecal excretion of the organism (Dean-Nystrom et al., 1999).

In cattle, intimin has been shown to play a key role in intestinal colonization of *E. coli* O157:H7 in colostrums-deprived neonatal calves and piglets, and the induction of colonic oedema and diarrhea (Donnenberg et al., 1993; Tzipori et al., 1995; Dean-Nystrom et al., 1998b); and has been demonstrated to influence colonization of adult cattle and sheep (Cornick et al., 2002). However, the *eae* gene is not always present in Shiga toxin-producing strains *E. coli* strains isolated from healthy cattle and diarrheic calves (Weiler et al., 1996; Sandhu et al., 1996; Jenkins et al., 2002), indicating that other unknown factors may be important in influencing persistence of STEC in the bovine intestine.

The secreted protein Tir (translocated intimin receptor) is injected into the host cell and becomes the receptor for intimin (DeVinney et al., 1999). The importance of the role of intimin-Tir interaction in binding of *E. coli* O157:H7and other EHEC and EPEC bacteria to the host cell during infection, has been demonstrated through studies involving inactivation of the *tir* gene (Kenny et al., 1997; DeVinney et al., 1999).

The *esp* genes encode the secreted proteins EspA, EspB, EspD, and EspF, and the *esp* along with *esc* genes are thought to encode the type III secretion machinery that facilitate the transport of the secreted Esp proteins and Tir (Nataro et al 1998; Roe et al., 2003a). EscC forms a pore in the bacterial outer cell membrane, and the EspA proteins form a hollow cylindrical translocation apparatus through which EspB, EspD, and Tir are translocated to the host cell. EspB and EspD form a pore in the host cell membrane, through which bacterial proteins are translocated into the host cell cytosol (Ebel et al 1998; Kenney et al., 1997; Frankel et al, 1998). EspB is also translocated into the host cell cytosol in order to trigger signal transduction events that mediate effacement of microvilli and replacement with pedestal-like structures, upon which the bacteria attach (Kenny et al., 1997).

Most of the STEC associated with outbreaks, including O157 and other serotypes, harbor the LEE pathogenicity island, or "LEE region". How STEC strains that do not contain the LEE region mediate colonization is not well understood.

Enterohemolysin (Ehx): The gene *hlyA* encodes a potent enterohemolysin (Ehx) located on 90 Kb plasmid referred to as pO157 that appears to be heterogeneous within O157:H7 serotypes (Barrett et al., 1992; Dorn et al., 1991).

The enterohemolytic phenotype (Ehx) is characterized by small hemolysis zones on sheep blood agar containing washed erythrocytes (LeBlanc et al., 2003). Ehx induces strong cytolytic activity on human and animal cells, and it has been suggested that the role of Ehx would be to lyse erythrocytes, releasing heme from hemoglobin, creating an iron source which would stimulate *E. coli* growth; however the implication of Ehx in pathogenesis remains controversial (Nataro et al., 1998; Torres et al., 1997; Law et al., 1995).

Other potential virulent factors harbored on a plasmid (pO157): Other putative virulence factors harboured by this plasmid (pO157) include a katalase-peroxidase and a serine protease, encoded by *katP* and *espP* genes. Their involvement in STEC O157 pathogenesis is unclear. Although, it has been proposed that the role of KatP, encoded by *katP*, is to enhance the virulence of the bacteria by protecting it from oxidative stress derived from the host immune cells (Nataro et al., 1998); and *espP*, encoding the espP protein, facilitates access of the bacteria to host cells through its cytolytic activity and tissue destruction mediated by cleavage of the pepsin A and human coagulation factor V (Brunder et al., 1996 and 1997). The *katP* and *espP* genes have been detected in only two thirds of the STEC O157 strains (Brunder et al., 1999; Schmidt et al., 2001). Due to the dynamic structure of pO157, that includes different mobile genetic elements such as transposons, prophages, and parts of other plasmids assembled by recombination events, these plasmid-encoded virulence factors are unevenly distributed among STEC O157 strains.

Shiga toxins (Stx): EHEC and STEC strains express one or more potent cytotoxins called Shiga toxins (Stx). Stx produced by *E. coli* O157:H7 are the major virulence factors in the pathogenesis of STEC disease (Kimmitt et al., 2000). *Escherichia coli* Stx consist of two families; Stx1 and Stx2 that are located in the genome of temperate lambdoid bacteriophages that can stably integrate into the host chromosome and be passed on from generation to generation. Stx1 is nearly identical in amino acid sequence to the toxin of *Shigella dysenteriae* type 1, and cannot be distinguished from it serologically (Nataro et al., 1998; Karmali 1989). Stx2 is less related to the Shiga toxin of *Shigella* (55% homologous) and shares less than 60% amino acid sequence homology with Stx1 (Melton-Celsa et al.,1998). A STEC strain can produce either Stx1 or Stx2 (or variants of Stx2) or both; however, severe human disease has been epidemiologically linked to the presence of Stx2 (Boerlin et al., 1999; Ostroff et al., 1989). Whereas, Stx1 shows little nucleotide sequence variations, Stx2 has multiple variants (Stx2, Stx2c, Stx2d, Stx2e, and Stx2f) that are in general closely related to each other in nucleotide sequence, but with altered antigenic or biological characteristics (Schmitt et al., 1991; Weinstein et al., 1988; Schmidt et al., 2000). Among the Stx variants, Stx2 and Stx2c are frequently found in strains isolated from patients with HUS. Strains producing Stx2d are usually isolated from cases of uncomplicated diarrhea (Friedrich et al., 2002). The variants Stx2e and Stx2f are produced by strains of animal origin and are rarely observed in human isolates; Stx2e is found in STEC causing oedema disease in pigs (Mainil, 1999), and Stx2f appears to be closely associated with STEC of avian origin (Schmidt et al., 2000).

There are some *E. coli* O157:H7 strains originating from both humans and animals that are *stx* negative (incapable of producing Stx) (Feng et al., 2001; Genevieve et al., 2004; Schmidt et al., 1999). Typically the strains of *E. coli* O157 and other STEC that cause illness in humans also possess genes that aid in intestinal colonization through intimin-mediated attaching-effacing lesions (Freidrich et al., 2002).

Shiga toxins are protein synthesis inhibiting toxins specific for glycosphingolipid globotriaosylceramide (Gb3) receptors found on the surfaces of epithelial enterocytes, vascular endothelial cells, smooth muscle cells, erythrocytes, and renal endothelial cells (O'Brien et al., 1992; Bast et al., 1999; Lingwood 1993). Stx1, Stx2, and Stx2 variants bind preferentially to the Gb3 receptors, with the exception of Stx2e, which binds preferentially to globotetraosylceramide (Gb4). It has been shown that cells lacking Gb3 are resistant to the toxins (Weinstein et al., 1989). Cross-linking analysis and crystallographic studies have confirmed that Stx possess an A_1B_5 structure, composed of a pentameric binding (B) component that plays a crucial role in the binding to Gb3 receptor, and a monomeric catalytic (A) subunit responsible for the enzymatic activity (Stein et al., 1992; O'Brien et al., 1987). The A chain of Stx has two domains: A1 domain cleaves the N-glycosidic bond of a specific adenine residue on the 28S of eukaryotic rRNA, and an A2 domain that serves a structural role in securing the A1 chain to the B pentamer (Endo et al., 1988; Saxena et al., 1989; Paton et al., 1998; Sandvig et al., 1996 and 1994). STEC/EHEC are not considered to be highly invasive, so toxin synthesis is thought to occur during colonization of the colon and that Shiga toxins must be absorbed from the intestine to cause disease (Slutsker et al., 1997).

How this occurs during STEC/EHEC infection is unknown, but it is suggested that after the Shiga toxins are synthesized and secreted, they are transported by transcytosis through the lumen and spread to their primary target organs, the kidney and central nervous system tissue, in the blood (Tzipori et al., 1988). Recent research has shown in vitro that induced neutrophil migration enhances Shiga toxin uptake across the intestinal epithelium, suggesting that inflammation occurring within the host GI tract during STEC infection may promote systemic Shiga toxin uptake (Hurley et al., 2001). After binding to the Gb3 receptor, the toxins are internalized by receptor-mediated endocytosis and transported to the Golgi apparatus and endoplasmic reticulum, where the A subunit is proteolytically cleaved and translocated to the cytoplasm. In the cytoplasm the A subunit catalytically inactivates the ribosomes, resulting in death of the cell by apoptosis (Yoshida et al., 1999). In an adult rabbit model, intravenously injected Stx1 was shown to localize in the same organ in which Gb3 receptors were expressed, and in a baboon model it was shown to induce the clinical features of HUS (Richardson et al., 1992; Siegler et al., 2001).

The mechanism behind translocation of Stx, produced by *E. coli* O157:H7 and other STEC, from the intestinal lumen to the bloodstream and to vascular targets in the brain, kidney and colon of humans, is not well understood. However, a few studies support the hypothesis that, in humans, polymorphonuclear leukocytes (PMN), serve as a carrier for Stx in the circulation from the intestines to target organs; Stx2 was shown to be bound exclusively to PMN in patients with HUS (Maroeska et al, 2001), and when Sxt1 experimentally incubated with whole blood, it bound solely to human PMN (Maroeska et al., 2000).

In cattle, the role of Stxs, if any, in intestinal colonization and disease is still unknown. Cattle lack the receptors for *E. coli* O157:H7 Stxs, and this is why cattle are thought to be resistant to *E. coli* O157:H7-related disease (Pruimboom-Brees et al., 2000). In Great Britain, the majority of *E. coli* O157:H7 strains isolated from cattle and sheep at slaughter contained the stx_2 gene (97.8% of cattle isolates, and 100% of sheep isolates), and 19.9% of cattle isolates and 1.4% of sheep isolates, respectively, contained both stx_1 and stx_2 genes (Paiba et al., 2002), suggesting a strong correlation between possession of *stx* genes and colonization of the ruminant host. Few studies have addressed the immune respsonse of cattle to *E. coli* O157:H7. Of those studies, anti-O157 serological responses of cattle have demonstrated that calves inoculated with two doses of *E. coli* O157:H7 (second inoculation was administered 13-14 weeks after the last positive fecal sample from the first inoculation) excreted less bacteria than those calves only inoculated once (Cray and Moon, 1995), suggesting that previous infection does not prevent reinfection by the same strain and that an immune response to the organism may have resulted in lower levels of fecal excretion during the second inoculation. A study by Laegrid et al. (1998) showed that the prevalence of anti-O157 antibodies in the serum of naturally infected range beef cattle was high, 63-100% of individual cattle within herds were seropositive. Sanderson et al. (1999) also suggests that cattle may develop some level of immunity that decreases fecal excretion following infection. A study looking at the length of fecal excretion in four 1-week-old calves following three separate oral inoculations of *E. coli* O157:H7 showed that the calves progressively excreted fewer organisms in their feces following each challenge (Sanderson et al., 1999).

Shiga toxin genes are widely disseminated among *E. coli* populations as a consequence of their phage encoded characteristic (Benson, 2003). Shiga toxin genes, in most cases, have been found encoded on the genome of temperate lambdoid double-stranded DNA phages (*stx*-phages) (Unkmeir et al., 2000; Karch et al., 1999; Makino et al., 1999; Neely et al., 1998; O'Brien et al., 1984). These lambdoid phages can exist in two phases, called "lyosgenic" and "lytic". In the lysogenic phase, the bacteriophage is incorporated into the host bacterial chromosome, and replicates along with the host DNA as prophage. In the lytic phase, the bacteriophage is induced from the host chromosome and replicates independently of the host DNA in order to produce many copies of itself, and then released from the host cell by lysis. The *stx*-phages are inducible from the host strain by DNA damaging agents such as UV light or mitomycin C, or antibiotics, as well as other environmental stresses (Kimmitt et al., 2000; Kohler et al., 2000; Zhang et al., 2000). As a result of the phage induction process, bacterial host cells lyse and release free phage particles that are able to infect other bacteria (Muniesa et al., 2004).

The comparison of lambdoid phage genomes revealed that the Shiga toxin containing phages have a common arrangement of functionally similar genes and share a strategy for regulating gene expression (Figure 1.2) (Waldor and Friedman, 2005). Indentification of *stx*-encoding genes downstream of the phage P_R promoter and upstream of the lysis genes, suggests that regulatory mechanism of the phage controls the expression of Stx. Basically, the lambdoid prophage remains in a quiescent state due to binding of a *c*I-encoded repressor protein to the right and left of operator sites, inhibiting transcription from the phage early promoters P_R and P_L.

When the phage lytic cycle is activated by some DNA-damaging event that activates the bacterial SOS response causing RecA-mediated cleavage of the repressor, transcription initiates at early promoters P_R and P_L. The cascade of regulatory events, begin with expression of the N transcription antitermination protein. Terminator read-through mediated by the N protein results in expression of the delayed early genes that encode products involved in replication and prophage excision, and the Q antitermination protein (Q). Q acts at a site called *qut* located within the late phage P_R that modifies RNA polymerases to a highly processive form that will read through downstream terminators. Thus, the Q protein acts at the P_R to turn on Stx expression and because Q production requires transcription from the early P_R promoter, expression of Stx is thought to require prophage induction (as reviewed in Waldor and Friedman, 2005).

Lysogeny of non-Shiga-toxin producing *E. coli* has been demonstrated to occur in vivo in a mouse model (a Stx1-encoding phage H-19B was shown to be transmitted to an *E. coli* recipient strain MC4100, the resulting lysogens were recovered in stool samples) (Acheson et al., 1998). Lysogeny of non-shiga-toxin producing bacteria has important implications for the emergence of new pathogenic strains. Lytic infection of non-O157 *E. coli* can lead to production of Stx and phage. Gamage et al. (2003) showed that lytic infection of non-toxin-producing *E. coli* with Stx-encoding phage can increase Stx production in vitro by more than 1,000-fold, demonstrating that non-toxin-producing *E. coli* can amplify Shiga toxin if they are susceptible to infection by Shiga toxin-encoding phages.

The role of Stx is undoubtedly an important virulence factor in the pathogenesis of *E. coli* O157:H7 and other STEC; however their expression during infection or entry into circulation is still unknown. It is evident though that there are other mechanisms involved in the pathogenesis of *E. coli* O157:H7 because *stx* negative strains can still cause diarrhea in humans. Human intestinal flora is highly variable and so is the host range of Stx-encoding phages, and it is still unknown how often or which intestinal microflora are susceptible to lysogeny with Shiga toxin-encoding bacteriphages.

HUS Treatment and Prevention

The purpose of treatment for symptomatic patients infected with *E. coli* O157:H7 and other STEC strains is to first of all, decrease the severity and duration of symptoms, secondly, to prevent complications such as HUS, and third of all, to prevent further transmission. The range of therapeutics offered to accomplish these purposes include, antibiotics, anti-motility agents, anti-diarrheal agents, and hydration treatments

Antimicrobial treatment of STEC infections is controversial because treatment of STEC infection in the diarrhea phase with certain antimicrobials (specifically DNA-damaging antibacterials) has been associated with HUS (Guerrant et al., 2001). However, treatment with other antimicrobials have been associated with protection from HUS (Safdar et al., 2002). A retrospective study by Wong et al., showed that children treated with antibiotics had a higher risk of developing HUS compared with children who did not receive antibiotic therapy.

It is thought that antibiotic treatment causes the release of Stx from injured bacteria in the intestine due to their DNA damaging or inhibition of replication (such as quinolones and trimethoprin) leading to induction of the bacteriophage into the lytic cycle. This is supported by *in vitro* studies showing that subinhibitory concentrations of different antibiotics increase the release of free Stx (Karch 1986; Walterspiel et al., 1992; Wolf et al., 1997); these observations have been confirmed *in vivo* as well, in a murine model (Zhang et al., 2000). In this model, mice with induced STEC infection and treated with ciprofloxacin (DNA active agent) had higher levels of both bacteriophage and Shiga toxin in their stools, than the control mice, and was associated with mouse death. However, the antimicrobioal, fosfomycin (a cell wall active agent), did not increase the levels of Shiga toxin in the stools or mortality, compared to the control mice.

There are many problems with the published studies on the effect of antibiotics on STEC infection; most studies were retrospective with a small number of patients analyzed, different degrees of severity of disease, and many different antibiotics were used and administered at different times during the illness (Neill, 1998). Therefore, in the absence of data from a nationwide, prospective, randomized, controlled trial study, antimicrobial use is currently not recommended to be used during STEC infection by the Infectious Society of America 2001 guidelines (Guerrant et al., 2001).

Due to the fact that STEC are noninvasive, one tactic for preventing HUS from developing involves uptake of the Shiga toxin from the gut during the diarrhea phase or once HUS has developed. These strategies rely on the administration of a molecular decoy bearing a Gb3 receptor with the highest affinity for Shiga toxin.

Data from a recent randomized controlled trial, using SYNSORB-Pk (Synsorb Biotech), a nonabsorbable silicon-based binding matrix linked to Gb3, showed that patients from 6 months to 18 years of age who already developed diarrhea-associated HUS, and received this compound had a similar mortality rate or serious renal complications requiring dialysis, as those not receiving the compound (Trachtman et al., 2003). SYNSORB-Pk was shown to be very safe, but evidence for its efficacy in preventing HUS was not conclusive.

New treatments for HUS being studied include Shiga toxin-neutralizing monoclonal antibodies and Shiga toxin absorbers. There is no current treatment for HUS, only supportive therapy with close attention to fluid, electrolyte and metabolic balance, nutrition and blood pressure, and early initiation of dialysis for acute renal failure (Robson et al., 1993).

LABORATORY METHODOLOGIES

Detection and Diagnosis of Escherichia coli O157:H7

The current methods used for the detection and diagnosis of *E. coli* O157:H7 in animals stem from modifications made to those first used to detect the organism in food and human patients, and methods used between animal species is quite similar (Moxley, 2003). Because *E. coli* O157:H7 is often found in low numbers in food, and the environment, and often excreted in low numbers in the feces of cattle, sensitive enrichment culture methods are required for detection.

Although, there is controversy over whether cattle excreting low numbers (most adult cattle excrete less than 100 CFU/g of feces) of organism contribute significantly to the overall contamination of the environment or food relative to the contributions of cattle excreting large numbers of organisms. Omisakin et al., has proposed that magnitude of fecal excretion of *E. coli* O157:H7 is more important than the prevalence for identify high-shedding cattle for developing on-farm control measures.

A number of culture methods for screening fecal samples for *E. coli* O157:H7 are currently available, and in fact still remain controversial as to the most sensitive method(s) to use. Feces can either be directly plated onto selective and/or differential agars (Chapman et al., 1994; Smith et al., 1993), or selectively enriched in broth followed by plating onto selective, differential agars (Sanderson et al., 1995). This enrichment step can also be followed by immunomagnetic separation (IMS) with beads coated with O157-specific antibody prior to plating onto agar (Chapman et al., 1994). Various researchers also use polymerase chain reaction (PCR) techniques to screen broth enrichment cultures for the presence of genes associated with *E. coli* O157:H7 that include Stx-encoding genes, O157 antigen-encoding gene, and the H7 flagellum-encoding gene (Gannon et al., 1997; Gannon et al., 1997; Smith et al., 1998). A wide variety of methods are available to detect and isolate *E. coli* O157:H7 but there is no standard protocol recommended, and the evaluation of the various bacterial culture methods for the recovery of *E. coli* O157:H7, the volume and number of samples required for processing, technique for collecting the sample, and the length of pre-enrichment incubation remain ongoing research areas for scientists.

The common method used by most researchers for identifying *E. coli* O157:H7 in human fecal samples is culture of the stool on sorbitol-MaConkey agar (SMAC) or SMAC contaning cefixime and tellurite (CT-SMAC), accompanied by Shiga-toxin detection assay, done on a broth culture of the stool sample (Tarr et al., 2005). In agricultural animal fecal samples, such as cattle samples, the traditional method used for identifying *E. coli* O157:H7 involves enrichment cultures, followed by IMS and subsequent plating onto selective/differential (i.e.CT-SMAC) agar for selection of sorbitol-negative colonies. Following the isolation of *E. coli* O157:H7 culture-positive isolates, biochemical and antigenic characteristics, which include metabolism of 4-methylumbelliferylbeta-D-glucuronide, lactose fermentation on MacConkey agar (MAC), and reactivity with a commercially available latex agglutination kit specific for the O157 antigen (Oxoid, Basingstoke, Hampshire, United Kingdom) are used to screen the isolates. Further confirmation of *E. coli* O157:H7 is determined by multiplex PCR assays for the detection of *hylA*, *eaeA*, *rfbE, fliC,* stx_1, stx_2 , and stx_2 variants (2, 2c, 2d, 2e, 2f) (Wang et al., 2002). Cultures can be further typed for Stx by use of cell culture and neutralizing antibodies to the toxins (Smith et al., 1993), or enzyme-linked immunosorbent assay (ELISA) (Premier EHEC Shiga toxin ELISA, Miridian Biosciences, Inc.).

Direct Plating vs Enrichment Culture: Direct plating is a suitable technique for the isolation of *E. coli* O157:H7 from the feces of patients with acute illness, where the organism is likely to be the predominant one in the sample. However, more sensitive methods are required for the isolation of *E. coli* O157:H7 from animal feces and environmental samples, where the organism may be in low numbers.

Some laboratories have success in detecting *E. coli* O157:H7 by direct plating of fecal dilutions onto sorbitol-MacConkey (SMAC) agar plates (Chapman et al., 1994; Smith et al., 1993). Sorbitol was initially added to to MacConkey (MAC) medium in place of lactose to differentiate *E. coli* O157:H7 strains, most of which do not ferment sorbitol, from other *E. coli* serogroups, which are predominately sorbitol-fermenters (SF) (Farmer et al., 1985; March et al 1986). These SF strains cannot be distinguished from commensal *E. coli* on SMAC agar and are thus missed by laboratories that use SMAC as their only procedure for the detection of STEC O157. Further sensitivity improvements in SMAC media resulted from the addition of the antibiotic cefixime, a *Proteus* spp. growth inhibitor, and the mineral tellurite, which differentially inhibits non-O157 *E. coli* and other organisms (Chapman et al., 1994). *E. coli* O157:H7 strains carry a pathogenicity island termed TAI (tellurite resistance and adherence-conferring island) that contains genes encoding tellurite resistance (Tarr et al., 2000). A study by Sanderson et al., compared SMAC, SMAC supplemented with cefixime (C-SMAC), and SMAC supplemented with cefixime and tellurite (CT-SMAC) for the isolation of *E. coli* O157:H7 from both artificially inoculated bovine feces, naturally contaminated bovine feces, and feces from orally inoculated calves, and confirmed that CT-SMAC was the most sensitive plating medium. Although in detecting SF *E. coli* O157:H^-, these strains are susceptible to tellurite and cannot be isolated on CT-SMAC agar (Karch et al., 1996).

A diagnostic approach for detecting SF *E. coli* O157:H$^-$ combines a direct culture or the IMS-enriched culture on SMAC (use IMS on stool culture follwed by plating on SMAC media) with screening for the O157 antigen in stool using a commercial enzyme-linked immunosorbent assay (ELISA) and detection of *stx*-containing bacteria by PCR (as reviewed in Karch and Bielaszewska, 2001).

Selective pre-enrichment prior to plating onto selective, differential agars has increased the sensitivity of detection of *E. coli* O157:H7 in samples where their numbers are low (McDonough et al., 2000). In fact one study showed that enrichment was necessary to detect low fecal shedding levels in calves after twenty days post inoculation with a naladixic acid-resistant *E. coli* O157:H7 strain (Brown et al., 1997). For pre-enrichment of 10-g fecal samples from bovine and other farm animals, the selective enrichment media tryptic soy broth (TSB) containing cefixime (0.05 μg /ml) and vancomycin (40 μg /ml) prior to plating onto CT-SMAC plates has been shown to increase the sensitivity of *E. coli* O157:H7 detection compared to non-selective pre-enrichment. For the pre-enrichment of 1-g bovine fecal samples followed by the method of immunomagnetic separation (IMS), buffered peptone water (BPW) has been shown to further increase the sensitivity of IMS (Chapman et al., 1994; Foster et al., 2003). Although sensitivity of detecting *E. coli* O157:H7 in pre-enrichment cultures has been shown to decrease with prolonged enrichment times, possibly as a result of an increase of the contaminating microflora (De Boer and Heuvelink, 2000; Tutenel et al., 2003).

Smaller fecal samples are used for pre-enrichment in conjunction with IMS because IMS has been reported to improve the sensitivity of *E. coli* O157:H7 detection in human and bovine feces, as few as a single organism present in the initial sample can theoretically be detected. Other types of pre-enrichment broths have been used for the recovery of *E. coli* O157:H7 from cattle feces (Vold et al., 1998; Conedera et al., 1997; Heuvelink et al., 1998b; Wallace et al., 1996; Johnson et al., 1995).

Immunomagnetic Separation (IMS): The introduction of pre-enrichment followed by IMS significantly increased the sensitivity of detection rates of *E. coli* O157:H7 from various sample materials including cattle feces (Chapman et al., 1998; Synge et al., 1996; Vold et al., 1998), other animals (Heuvelink et al., 1998a), and humans (Chapman et al., 1996). IMS consists of magnetic beads coated with an antibody against *E. coli* O157 (Dynabeads anti-*E. coli* O157; Dynal, Oslo), and increases sensitivity by concentrating *E. coli* O157 relative to background microflora, which may overgrow or mimic O157 STEC cells on selective agars . When compared with direct culture of experimentally inoculated bovine fecal suspensions, enrichment culture followed by IMS was 100-fold more sensitive for detection of the organism than direct culture on CT-SMAC (Chapman et al., 1994). However, in the laboratory, certain variables can impact the sensitivity of detection of *E. coli* O157:H7 in fecal samples using IMS, many variations in methodology exist, including sample volume (Sargeant et al., 2000), type of pre-enrichment broth (Chapman et al., 1994; Tutenel et al., 2003; Foster et al., 2003), and length of pre-enrichment incubation time (Tutenel et al., 2003; Chapman et al., 1994; Sargeant et al., 2000).

Sample Collection and Pre-Enrichment Incubation: In sample collection many variables such as, the freshness of the fecal sample, sample size, and the sampling technique used to collect the feces, can all impact the sensitivity of the method used for detection of *E. coli* O157:H7 in animal feces. For bovine fecal samples, a variety of sampling techniques have been used to collect samples, including rectal swabs, rectoanal mucosal swabs (RAMS) (Hancock et al., 1997; Mechie et al., 1997; Rice et al., 2003), rectal grab samples (Laegrid et al., 1999; Zhao et al., 1995), and grab or swab samples from fecal pats (Hancock et al., 1994 and 1997; LeJeune et al., 2005). The impact that sample size has on the culture detection method used for isolation of *E. coli* O157:H7 is debated in the literature; however, sample size determination seems to be highly dependent on the number of samples to be screened, as well as whether the animal is naturally or experimentally infected, and the sampling technique used for collecting the fecal sample. One study showed that the culture of 0.1-g fecal samples on cotton tipped swabs with TSBcv for enrichment followed by plating onto CT-SMAC is a sensitive method for large-scale studies involving hundreds of samples collected weekly, whereas larger volume (10-g) fecal samples enriched in culture prior to IMS only provided a relatively small increase in sensitivity for the extra amount of work required to process the samples (Sanderson et al., 1995). RAMS are just as sensitive and usually more sensitive (70.3% positive) than culture of 10-g fecal cultures (46.8% positive) at detecting *E. coli* O157:H7 in cattle, and RAMS samples are less contaminated with fecal material containing high titers of other bacteria (Rice et al., 2003).

In experimentally exposed Holstein bull calves (exposed by penning them with *E. coli* O157:H7-positive calves), prior to bacterial colonization (1-14 days postexposure), enriched fecal cultures were more sensitive at detecting *E. coli* O157:H7 than enriched RAMS cultures, however after colonization (>40 days postexposure) enriched RAMS cultures were more sensitive at detecting *E. coli* O157:H7 than enriched fecal cultures. The RAMS sampling technique is suggested to differentiate colonized from transiently positive cattle, if *E. coli* O157:H7 primarily colonizes the mucosa of the rectoanal junction. RAMS sampling technique has several benefits, in addition to approved sensitivity: small volumes of media, minimal hands-on technician time, no expensive equipment, and only a small number of reagents are required.

In contradiction to using smaller sample sizes, another study showed that incubation of 10-g fecal samples in TSBcv and subsequent plating on CT-SMAC detected more positive samples of *E. coli* O157:H7 from naturally infected cattle feces than incubation of fecal swab samples (approximately 0.1-g samples) in TSBcv with subsequent plating on CT-SMAC; however, the difference was not statistically significant (Sanderson et al., 1995).

The distribution of *E. coli* O157:H7 in fecal samples can play an important role in the sensitivity of detection of *E. coli* O157:H7. The density of *E. coli* O157 in bovine fecal pats is highly variable, with most positive samples having a density <100 CFU/g, which is the limit for reliable detection by IMS (Pearce et al., 2004). Sampling of multiple pats (1-g samples) from bovine fecal pats, showed that *E. coli* O157 could not be detected in all samples from the same pat using IMS, and that testing only 1-g samples per pat could result in a sensitivity as low as 20-50%.

This uneven distribution and low density of *E. coli* O157 in some fecal pats could lead to false-negative results and O157 could be less likely to be detected on farms with a low number of cattle excreting this organism than on farms with many cattle excreting *E. coli* O157. Therefore, it is recommended, if using the swab fecal pat sampling technique, to test at least two 1-g samples per pat rather than one 1-g sample (Pearce et al., 2004).

The incubation temperature and the length of time for incubation *E. coli* O157:H7 pre-enrichment broth used by researchers is another varying variable. Temperature can affect both the growth rate of the target organism, and the time it takes to reach detectable levels, and has an effect on the type of competitor organisms present in the enriched sample (Baylis et al., 2001). The length of pre-enrichment incubation time can be influenced by the type of broth used. In one study, bovine fecal samples enriched for 6 h in tryptic soy broth supplemented with novobiocin (mTSBn), followed by IMS detected more *E. coli* O157 culture-positive samples than the same samples enriched for 24 h; and pre-enrichment of the same bovine fecal samples in two different pre-enrichments, mTSBn and BPW, resulted in the detection of more positive *E. coli* O157 samples in mTSBn at 6 h, and BPW when incubated for 24 h (Tutenel et al., 2003). LeJeune et al. (2001) showed that the sensitivity of detecting *E. coli* O157 in water and water sediment samples was increased with the use of unsupplemented TSB (compared to TSBcv or Gram-neagtive broth) incubated for 18-24 h at 44.5ºC (compared to incubation at 37ºC for 5 h or 18-24 h, and TSB incubated at 44.5ºC for 5 h) with the use of IMS. Although, prolonged enrichment (> 24 h) for the detection of *E. coli* O157:H7 can affect the sensitivity of tests used for isolating *E. coli* O157:H7 as a result of decrease in the ratio of *E. coli* O157:H7 to background flora (Tutenel et al., 2003; LeJeune et al., 2001).

The storage and transport of samples is another important issue in detection of *E. coli* O157:H7 in bovine fecal samples. Studies of survival of *E. coli* O157:H7 in bovine feces have shown that *E. coli* O157:H7 can survive for longer periods at lower temperatures and in moist conditions (Wang et al., 1996; Fukushima et al., 1999), and much shorter periods if subject to drying, higher temperatures, or contained in bovine feces applied to grassland (Bolton et al., 1999). Therefore, to maximize recovery of *E. coli* O157:H7 in fecal samples, all samples should be collected fresh, refrigerated during transport to the laboratory, and processed within 48 h of collection (Chapman 2001).

Numerous methods for detecting *E. coli* O157:H7, at least in bovine fecal samples, have been developed and used by different laboratories. Therefore, the levels of sensitivity of detection being reported in the literature for these various methods vary greatly, making it difficult to compare data between studies using different detection methods with dissimilar levels of sensitivity. Based on the literature, we chose to pre-enrich fecal pat swabs in BPW for 18-24 h at 42ºC, followed by IMS and subsequent plating on CT-SMAC. Fecal pat swab sampling technique was chosen due to the large number of fecal pat samples, and environmental samples collected. We did not use the RAMS sampling technique because the paper was published after our study was completed. *E. coli* O157:H7 suspect colonies were screened for MUG- activity, and confirmed by a latex agglutination test for the presence of the O157 antigen, and multiplex PCR assays for the detection of *hylA*, *eaeA*, *rfbE, fliC,* stx_1, stx_2 , and stx_2 variants (2, 2c, 2d, 2e, 2f) (29).

Subtyping

Subtyping of *E. coli* O157:H7 isolates following an outbreak enables identification of the probable source of *E. coli* O157:H7 infections, and the development of measures to prevent and control further outbreaks. In research studies, bacterial subtyping methods provide information on the individual organism, its clonal type, inference on the genetic relatedness between isolates from different animal species, and description of the bacterial transmission dynamics. There are two categories of typing methods used by laboratories; (1) phenotypic methods (i.e., serotyping, biotyping, and bacteriophage typing), based on the products of gene expression, and (2) genotypic methods (i.e., plasmid fingerprinting, restriction fragment length polymorphism, pulsed-field gel electrophoresis, various polymerase chain reaction-based methods, and DNA sequencing), based on the analysis of the genetic structure of an organism, including DNA polymorphisms and the presence or absence of extrachromosomal DNA (Tenover et al., 1997). The selection of typing technique to be used relies on the question being asked and information required to answer the question, the equipment and expertise to perform the technique, as well as the time-frame needed to answer a question (Thomas-Carter 2001).

The traditional methods of strain typing, such as bacteriophage typing and serotyping, have been supplemented or replaced in many laboratories with molecular genotyping methods because of their higher discriminatory power to differentiate among epidemiologically unrelated isolates.

Numerous techniques have been used to genetically subtype *E. coli* O157:H7 isolates (Hahm et al., 2003), however pulsed-field gel electrophoresis (PFGE) is currently considered the "gold standard" and the most widely used DNA genetic fingerprinting method for detecting both the source of an outbreak of *E. coli* O157:H7 (CDC 1996 and 1997; Johnson et al, 1995; Keene et al., 1997; Roberts et al., 1995), and surveillance of *E. coli* O157:H7. It has also been used in many studies to show clonality between isolates of *E. coli* O157:H7 from different cattle herds (Shere et al., 1998; Lee et al., 1996). The National Foodborne Surveillance Network (PulseNet), sponsored by the Centers of Disease Control and Prevention in the United States and Canada (CDC 2001) established a standard protocol to be used state wide, with the objective of sharing data between state and local public health monitoring laboratories to allow for the rapid access of DNA fingerprints of isolates from disease outbreaks and follow-up isolates from cases that are geographically and temporally unrelated. The method of PFGE uses restriction endonucleases to cut chromosomal DNA infrequently, generating from 10-20 fragments. These fragments are then subsequently separated into discrete bands (1 kb to 1,000 kb in size) in an agarose gel by PFGE using an apparatus that switches the direction of current according to set switch times (determined by the organism and specific restriction endonuclease). Interpretation of DNA restriction patterns generated by PFGE data can be determined by a number of methods; (1) assess the relatedness of bacterial strains by determining the number of band differences between each pair of isolates through categorization of genetic and epidemiologic relatedness, and (2) calculation of the band-sharing similarity coefficients, where the restriction endonuclease fragment pattern (REDP) generated from each isolate is compared using a number of available software

(Singer et al., 2004). For the first method mentioned, the interpretation of the number of band differences between isolates is based on the minimum number of genetic mutational events that would result in the observed number of band differences in order to categorize them as: indistinguishable (no genetic events, resulting in 0 band differences), closely related (a single genetic event, resulting in 2-3 band differences), possibly related (two independent genetic events that result in 4-6 differences), or as unrelated (three or more independent genetic events leading to >7 band differences) (Tenover et al., 1995). For the second method, software such as Bionumerics (Applied Maths, Inc., Austin, Tx) contains different algorithms for the importation of gel images and normalization of the lanes in the PFGE gel that can be used to calculate the similarity among isolates, and to construct dendrograms that graphically depict the relatedness of isolates. Similarities between isolates are calculated by pairwise comparison of the number of bands in each REDP and the number of shared bands for all isolates in order to create a matrix of band-sharing coefficients between all pairwise comparisons. This matrix of band-sharing coefficients is then used in a cluster analysis and graphically shown on a dendrogram (Singer et al., 2004).

Eventhough, PFGE is currently the most widely used molecular subtyping method for outbreaks of *E. coli* O157:H7, and studying the ecology and epidemiology of *E. coli* O157:H7 in livestock farms, there are contradictory findings in the literature as to the advantages of using this method.

The PFGE method while simple and highly discriminatory, has many limitations: takes several days to complete; often produces results that are suboptimal for inter-laboratory comparison; has limitations in the power to resolve bands of nearly identical size (Davis et al., 2003); the interpretation of banding patterns is subjective (Noller et al., 2003); and if used as a tool in establishing genetic relatedness of *E. coli* O157:H7 strains, the use of six or more restriction enzymes are required (Davis et al., 2003). However, the discriminatory power of PFGE has compared favorably and even higher to that of other subtyping methods for typing *E. coli* O157:H7. When compared to the methods Amplified-Fragment Length Polymorphism (AFLP), which looks at specific sets of genomic restriction fragments by PCR, AFLP was shown to be faster but PFGE offered the best resolution for typing *E. coli* O157:H7 (Heir et al., 2000); and Multilocus Sequence Typing (MLST), a DNA sequencing-based approach, that analyzes the internal fragments of housekeeping genes to establish genetic relatedness between isolates, also proved to not be better than PFGE due to the lack of genetic diversity in the housekeeping genes of *E. coli* O157:H7 (Noller et al., 2003). However, the method multilocus variable-number tandem repeat (MLVA), that analyzes multiple-variable number tandem repeat (VNTR) loci located in the areas of the bacterial genome that evolve quickly, was shown to have a sensitivity equal to that of PFGE and a specificity superior to that of PFGE for distinguishing *E. coli* O157:H7 strains associated with an outbreak (Noller et al., 2003), as well as being far less labor intensive and faster (Lindstedt et al., 2003).

In conclusion, PFGE is the current choice of molecular subtyping tool used in outbreak investigations, and surveillance, due to its high discriminatory power. Although, for more accurate investigation of epidemiological relationship between isolates, especially in making inferences about routes of pathogen transmission, one must use multiple enzymes, or combine the use of PFGE with that of another genetic subtyping method, such as phage typing or ribotying (Avery et al., 2002; Hahm et al., 2003). PFGE was chosen to be used in this study in the event we desired to compare our animal *E. coli* O157:H7 isolates with human isolates from the CDC PulseNet database in the near future.

EPIDEMIOLOGY AND ON-FARM ECOLOGY

Humans

Temporal trends: *Escherichia coli* O157:H7, based on increased reporting of O157-related illnesses, is an emerging disease pathogen in humans (Armstrong et al., 1996). It is still unclear as to whether the increased reporting of *E. coli* O157 illness is due to the increased awareness and better diagnostic methods, or due to an increase in infection, or both. The incidence of HUS has been used to study trends in the occurrence of O157-related disease because the clinical presentation of HUS is very distinctive and less likely to be misdiagnosed than other related illnesses (Tarr et al., 2005). The frequency of HUS and rates of isolation of *E. coli* O157:H7 have been correlated with the distance from the equator in the northern hemispshere (Cummings et al., 2002).

However, this correlation is not absolute; for example Scotland has a high incidence of HUS and *E. coli* O157:H7 infections, but Denmark does not (Thomas et al., 1993; Nielsen et al., 2002); and in the southern hemisphere, Buenos Aires in Argentina has a very high incidence of HUS. Sporadic cases of HUS in North America is about 2-3 cases per 100,000 children under 5 years of age, in contrast to a 10-fold higher incidence in this age group in Argentina (Griffin, 1995). In fact, Argentina has probably the highest incidence of HUS in the world, with approximately 250 cases each year, and the annual incidence of HUS in Buenos Aires in children under 4 years of age is approximately 22 cases per 100,000 (Lopez et al., 1998). In the United States and England, the incidence of diagnosed *E. coli* O157:H7 infections seems to be greater among rural than urban populations (Tarr et al. 2005; Trompeter et al., 1983). In North America, seroepidemiological surveys have shown higher frequencies of antibodies to the O157 lipopolysaccharide among residents of rural areas than among those living in urban areas (Reymond et al., 1996; Haack et al., 2003). Visits to cattle farms have been implicated as sites for human *E. coli* O157:H7 infections in Finland (Lahti et al., 2002) and the United States (Crump et al., 2002). The reasons for these demographic differences in the rates of *E. coli* O157:H7 human infections and HUS are not known.

The majority of *E. coli* O157:H7 infections and HUS occur in the summer and autumn months (Gerber et al., 2002; Kaplan et al., 1975; Tarr and Hickman, 1987; Slutsker et al., 1997; Klein et al., 2002). For non-O157:H7 STEC infections and HUS in Australia and Montana, USA, the same seasonality pattern has been reported to occur (Elliot et al., 2001; Jelacic et al., 2003).

Modes of Transmission: Humans may become infected with *E. coli* O157:H7 either through (1) direct contact with contaminated animal or human feces, (2) indirectly after the consumption of contaminated food and water sources, (3) contact with environments which have been contaminated with bovine feces, or (4) by transmission in the laboratory (Feng et al., 1995).

Outbreaks: An outbreak is defined as ≥ 2 cases of STEC infection with a common epidemiologic exposure, and a case is usually defined as a stool culture yielding STEC, or bloody diarrhea, or HUS (Rangel et al., 2005).

Human STEC infections have been a greater problem in industrialized countries than in non-industrialized countries, probably due to differences in the production and distribution of food. Although, reports of STEC-associated outbreaks from countries such as Africa, and the difficulties in performing laboratory diagnosis, suggest that the extent of STEC infections in non-industrialized countries may be underestimated (Tozzi et al., 2001).

Europe: In Europe, the United Kingdom and Scotland have the highest reported incidences of *E. coli* O157:H7 infections (surveillance is based on the reporting of *E. coli* O157:H7 isolates), and in the period from 1990-1996, the national incidence of infection with *E. coli* O157:H7 per 100,000 population ranged from 1.5 in England and Wales to more than 5 in Scotland (Smith et al., 1998). In the UK, foodborne outbreaks associated with *E. coli* O157:H7 have involved beef, cooked meat, raw vegetables, cheese, milk, and yoghurt, many of these cases were due to cross-contamination (Tozzi et al., 2001).

Other non-O157 serogroups including O26, O103, O111, and O145 have been frequently associated with HUS in Europe, and soribitol-fermenting strains of *E. coli* O157:H$^-$ have caused outbreaks of HUS in Germany, Finland, Hungary, and the Czech Republic (Tozzi et al., 2001). With regard to northern Europe, in Scandinavian countries, STEC infections seem to be rare. In Norway, for example, between 1992 and 2003 only 96 human cases of disease caused by *E. coli* O157:H7 or other STEC were reported, an incidence of 0-0.4 per 100,000 inhabitants (Hofshagen et al., 2004). In the Mediterranean area of Europe, there is a low incidence of STEC infections. In Italy and Spain, STEC infection has been diagnosed in 0.8% and 1.9% of persons with diarrhea, respectively; however, *E. coli* O157:H7 has rarely been isolated from human stool samples routinely submitted to clinical laboratories (Blanco et al., 1998; Caprioli and Tozzi, 1998). *Escherichia coli* O157:H7 has been isolated in Croatia, Slovenia, and Turkey and Isreal (Tozzi et al, 2001).

South America: High incidences of HUS have been reported for Argentina (as previously mentioned), Uruguay (5 cases per 100,000 population in children < 5 years of age), and in Chile (3-4.2 cases per 100,000 population in children < 5 years of age) (Tozzi et al., 2003), however *E. coli* O157:H7 is an uncommon serotype in Latin American countries. The high incidences of STEC infection in Argentina may be due to several reasons: the very large numbers of cattle raised and a 16.3% prevalence of STEC in beef cattle and 21.3% prevalence of STEC in bovine meat samples; the high rate of bovine meat consumption (about 60 kg per person per year); and the widespread consumption of undercooked meat (Lopez et al., 1998).

Asia: Most information on STEC outbreaks comes from Japan, where the first outbreak due to *E. coli* O145 was reported in 1984 (Kudoh et al., 1994). Between 1985 and 1995, numerous outbreaks of *E. coli* O157:H7 and several outbreaks of *E. coli* O111 were reported to occur mainly within families and school settings (Kudoh et al., 1994; Michino et al., 1998). Then in 1996, multiple outbreaks of *E. coli* O157:H7 infection involving over 11,000 cases and 12 deaths occurred associated with different foods (fish flake salad, salad with seafood sauce, potato salad with fish sausages, and radish sprouts) (Michino et al., 1998; Michino et al., 1999). One of these outbreaks was the largest known *E. coli* O157:H7 outbreak in the world, involving more than 6,000 children in 47 primary schools in Sakai City that was linked to the consumption of uncooked white radish sprouts (Michino et al., 1999). Other Asian countries have observed high rates of STEC infection in both diarrheal and asymptomatic people in Thailand and Iran; however none of the strains described belonged to serotype O157:H7 (Tozzi et al., 2001).

Australia and New Zealand: A low frequency of *E. coli* O157:H7 has been found in surveys for STEC in Australian children compared with that in similar industrialized countries (the incidence of sporadic cases of HUS in 1994-1995 was 0.62 cases per 100,000 children of 0-15 years of age). The only outbreak of *E. coli* O157:H7 reported in Australia occurred in 1996 and involved three children with diarrhea that ate food from a delicatessen where the food handlers were infected with *E. coli* O157:H7 (Desmarchelier, 1997; McCall et al., 1996).

The United States: *Escherichia coli* O157:H7 is the predominant STEC serotype in causing human disease in both North America and Europe (Sargeant and Smith, 2003). Published data on the surveillance results of *E. coli* O157 outbreaks in the United States reported to the CDC from 1982 through 2002, showed that 49 states reported 350 outbreaks, representing 8, 598 cases, 1,493 hospitalizations, 354 HUS cases, and 40 deaths. The number of reported outbreaks began to increase in 1993, probably due to increased detection methods and establishment of *E. coli* O157 as a nationally notifiable pathogen, and peaked in 2000. Most outbreaks occurred from May to November with the state of Minnesota reporting the most outbreaks (43 outbreaks), followed by Washington, New York, California, and Oregon (18 outbreaks). Among the 350 *E. coli* O157:H7 outbreaks, transmission routes for 183 were foodborne, 74 unknown, 50 person-to person, 21 recreational water, 11 animal contact, 10 drinking water, and 1 laboratory-related. However, these numbers might be skewed due to unrecognized outbreaks not captured by the CDC's surveillance system. In addition, trends of outbreak reporting should be interpreted carefully, do to the changing factors (increased awareness of disease, and improved detection and subtyping methods) over time that may impact outbreak detection and reporting (Rangel et al., 2005).

Escherichia coli O157:H7 foodborne outbreaks during this period occurred most frequently in communities such as restaurants, and schools with ground beef being the most common vehicle among the outbreaks prior to 1995 (75/183). However, with major improvements in meat safety in the U.S. fast food industry, the last hamburger-associated fast-food restaurant outbreak was reported in 1995.

Fruit and Vegetable-associated outbreaks, first reported in 1991, accounted for 38 out of the 183 *E. coli* O157:H7-related foodborne outbreaks, and remain the prominent food vehicle as of 2002. Most *E. coli* O157:H7 outbreaks during 1982 through 2002 were due to kitchen cross-contamination, and already-made or packaged produce, including cabbage, lettuce, sprouts, apple cider, and apple juice (Ackers et al., 1998; Mahon et al., 1997; Besser et al., 1993; Cody et al., 1999). An outbreak of *Escherichia coli* O157:H7 in 1996 involving the consumption of unpasterized apple juice, causing over 70 illnesses (children mostly) with HUS development in 20% and one death, led to the federal regulation that all unpasteruized apple juice or apple cider must carry a warning label advising consumers of potential harmful bacteria in the product (US Food & Drug Adm. 1998). Since 1988, there have only been two published reports of outbreaks associated with unpasteurized apple cider; one at a local fair and one from a locally produced cider (Rangel et al., 2005).

Outbreaks of *E. coli* O157 associated with person-to-person were shown to be spread by the fecal-to-oral route in child daycare centers, individual residencies, nursing homes, and schools from 1982 through 2002 (Rangel et al., 2005). Animal contact outbreaks of *E. coli* O157 were first reported in 1996, and are one of the newest recognized transmission routes.

Animal-to-person transmission usually occurs via fecal-to-oral route either through the touching of animal hides contaminated with *E. coli* O157 from fecal matter, or by touching fecal-contaminated surfaces in the animal's environment.

Out of the11 animal contact outbreaks that were reported by Rangel et al. (2005) between 1996-2002, five occurred on farms, two at county fairs, two at petting zoos, one at a barn dance, and one at a camp (Rangel et al., 2005). The numbers reported by Rangel et al. for animal contact outbreaks from county fairs in the U.S., was found to be slightly higher by LeJeune and Davis (2004), with the number reaching 5 outbreaks of *E. coli* O157:H7.

Escherichia coli O157-associated waterborne outbreaks were first reported in 1991 and since then 31 outbreaks have occurred. Fourteen outbreaks occurred in lakes or ponds; 7 in swimming pools, 4 were attributed to local well water systems, 3 involved municipal water supply systems, 1 from spring water and residential faucet water. Drinking and recreational water have great potential to infect many persons, and most outbreaks due to contaminated drinking water tended to be larger than all other outbreaks. In 1999, the largest U.S *E. coli* O157 outbreak occurred at a county fair due to contaminated drinking water that involved 781 persons; 9% were hospitalized and of those, 2% developed HUS and 2 died. The contaminated water source implicated in the outbreak was a temporary unregulated well located at the fairground (CDC 1999).

The most recent preliminary FoodNet data (part of the CDC's Emerging Infections Program) on the estimated incidence of infection with *E. coli* O157:H7 for 2004 when compared to data from 1996-1998 in the US, shows a significant decline (42%) in the incidence of infections. This substantial decline, first noted in 2003 by FoodNet, and sustained in 2004, is also consistent with the decline in *E. coli* O157 contamination of ground beef reported by the U.S. Department of Agriculture Food Safety and Inspection Service (FSIS) for 2003 (CDC 2005).

The recognition of outbreaks due to other STEC strains in the United States are uncommon, partly because of limitations in surveillance. Because large numbers of outbreaks of human illnesses are attributed to *E. coli* O157:H7, the availability of selective media is designed for isolation of this serotype and many laboratories have limited their screening of human or animal feces to *E. coli* O157:H7 (Bettelheim, 2003). Other serotypes in *E. coli* that have been demonstrated to cause human outbreaks in the U.S include, O103:H2, O104:H21, and O111:48 (Bettelheim, 2003).

Cattle are considered the primary reservoir of *E. coli* O157:H7, and therefore initially undercooked beef and unpasteurized dairy products were considered the primary routes of human infection. However, it has become progressively more apparent that routes other than foods of bovine origin can contribute to the epidemiology of human disease, such as environmental contamination with this pathogen (Chalmers et al., 2000; Renwick et al., 1994; Strachan et al., 2002), and produce that has been in contact with *E. coli* O157:H7-contaminated manure (fertilizer) or irrigation water during stages of production and processing (Hilborne et al., 1999).

Animals

Cattle: The identification of bovine sources in human cases of *E. coli* O157:H7 illness has led to a considerable amount of resources and time devoted to elucidating the distribution and patterns of fecal excretion by cattle in hopes to identify control measures for this pathogen in cattle operations. As previously mentioned, differences in sampling methods, and laboratory techniques, comparing prevalence values is difficult.

Initial, point-in-time prevalence surveys in North America reported a prevalence of fecal excretion in individual cattle of less than 6% in dairy cattle (Wells et al., 1991; Hancock et al., 1994), range beef cattle (Hancock et al., 1994; Sargeant et al., 2000), and feedlot beef cattle (Hancock et al., 1994; Galland et al., 2001), with similar prevalence levels reported in surveys in European cattle herds (Blanco et al., 1996; Vold et al., 1998; Lahti et al., 2001). However, more recent studies have found a much higher prevalence of fecal excretion of *E. coli* O157:H7 in individual cattle, more than likely do to the development of more sensitive detection methods; approximately 7.4% in some cattle herds (Laegrid et al., 1999); 28% prevalence was reported in a study of fed cattle at slaughter (Elder et al., 2000); and a range of 10-23% have been reported in feedlot cattle (National Animal Health Monitoring System 2001; Sargeant et al., 2001). Many animal, herd, environmental, and production factors may affect prevalence of *E. coli* O157:H7 in cattle. The prevalence of fecal excretion of *E. coli* O157:H7 varies by age, with higher prevalence values reported in younger animals (2 to 24 months of age) than adults in field studies (Hancock et al., 1994; Wells et al., 1991; VanDonkersgoed et al., 1999). The reasons for age-related differences are unclear, but it may be due to ruminal development differences, differences in microbial flora in gastrointestinal tract, or management differences such as dietary factors (Meyer-Broseta et al., 2001). At the herd level, *E. coli* O157:H7 has been isolated from cattle feces from the majority of, if not all, dairy and beef cattle operations studied longitudinally (Hancock et al., 1997a; Sargeant et al., 2000). Estimates of point-in-time prevalence at the herd/pen level have been very high; ranging from 63% to 100% (Dargatz et al., 1997; Laegrid et al., 1999; Elder et al., 2000; Smith et al., 2001).

Fecal excretion of *E. coli* O157:H7 often occurs simultaneously among cattle co-housed in the same group that results in peaks or bursts of prevalence (Hancock et al., 1997).

Longitudinal and observational studies in cattle have shown that fecal excretion of *E. coli* O157:H7 by either naturally infected or experimentally infected cattle, is typically transient or intermittent ranging from a few days to a few months, but most frequently limited to a few weeks (Besser et al., 1997 and 1999; Zhao et al., 1995; Cray and Moon 1995). Cattle also excrete *E. coli* O157:H7 in their feces in variable magnitudes, with high doses early on in infection and lower doses for extended periods of time, but rarely exceed 10^6 CFU per gram of feces (Besser et al., 2001; Mechie et al., 1997). Long term carriers of *E. coli* O157:H7 have not been detected in cattle populations, and this has led many to question whether cattle are true reservoirs of *E. coli* O157:H7 or simply carriers that are intermittently exposed to this organism (Rasmussen and Casey 2001). Several reasons have been proposed for why intermittent shedding occurs among individual animals such as, latent infections and/or excretion of the bacteria at levels below the detection threshold of a diagnostic assay (Cray and Moon 1995), or reinfection after the initial infection (Zhao et al., 1995).

Infection of the gastrointestinal tract of adult cattle, and weaned calves with *E. coli* O157:H7 is asymptomatic (reviewed in Stevens et al., 2002). Histological analysis of intestinal epithelial from cattle and calves infected with *E. coli* O157:H7 has shown intimate bacterial adherence, in some but not all cases, and an inflammatory response characterized by diffuse infiltration of neutrophils into the lamina propia (Stevens et al., 2002).

One study demonstrated infection of neonatal colostrums-deprived calves (less than 36 h old) with *E. coli* O157:H7 and development of enterocolitis within 18 h of inoculation and attaching and effacing lesions in the large and small intestine at necropsy (Dean-Nystrom et al., 1997). However, other challenge studies in pre-weaned calves inoculated with *E. coli* O157:H7 have not reported illness (Cray and Moon 1995; Sanderson et al., 1999; Woodward et al., 1999).

Fecal excretion of *E. coli* O157:H7 by cattle occurs in a seasonal pattern, with higher prevalence levels occurring in the summertime or early fall; and of which coincides with the seasonal variation in disease incidence seen in humans, with higher rates also observed during the summer months (Chapman et al., 1997; VanDonkersgoed et al., 1999; Wallace et al., 2000). In nine herds sampled for approximately one year, the prevalence of *E. coli* O157:H7 in the months of June through October were several times that observed in December through March (Hancock et al., 1994). Observed seasonal effects of *E. coli* O157:H7 excretion in cattle could be due to confounded factors, such as differences in the microbial flora of the gastrointestinal tract in cattle during the summer verses the winter months due to changes in diet, or related to condusive conditions for multiplication of the bacteria in environmental niches. It is not known whether a direct relationship exists between the seasonal patterns of fecal excretion in cattle and disease incidence in humans, or whether the increase in human cases of *E. coli* O157:H7 infection during the warmer months is caused by behavioral factors in humans.

Such behavioral factors might include, an increase in the consumption of bovine foods or fruits/vegetables (possibly contaminated with *E. coli* O157:H7 from fertilization with contaminated manure), or an increase in visitors at petting zoos and agricultural fairs (where outbreaks have been shown to occur) during the summer months.

In additional to seasonal patterns of *E. coli* O157:H7 prevalence in cattle populations, there appear to be temporal effects; such that a high percentage of animals excrete the organism for a short period of time followed by longer periods of low prevalence within herds. This temporal effect of fecal excretion has been identified in both dairy (Hancock et al., 1997; Shere et al., 1998) and beef (Sargeant et al., 2000; Smith et al., 2001) cattle herds. Horizontal transmission of *E. coli* O157:H7 between cattle has also been shown to ocurr, both experimentally and naturally (Besser et al., 2001; Shere et al., 2002; Faith et al., 1996). The fact that some cattle can excrete large numbers of *E. coli* O157:H7 for several weeks at a time following exposure to a low dose (200 CFUs) of the bacteria, and that the organism can be isolated from the mouth and hide of cattle at a high rate (Keen and Elder, 2002; Besser et al., 2001), suggests that temporal clustering of *E. coli* O157:H7 may result from an initial exposure of a small number of animals followed by horizontal transmission to a large number of animals.

Escherichia coli O157:H7 has been found in cattle feces from most areas of North America, Cananda, the United Kingdom, Scotland, The Netherlands, Finland, Italy, Japan, France, South America, and Australia suggesting that this organism is ubiquitously present on cattle farms. There are however, other regions, such as Scandinavia, Africa, and Norway that report a very low prevalence of *E. coli* O157:H7 (Lahti et al., 2001; Johnsen et al., 2001; Vold et al., 1998).

This may be due to climate factors, or farm management practices less conducive to cattle being colonized with *E. coli* O157:H7, or the possibility that this organism has not yet been introduced into these regions (Besser et al., 2003).

Other Domestic Species. In addition to colonizing the gastrointestinal tract of cattle, *E. coli* O157:H7 has been sporadically isolated from the feces of a wide variety of other animal species, including sheep, goats, pigs, dogs, cats, horses, and poultry (Hancock et al., 1998; Heuvelink et al., 1999; Feder et al., 2003; Shere et al., 1998; Chapman et al., 1997). The epidemiology of *E. coli* O157:H7 in sheep is similar to that of cattle: transient fecal excretion in individual sheep, with a higher prevalence detected in the summer, ranging in one study from 31% positive in June to undetectable levels in November (Kudva et al., 1996). Prevalence levels for individual sheep in the United Kingdom range from 2.2 to 7.4% (Chapman et al., 1997b, 2001). Sheep may shed more than one strain of *E. coli* O157:H7 at the same time, the strains excreted by individual animals change over time, and recent excretion of *E. coli* O157:H7 does not prevent recolonization of sheep with *E. coli* O157:H7 (Kudva et al., 1996; Kudva et al., 1997). *Escherichia coli* O157:H7 have been isolated from swine and poultry carcasses, but not consistently, and with very low prevalence (Doyle and Schoeni 1987; Chapman et al., 1997; Heuvelink et al., 1999; Pilipcinec et al., 1999; Nakazawa et al., 1999; Johnsen et al., 2001).

With regard to poultry and turkeys, a limited number of surveys have been published on the prevalence of *E. coli* O157:H7 in chicken and turkey flocks, and on processed poultry meat.

One study, by Pilipcinec et al. (1999), reported that 20 out of 216 cloacal swabs taken from chicken at a slaughterhouse in Slovakia were positive for Shiga toxin-producing *E. coli* O157:H$^-$. In contrast, Chapman et al. (1997) and Heuvelink et al. (1999) did not detect any *E. coli* O157:H7 in fecal samples taken from 1,000 and 501 chicken layer flocks respectively. Doyle and Schoeni (1987) detected the presence of *E. coli* O157:H7 on 1.5% of poultry carcasses from the state of Wisconsin in the US; whereas several other surveys of poultry carcasses failed to detect *E. coli* O157:H7 (Irvin et al., 1989; Read et al., 1990; Smith et al., 1991). A 10-year study carried out by the US Federal Government in ready-to-eat poultry containing meals, resulted in the failure to detect any *E. coli* O157:H7 organisms in the meals (Levine et al., 2001). In the rearing stages of turkey flocks, one study isolated *E. coli* O157 from 2 out of the 11 flocks tested, and at the processing stage *E. coli* O157:H7 could be detected in 8 out of the 11 flocks, indicating that cross contamination in the processing plant can occur (Hafez et al, 2001). Another study by Heuvelink et al. (1999) isolated *E. coli* O157 strains from six of 459 (1.4%) pooled fecal samples from turkey flocks. In spite of current data suggesting that the prevalence of *E. coli* O157:H7 is low in poultry, several studies into the behavior of *E. coli* O157:H7 in poultry have demonstrated the ability of *E. coli* O157:H7 to colonize certain age groups of poultry for long periods of time. Artificially inoculated 1-day-old specific pathogen free (SPF) chickens by crop canulation can be colonized with *E. coli* O157:H7 without developing clinical signs of illness over a 90-day postinoculation period; however, histological examination showed the bacteria attached to the cecal mucosa in an A/E manner and induced mild mucous membrane damage in the proximal cecae at 14 to 28 days postinoculation (Beery et al., 1985).

Subsequent studies of SPF 1-day-old chickens, have demonstrated that inoculation of either 26 CFU by oral gavage or 10^8 CFU was sufficient to establish colonization of the cecum for approximately 90 days and 10 to 11 months respectively, and *E. coli* O157:H7 was cultured from egg shells from those hens excreting the organism for up to 11 months (Schoeni et al., 1994). Another study by Sueyoshi and Nakazawa (1994) demonstrated the susceptibility of SPF 1-day-old chickens to A/E lesions in the cecum with different strains of *E. coli* O157:H7 isolated from the feces of calves (O26:H11, O5:NM), pigs (O15:H10), chickens (O103:NM), and humans (O111:NM, O157:H7); the O5:NM strain isolated from a calf was the only one which did cause A/E lesions after inoculation.

In swine, *E. coli* O157:H7 has only been occasionally isolated from healthy animals, with reports of isolation in swine from Japan, Netherlands, Sweden, Canada, Norway, Mexico, Chile and the United States (Erikkson et al., 2003; Feder et al., 2003; Heuvelink et al., 1999; Johnsen et al., 2001; Callaway et al., 2004a, Borie et al., 1997). The prevalence of *E. coli* O157:H7 in these studies ranged from 0.1 to 10.8%. In Chile, the prevalence of *E. coli* O157:H7 in pigs (10.8%) is reported to be higher than that reported in cattle (2.9%), suggesting that swine may be an important source of this organism in some countries (Borie et al., 1997). The infectious dose of *E. coli* O157:H7 for 3-month-old pigs was shown to be 6 x 10^3 CFU in vitro-grown bacteria (Cornick and Helgerson, 2004), and experimentally infected market weight pigs excreted *E. coli* O157:H7 in their feces for at least 2 months (Booher et al., 2002).

Using gnotobiotic pig models, *E. coli* O157:H7 has been demonstrated to attach intimately to intestinal epithelial cells, primarily in the large intestine with A/E lesions at the site of attachment. Gnotobiotic pig model is currently the only animal model in which CNS vascular lesions (lesions in the brain) have been induced by infection (orally inoculated) with Shiga toxin-producing *E. coli* O157:H7, and identical to the lesions reported in human cases of HUS (Moxley and Francis, 1998). To date, no human outbreaks have been specifically traced to pork. One *E. coli* O157:H7 outbreak did occur in which sausage containing both beef and pork was implicated as the source of infection (Paton et al., 1996).

Insects: *Escherichia coli* O157:H7 has been isolated from flies on cattle farms, but without detection methods determining whether the isolates were from the body surface or the gut of the flies (Rahn et al., 1997; Hancock et al., 1998; Iwasa et al., 1999). Experimental studies have shown that *E. coli* O157:H7 can proliferate in mouthparts of the house fly and be excreted for at least three days after feeding (Sakai et al., 2000; Kobayashai et al., 1999). It is not known whether the amount excreted by flies is sufficient to infect cattle or replicate in cattle feed and water sources. Therefore, further studies on flies and other insects are required to determine the extent, if any, to which they are involved in the ecology of *E. coli* O157:H7 in cattle farms.

Wildlife. Since many cattle spend a lot of their time outside, the potential to have contact with non-domestic animal species is greater than many other livestock species. National surveys of feedlot and dairy producers have reported that many wild animals, such as rodents, deer, small mammals, and wild birds, to be a problem on their cattle operation (National Animal Health Monitoring System 1996, 1998, 2000).

Escherichia coli O157:H7 has been sporadically isolated from a number of wildlife species inhabiting livestock environments, including rats (Cizek et al., 1999; Nielsen et al. 2004), wild birds (Hancock et al., 1998; Nielsen et al., 2004), opossums (Renter et al., 2000 and 2003), raccoons (Shere et al., 1998), and deer (Rice et al., 1995; Keene et al., 1997; Sargeant et al., 1999; Renter et al., 2001).

Two human outbreaks of *E. coli* O157:H7 from venison (Keene et al., 1997) and unpasteurized commercial apple juice where the apples were ground-contaminated with deer feces (Cody et al., 1999) were the first to implicate deer as a possible source of contamination. Since then, many epidemiological studies were performed to determine the prevalence of *E. coli* O157:H7 in deer, which appears to be quite low (less than 3%) (Sargeant et al.,1999; Fischer et al., 2001; Renter et al., 2001). Fecal excretion patterns of *E. coli* O157:H7 in deer, as shown by challenge studies, occurs in transient patterns similar to those seen in experimentally infected calves, and horizontal transmission does occur among deer (Fischer et al., 2001).

The experimental infection of laboratory rats and pigeons infected with a natural isolate obtained from the colon of a rat trapped on a cattle farm, resulted in a duration and magnitude of *E. coli* O157:H7 fecal excretion of 14 days when infected with 10^5 CFU and 20 days respectively when infected with 10^9 CFU in domestic pigeons, and laboratory rats infected with the same doses shed the organism for 2 days and 10 days respectively (Cizek et al., 1999).

A prevalence of 0.5% (1 out of 200) was detected in wild birds inhabiting 12 different cattle farms in Northwestern USA (Hancock, et al., 1998), and a prevalence of 2.9% in fecal samples from gulls inhabiting intertidal sediments, and 0.9% prevalence in gulls residing at an urban landfill site (Wallace et al., 1997). The low prevalence seen in wild birds could be attributed to both the small number of studies performed and the low number of samples examined in any one study, thus precluding a precise determination of prevalence in wild birds.

Conclusion. Non-domestic species capable of excreting *E. coli* O157:H7 in their feces can provide either a direct or indirect source of contamination to humans, and may be involved in the on-farm ecology of *E. coli* O157:H7 due to sharing of water, feed or other environmental sources located on cattle operations (Sargeant and Smith 2003). Despite the evidence that non-domestic species may be involved in the on-farm ecology of *E. coli* O157:H7, the routes of dissemination and persistence of this organism within cattle herds has not been elucidated, and it is not known if these non-domestic species represent an incidental carriage or containment of *E. coli* O157:H7, or their role if any in the dissemination of this organism from farm to farm.

Environment

There has been debate as to whether cattle are the main reservoir species, defined as the host without which the agent cannot perpetuate, or simply a source of *E. coli* O157:H7.

Other reservoirs or niches have been suggested to exist to sustain *E. coli* O157:H7 within livestock farm environments because of the transient nature of fecal excretion seen in individual cattle, the persistence of specific strains within farms, and the distribution of genetic strains across large geographic distances (Sargeant and Smith 2003). Cattle are also known to excrete a large number of *E. coli* O157:H7 in their feces each day; i.e an individual cattle may excrete 10^5 CFU of this organism per gram of feces, and most mature cattle produce over 20 kg of manure per day (Shere et al., 2002; Overcash et al., 1983). Therefore, considerable research efforts have been devoted to determining the ecology and epidemiology of *E. coli* O157:H7 in the environment of cattle farms, such as agricultural land, water, free-stall bedding, and feed.

Agricultural Land. Effluents from farming operations include raw manure, untreated slurry (a mixture of manure, urine, feed debris, and water that is held without aeration), and treated slurry or aerated slurry that is filtered to separate the solid fraction from the liquid fraction (Woolcock, 1991). Often these effluents are applied as fertilizer to land used for silage, grazing, or cultivation, and unless appropriately processed manure is a potential biohazard capable of transmitting *E. coli* O157:H7 to both humans and animals. A variety of conditions, including temperature, solid content, pH, bacterial concentration, aeration and the length of time that manure or slurry is held before being applied to pastureland can influence the survival *E. coli* O157:H7, as well as other pathogens (Wang et al., 1996). Laboratory experiments mimicking farm effluents in the environment showed that *E. coli* O157:H7 survived the longest in effluents with a high solid content incubated without aeration at temperatures below 23ºC, and was recovered less from effluents incubated at higher temperatures (70ºC).

Escherichia coli O157:H7 has been shown to replicate in bovine feces at 22ºC and 37ºC, and survive for long periods of time (up to 8 weeks), and at 5ºC does not replicate, but can survive for up to 11 weeks under experimental conditions (Wang et al., 1996). *Escherichia coli* O157:H7 can survive for more than 1 year in a nonaerated ovine manure pile, with initial concentrations of 10^5 to 10^8 CFU/g and final concentrations of 10^1 to 10^2 CFU/g of feces. However, in aerated ovine and bovine manure piles (by periodic mixing), the organism was only able to survive for 4 months at concentrations of $\leq 10^2$ CFU/g of feces (Kudva et al., 1998). *Escherichia coli* O157:H7 has been shown to survive for shorter periods of time (less than 9 days) under experimental conditions in slurry (Kudva et al., 1998). Animal waste and bedding composted for several days (reaching temperatures of 70ºC or more) before used as fertilizer has been shown to reduce the number of viable pathogens (Pell, 1997; Woolcock, 1991). The problem has arisen because composting is no longer a practical approach for processing cattle manure because farms now contain large numbers of animals per farm, and quicker and easier methods for disposal of waste have been devised. Most large farms wash animal feces, urine, and leftover feed into a slurry mixture in settling tanks, located away from where the animals are housed, that is untreated and unaerated for more than 1 month before being used as fertilizer. It has been recommended that farm effluents be contained in holding tanks with proper aeration for appropriate lengths of time (1 to 3 months or as required) prior to be used as fertilizers (Strauch, 1991).

Long term survival (greater than 100 days) has also been reported for *E. coli* O157 in soil (Maule 1999; Ogden et al., 2001), as well as leeching capabilities in soils (Gagliardi and Karns 2000).

There seem to be several avenues in which *E. coli* O157:H7 can enter the environment: (1) direct deposition of feces onto land by pastured cattle, (2) spreading of animal waste onto agricultural lands, (3) leeching of the organism from fertilized pastures into natural water systems, or (4) fertilization of agricultural crops with infected feces (Sargeant and Smith 2003).

Water. *E. coli* O157:H7 has been detected in various types of cattle water sources, including troughs, tanks, ponds and creeks and streams (free-flowing water systems) (Faith et al., 1996; Hancock et al., 1998; Sargeant et al., 2000; LeJeune et al., 2001a); and under an experimental cattle water trough microcosm, LeJeune et al. (2001b), found that *E. coli* O157:H7 could survive and remain infectious to calves for up to 8 months in microcosm sediments. *E. coli* O157:H7, depending on its persistence in cattle water sources, has the potential to act as a source of infection or reinfection for cattle and other species, including birds, flies and rodents, which in turn can act as vectors in the transmission of the organism within and between farms (McGee et al., 2002).

Free-stall Bedding. One study showed that a higher fecal prevalence of *E. coli* O157:H7 among dairy cattle was associated with the use of sawdust bedding material opposed to sand bedding material, and under experimental bedding microcosms, *E. coli* O157:H7 persisted at higher concentrations in used-sawdust bedding than in used-sand bedding (LeJeune and Kauffman 2005).

Feed. Livestock feed has been implicated in serving as a vehicle in the transmission of indistinguishable *E. coli* O157:H7 strains over widespread geographic regions (Lee et al., 1996; Rice et al., 1999). Under experimental conditions, *E. coli* O157:H7 has been shown to survive and multiply in a variety of feeds (Lynn et al., 1998).

The association of *E. coli* O157:H7, isolated from feed, with the prevalence of cattle excreting the organism has not yet been demonstrated (Smith et al., 2001). However, one study identified *E. coli* O157:H7 in 16.6% of 446 feed samples collected from the feedbunks of commercial feedlot cattle (Dodd et al., 2001). There are several possible routes for contamination of feed with *E. coli* O157:H7 that include: contamination of the feed components at the harvest stage due to applications of manure in the field, contamination at the feedmill, contamination during storage on the farm by rodent and wild bird feces, contamination of the equipment used to administer the feed to cattle, or contamination of the feed in the feedbunk with cattle feces (Sargeant and Smith 2003).

Conclusion. The role of environmental sources in the ecology and epidemiology of *E. coli* O157:H7 in cattle operations is still unclear; however, it's very probable that environmental sources are involved in the maintenance, multiplication, and/or transmission of this organism both within and between cattle farms.

MOLECULAR EPIDEMIOLOGY

The application of subtyping methods in studies of *E. coli* O157:H7 have provided knowledge into the distribution of this organism within and among cattle environments, showing a complex and dynamic molecular epidemiology of *E. coli* O157:H7 in cattle operations. Individual cattle can excrete multiple *E. coli* O157:H7 subtypes simultaneously, although excretion of *E. coli* O157:H7 is more often of the same subtype (Faith et al., 1996; Besser et al., 1997; Renter et al., 2003).

Escherichia coli O157:H7 genetic changes *in vivo* have been shown to occur rapidly in experimentally infected calves, with detectable genetic differences found as early as 1 day post-inoculation; however repeated isolation of *E. coli* O157:H7 subtypes in cattle herds for more than a year suggest that rapid genetic turnover rates does not necessarily occur with all strains of *E. coli* O157:H7 found on cattle farms (Akiba et al., 2000; Shere et al., 1998).

A diverse population of genetic subtypes of *E. coli* O157:H7 is found in dairy (Faith et al., 1996; Rice et al., 1999), beef feedlot cattle (Rice et al., 1999; Galland et al., 2001; Renter et al., 2001a), and free range cattle (Renter et al., 2003), ranging from 79 to 81 different *Xba*I-PFGE subtypes observed. Multiple subtypes of *E. coli* O157:H7 may be present within pens and within animals in both confined and pasture cattle production environments at a single point in time, and some subtypes appear to be unique to a specific herd (Keen and Elder 2002; Renter 2002; Laegrid et al., 1999; Rice et al., 1999). The probability of detecting multiple subtypes on a farm has been found in one study to be dependent on both the number of samples collected and the duration of time in collecting the samples (Renter et al., 2004); however, in contrast, Rice et al. found no correlation between the diversity of subtypes detected and the sample size or duration of time in collecting samples. Differences in these two studies could be attributed to differences in prevalence levels of the herds sampled, differences in sampling methods, detection methods, and subtyping methods, or the seasonal time at which the samples were collected because cattle have been shown to have a higher prevalence of *E. coli* O157:H7 in the summer months.

Specific subtypes of *E. coli* O157:H7 have been repeatedly detected on a cattle farm with a range of several months to two years (Faith et al., 1996; Rice et al., 1999; Renter et al., 2003; Shere et al., 1998). A study by Rice et al. (1999), showed that most *E. coli* O157:H7 subtypes appeared within a particular farm only briefly, and most farms had a succession of different subtypes, with seven different subtypes of *E. coli* O157:H7 found on one farm on a single sampling date.

Indistinguishable *E. coli* O157:H7 subtypes have been detected on cattle farms separated by more than 100 km and up to 640 km, and on cattle farms separated by large distances lacking any identifiable common sources (Faith et al., 1996; Renter et al., 2003; Rice et al., 1999). Rice et al. (1999) found no correlation between the introduction of new cattle in dairy herds and the number of detected *E. coli* O157:H7 subtypes. In one study, investigators found a greater number of *E. coli* O157:H7 subtypes per herd in feedlot cattle verses dairy cattle (Rice et al., 1999). Some researchers suggest that the diversity of *E. coli* O157:H7 subtypes in feedlots, known to have frequent outside additions to the cattle herd, is a reflection of cattle colonized prior to feedlot arrival rather than naïve cattle exposed to multiple subtypes on feedlot arrival (Laegrid et al., 1999; Galland et al., 2001). In fact two studies have shown that multiple subtypes of *E. coli* O157:H7 can exist in groups of pre-feedlot or pastured calves (Laegrid et al., 1999; Renter et al., 2002). However, other studies have shown that even in cattle operations which do not bring outside animals into the herd, multiple subtypes still exist at the same time with periodic addition and/or clonal turnover (genetic changes) of *E. coli* O157:H7 subtypes (Shere et al., 1998; Rice et al., 1999; Renter 2002).

A study by LeJeune et al. (2004a) showed that *E. coli* O157:H7 isolates obtained from cattle feces in 20 feedlot pens, where incoming animals in each lot were from multiple sources, when subjected to restriction endonuclease digestion and PFGE, were predominantly of a few subtypes that were persistent over the sampling period (4 months). These findings indicate the importance of the farm environment, and not necessarily the incoming cattle, as a potential reservoir or source of *E. coli* O157:H7 on feedlot farms. If incoming feedlot cattle were a major source for introduction of *E. coli* O157:H7 in the feedlot, one might have expected to detect much more diversity among the *E. coli* O157:H7 strain types during the 4 month sampling period.

Indistinguishable subtypes of *E. coli* O157:H7 have been demonstrated to be shared between cattle, the farm environment, and other non-bovine species (Rice et al., 1995; Faith et al., 1996; Hancock et al., 1998; Shere et al., 1998, Van Donkersgoed et al., 2001; Renter et al., 2003, 2004). Within cattle production environments, the sharing of indistinguishable subtypes has been shown to occur between cattle and water sources (troughs, ponds, and creeks) (Faith et al., 1996; Hancock et al., 1998; Shere et al., 1998, Van Donkersgoed et al., 2001; Renter et al., 2003). It has been suggested that water systems, in particular water troughs, can serve as a long-term reservoir and recurrent source of *E. coli* O157:H7 for cattle (Shere et al., 1998; LeJeune et al., 2001; Renter et al., 2004).

Indistinguishable *E. coli* O157:H7 subtypes have also been shown to be shared between cattle or cattle water sources and feed, pigeons, horses, dogs, wild birds, opposums, deer, rats, and flies (Shere et al., 1998; Hancock et al., 1998; Neilsen et al., 2004; Renter et al., 2003, 2004), indicating that *E. coli* O157:H7 strains are not specific to cattle and that the environment and non-bovine species could be involved in the maintenance or transmission of the organism within and among cattle farms.

The diversity, distribution and persistence of some *E. coli* O157:H7 subtypes in cattle operations indicate; that a large number of subtypes exist, that herds are repeatedly exposed to common subtypes, and that many different non-bovine sources that exist in the cattle production environment serve as reservoirs or vectors in the transmission of *E. coli* O157:H7 to cattle. Additional studies are needed to identify more of the factors, such as wildlife, or herd health management and biosecurity management practices that may influence transmission within and among cattle operations, in order to be able to control *E. coli* O157:H7 in cattle.

PRE-HARVEST CONTROL

The cattle industry and researchers have traditionally focused on improving the safety of meats at slaughter and post-slaughter. Hazard analysis and criticial control point (HACCP) policies implemented in slaughter plants, along with post slaughter sanitation methods have been shown to significantly reduce carcass contamination with *E. coli* O157:H7 (Elder et al., 2000).

Yet, in spite of the tremendous in-plant intervention strategies focused solely on reducing this organism and other foodborne pathogens in the finished food product, human illnesses caused by contaminated meat products still occur, and in-plant intervention strategiest do not address environmental contamination or direct and indirect animal exposure routes of *E. coli* O157:H7 to humans. Therefore, in order to reduce human *E. coli* O157:H7 illnesses by food and environmental exposure, greater emphasis has recently been placed on the development of intervention strategies that target the pathogen in live animals on the farm prior to slaughter, termed pre-harvest intervention/control.

Live animals are critical links in the production chain emphasized by the fact that fecal excretion of *E. coli* O157:H7 in cattle has been directly correlated with levels of carcass contamination (Elder er al., 2000), and the increase in human outbreaks associated with animal contact at fairs and petting zoos in the U.S. (Keen et al., 2003; LeJuene and Davis, 2004). Therefore implementing pre-harvest control strategies to reduce the number of *E. coli* O157:H7 infected cattle from entering the abattoir, agricultural fairs, and in cattle farm run-off, may significantly reduce the number of human exposures to this pathogen, thus reducing the number of related illnesses and deaths (Hynes and Wachsmuth 2000).

Unfortunately, several epidemiological features of *E. coli* O157:H7 represent constraints to the effectiveness of some strategies used for pre-harvest control of *E. coli* O157:H7. The widespread distribution of *E. coli* O157:H7, its persistence in environmental sources, the lack of resistance to re-infection in cattle, as well as its wide host range (including wildlife) make eradication of *E. coli* O157:H7 an unlikely task.

Most pre-harvest intervention strategies would seemingly be limited to reducing the magnitude of fecal excretion and the prevalence of *E. coli* O157:H7 in farm animals, rather than eradication. Pre-harvest testing and removal/segregation of *E. coli* O157:H7 infected cattle prior to slaughter until infections are cleared is problematic for several reasons: including the sporadic fecal excretion of *E. coli* O157:H7 such that an animal might test positive one day but not again for several days or weeks; hide contamination in the absence of fecal contamination (Hancock et al., 2001); and the expense and time put forth for testing large numbers of cattle, in which a percentage of those test-negative animals will become test-positive in the time between testing and sending to slaughter (Callaway et al., 2004; Besser et al., 2003). Therefore, pre-harvest intervention strategies must be economically viable and applicable to large groups of animals at different stages of on-farm production.

Pre-harvest intervention methods can be grouped into three approaches: (1) animal management strategies; (2) competitive enhancement strategies; and (3) direct anti-pathogen strategies. Some of these pre-harvest intervention strategies are available to be used by animal producers, some will be available in the near future, and other strategies still require more research into their effectiveness at controlling *E. coli* O157:H7 before they can be available to animal producers (Callaway et al., 2004).

Management Strategies. Cattle can be exposed to *E. coli* O157:H7 from consumption of contaminated feed, water sources, grooming activities of themselves and of other cattle, and from their environment (bedding, rails in the barn, or pasture land).

Considerable efforts have been made in identifying herd management practices and environmental factors that inhibit or contribute to infection of *E. coli* O157:H7 in cattle such as, diet effects, drinking water, animal factor (age, sex, herd size), and adequate bedding cleaning and type of bedding used.

Diet and feeding regime have been suggested to influence growth and survival of *E. coli* O157:H7 in the ruminant intestine of cattle by creating fermentation conditions that give rise to elevated volatile fatty acid concentrations, adverse pH conditions, and changes in the composition of the resident microflora (Duncan et al., 2000; Russel et al., 2000). Reports in the literature on the effect of diet and diet shifts on fecal excretion of *E. coli* O157:H7 in cattle have been conflicting. Grain feeding has been proposed as a risk factor for increased excretion of *E. coli* O157:H7 in cattle by several research groups (Diez-Gonzalez et al., 1998; Keen et al., 1999); however, Hancock et al. (1994) and Magnuson et al. (2000) found no difference in the amount or duration of *E. coli* O157:H7 excreted between cattle fed grain and forage diets. Experimentally inoculated calves with *E. coli* O157:H7, fed a grain-based diet have been shown to develop a lower rumen pH and excrete elevated numbers of acid-tolerant *E. coli* O157:H7 in their feces, than calves fed a high-roughage (hay-based) diet (Diez-Gonzalez et al., 1998; Tkalcic et al., 2000). These acid-tolerant *E. coli* O157:H7 strains may colonize other animals, and possibly humans, more efficiently since they can survive better in the acidic compartments of the intestine, facilitating the transmission of *E. coli* O157:H7 in the farm environment (Stevens et al., 2002).

Some researchers claim that briefly switching cattle from a grain-fed diet to hay prior to slaughter reduces the number of animals positive for *E. coli* O157:H7 entering the food chain (Diez-Gonzalez et al., 1998; Keen et al., 1999), whereas other studies contradict this finding (Hovde et al., 1999; Buchko et al., 2000). Based on the available literature, it appears that an abrupt change in diet from grain to hay in cattle can affect *E. coli* populations in cattle but the magnitude of this effect is not always consistent (Calloway et al., 2003); further studies are required to determine the influence of different diets on reducing the fecal excretion of *E. coli* O157:H7 in cattle.

Other feed components, including cottonseed, clover or Lucerne hay, and soymeal have been reported to reduce fecal excretion of *E. coli* O157:H7 in cattle (Dargatz et al., 1997; Garber et al., 1995), while corn silage, barley, and beetpulp appear to increase carriage rates of *E. coli* O157:H7 in cattle (Dargatz et al., 1997; Herriot et al., 1998; Schouten et al., 2001; Berg et al., 2004).

Interventions at the water and environmental hygiene levels are other suggested areas for pre-harvest control of *E. coli* O157:H7. Water sources for cattle are frequently contaminated with relatively high numbers of *E. coli,* and in some studies, researchers have demonstrated that cattle water troughs can be reservoirs for dissemination of *E. coli* O157:H7 (LeJeune et al., 2001). The significance of this route in horizontal transmission remains to be proven in cattle, but interventions such as, chlorination, ozonation, frequent cleaning, and screens that decrease organic solids in the troughs, offer great potential to decrease *E. coli* O157:H7 contamination and cross-contamination of animals (LeJeune et al., 2001).

One study even showed that the position of water troughs on the farm can affect prevalence of *E. coli* O157:H7 in cattle, with water troughs located close to feed bunks having a higher number of *E. coli* O157:H7 in them compared to those placed farther apart (LeJeune et al., 2001a). Housing of calves (more susceptible than adult cattle to *E. coli* O157:H7 infection) separately from the rest of the herd has been shown to have some effects in reducing the prevalence of *E. coli* O157:H7, and one study by LeJeune et al. (2005), showed that the type of free-stall bedding can affect the fecal prevalence of *E. coli* O157:H7 among dairy cattle, with a higher prevalence seen in herds using sawdust bedding material opposed to sand bedding material.

Competitive Enhancement Strategies. Competitive enhancement strategies involve the use of probiotics, prebiotics and competitive exclusion (CE), all with the goal of promoting the growth of groups of beneficial bacteria to fill all ecological niches in the gastrointestinal tract in order to prevent the establishment of, or cause the displacement of, pathogenic bacteria (Fuller 1989).

Probiotics are defined as 'a preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the micro-flora in a compartment of the host and that exert beneficial health effects in this host'(Schrezenmeir and De Vrese 2001). Probiotic preparations generally consist of individual bacterial species or mixtures of lactic acid bacteria or yeasts that do not have to be of animal origin (Weiman 2003). Various probiotics have been used in the cattle industry, but without much benefit in reducing pathogen levels (Tournut 1989; Dawson et al., 1999; Yoon and Stern 1996; Keen and Elder 2000), until the recent discovery of the use of *Lactobacillus acidophilus* as a probiotic.

Researchers have shown that addition of *Lactobacillus acidophilus* cultures to the diet of finishing cattle decreased *E. coli*O157:H7 excretion by more than 50% (Brashears and Galyean 2002; Brashears et al., 2003a,b). This product is currently available on the commercial market, and is being used by the cattle industry (Calloway et al., 2003).

Prebiotics are organic compounds (i.e fructo-oligosaccharides) that are unavailable to or indigestible by the host animal, but are digestable by a specific group of bacteria in the microbial population (Walker and Duffy, 1998; Schrezenmeir and DeVrese, 2001). The use of prebiotics have been used more in humans to promote intestinal health, and in pigs to improve nutrition, than in cattle (reviewed in Callaway et al., 2004). Therefore, not many studies have been reported on the use in cattle.

Competitive exclusion (CE) involves the addition of a nonpathogenic bacterial culture to the intestinal tract of farm animals in order to compete with pathogenic bacteria in the gastrointestinal tract. CE cultures may be composed of one or more strains or species of bacteria and should be derived from the animal of interest. Originally CE was not considered a good technique for reducing pathogens in cattle because of the vast microbial reservoir of the rumen. But recently researchers isolated a population of several *E. coli* strains from cattle capable of producing colicins (proteins that specifically target *E. coli*) that in vivo can displace establishment of *E. coli* O157:H7 populations in adult cattle (Zhao et al., 1998), and reduce fecal excretion of EHEC in neonatal calves (Zhao et al., 2003) and in weaned calves (Tkalcic et al., 2003). This CE culture specific to cattle was being developed in 2003, with field trials expected to be underway in 2004 (Calloway et al., 2003).

Direct Anti-pathogen Strategies. The goal of direct anti-pathogen strategies is to specifically target and kill pathogenic bacteria through the use of antibiotics, bacteriophage, specific physiology targeting compounds, or vaccination.

Antibiotic use as growth promotants has become highly controversial in the recent years because of its extensive use in both humans and agricultural animals contributing to the widespread dissemination of antibiotic resistance genes among bacterial species. Because of concern over the spread of antibiotic resistance, the prophylactic use of antibiotics as growth promotants in agricultural animals will more than likely become highly regulated or possibly prohibited in the U.S. in the future (Calloway et al., 2003). The ability of antibiotics to disrupt the gastrointestinal microbial ecosystem such that opportunistic pathogens can occupy niches from which they would ordinarily be excluded is another drawback to the use of antibiotics in agricultural animals (Phillips 1998; Witte 2000). However, in spite of these drawbacks, some antibiotics have been shown to decrease the intestinal populations of pathogenic bacteria. The antibiotic, neomycin sulfate, has been demonstrated to significantly decrease fecal populations and excretion of *E. coli* O157:H7 in cattle (Elder et al., 2002; Ransom et al., 2003). Neomycin is a good candidate for use in the cattle industry for decreasing *E. coli* O157:H7 populations in finishing cattle because of its 24 h withdrawal, and the fact that it's not used in human medicine. However, it is closely related to other antibiotics from the same family (e.g. streptomycin, kanamycin, and gentamycin) that are used to treat some human infections, and therefore its use in cattle is recommended only until less controversial intervention strategies become available (Calloway et al., 2003).

Bacteria are subject to lysis and killing by a large number of viruses called bacteriophages, commonly found in intestinal microbial flora of food animals (Klieve and Bauchop 1988; Klieve and Swain 1993). Some bacteriophages are highly specific to act against a single strain of bacteria (Barrow and Soothill 1997), and therefore have been suggested to be used in cattle and other food animals to eliminate specific pathogens from a mixed microbial population. The effectiveness of bacteriophage treatment to decrease *E. coli* O157:H7 in cattle has been variable and limited in the literature; therefore more research needs to be done before bacteriophages can be considered an effective method in controlling *E. coli* O157:H7 in cattle.

The inhibition of specific pathogens via metabolic pathways, using the compound sodium chlorate experimentally administered in feed and drinking water, has been shown to be successful in reducing *E. coli* O157:H7 populations in both the feces and intestinal content of cattle and sheep (Anderson et al., 2000; Edrington et al., 2003; Callaway et al., 2002). The beneficial effects of sodium chlorate are ascribed to the anaerobic reduction of this chemical by nitrate reductase to chlorite, a bacteriocidal metabolite. The use of sodium chlorate in cattle and other food animals has not yet been approved for use in the U.S. (Calloway et al., 2003).

The use of vaccination to prevent pathogen colonization and fecal excretion in agricultural animals is based on the priming of the animal's immune system against antigens expressed by *E. coli* O157:H7 to prevent the colonization of this organism in the gastrointestinal tract. Specific immunization against pathogenice bacteria has only recently been used to attempt to decrease pathogenice bacteria in cattle.

Because many foodborne pathogenic bacteria, including *E. coli* O157:H7 do not cause illness in cattle, development of vaccines against these pathogens has been a difficult process. Vaccines have been made against the proteins intimin (required for bacterial adherence to mammalian cells and the intestinal mucosa of calves, and piglets), Esps, and Tir (Type III secreted proteins), and the O157 lipopolysaccharide. Only recently, has vaccination of feedlot cattle, using a vaccine targeted against the Esp and Tir, been shown to reduce the prevalence of *E. coli* O157:H7 in a clinical trial. Preliminary results indicated that this vaccine reduced the duration, frequency, and quantity of *E. coli* O157:H7 excreted in cattle feces in feedlot cattle both experimentally challenged and naturally exposed to the organism (Potter et al., 2004). However, a more recent study by Donkersgoed et al. (2005) contradicts this finding. Donkersgoed et al. (2005) showed that this *E. coli* O157:H7 vaccine did not significantly reduce the proportion of feedlot cattle excreting *E. coli* O157:H7 in their feces during a trial of 218 pens of feedlot cattle in 9 feedlots in Canada. Although, differences seen between the two studies in the efficacy of the vaccine to reduce fecal excretion of *E. coli* O157:H7 in cattle, could have been attributed to different vaccination strategies used (interval between vaccinations and the number of doses), and different methods for producing the vaccination. In the feedlot trial by Potter et al. (2004), the size of pens of feedlot cattle were smaller (8 animals) than those used in Donkersgoed et al. feedlot trial (250 animals per pen). Three doses of the vaccine were admistered at 3-week intervals in the first study, whereas the second study only used 2 doses of the vaccine administered at arrival and 73 to 103 d postarrival.

Eventhough, the study by Potter et al. (2004) showed that the vaccination of cattle using his strategy can decrease the level of *E. coli* O157:H7 fecal excretion for the purpose of reducing the risk of human disease, vaccination of cattle 3 times within a 3-week interval post-arrival, does not fit many feedlot management protocols. Therefore, currently the use of vaccines to decrease *E. coli* O157:H7 in cattle in field studies is contradicted, but further research could hold promises for the use of vaccines in the near future.

Conclusion: Currently, only one pre-harvest control for *E. coli* O157:H7 in cattle has been proven to be effective for use at reducing the prevalence of *E. coli* O157:H7 in cattle (the probiotic *Lactobacillus acidophilus*). Progress is being made in the direction of pre-harvest control strategies in cattle. More research into the effectiveness of parallel and simultaneous application of one or more pre-harvest control strategies, as well as the identification of new pre-harvest control techniques, may provide practical means to substantially reduce the incidence of human *E. coli* O157-related illness by intervening at the farm level.

CONCLUSION

Escherichia coli O157:H7 is an increasingly recognized cause of diarrhea in humans and the most common bacterial pathogen isolated from visibly bloody stool specimens in many countries including Europe, the United States, and Canada. *Esherichia coli* O157:H7 causes a distinct syndrome of diarrheal disease, known as hemorrhagic colitis and the life-threatening post-diarrheal sequelae, HUS.

There is no current treatment of the disease in humans, making many cases of *E. coli* O157:H7-related disease a debilitating one in young children and the elderly. Transmission can occur from animal-to-person, person-to-person, water-borne and environmental-borne by direct contact with contaminated animal or human feces, indirectly after the consumption of contaminated food and water sources, or contact with environments which have been contaminated with animal feces. Cattle feces are recognized as a primary source from which the food supply and environment become contaminated with *E. coli* O157:H7 by direct culture of the organism from foods of bovine origin, prevalence studies in cattle, and by epidemiologic associations with outbreaks of *E. coli* O157:H7 associated disease in humans. Therefore, tremendous research efforts have been devoted to studying the ecology and epidemiology of *E. coli* O157:H7 in cattle production environments, in an effort to enhance food/environmental safety.

The following key features about the ecology and epidemiology of *E. coli* O157:H7 in cattle have shown the complexity and dynamics of this organism in cattle operations:

- Lack of host specificity with the organism sporadically isolated from a variety of species and the environment.
- The organism can be found on most cattle farms, if sampled long enough; asymptomatic carriage and transient residence in the gastrointestinal tract of cattle and other animals.
- Temporal clustering at the population level with most fecal excretion confined to sharp peaks in a high percentage of animals, separated by longer periods of low prevalence.

- Higher prevalence is seen in younger animals compared to adult cattle, and a higher prevalence of *E. coli* O157:H7 detected in cattle during the warmer months.
- Prolonged survival and replication of *E. coli* O157:H7 in water trough sediments, feed, soil, and manure.
- *E. coli* O157:H7 subtypes can persist on cattle farms for years.
- Indistinguishable subtypes have been found to be shared between cattle and the environment and cattle and other species of animals.
- Several *E. coli* O157:H7 subtypes often exist on a farm simultaneously with periodic additions and turnovers, even on farms which do not bring cattle into their herd from outside sources.
- Long distance transmission (200 Km) of *E. coli* O157:H7, not associated with animal movements, between farms has been show to occur.

Many research efforts have been targeted at trying to control *E. coli* O157:H7 at the pre-harvest stage, on the farm prior to slaughter. Unfortunately, currently only one effective pre-harvest control strategy for *E. coli* O157:H7 in cattle has been proven to be used in the cattle industry. Many of the known epidemiologic and ecologic characteristics of *E. coli* O157:H7 in cattle can place restraints on pre-harvest strategies, such that eradication of the organism is highly unlikely, and pre-harvest strategies would be limited to reducing prevalence of *E. coli* O157:H7 in cattle instead.

Although *E. coli* O157:H7 has been isolated from many non-bovine species and environmental sources, their roles and the role of yet unidentified sources in the transmission of this organism have not been elucidated.

The multiplicity of sources of *E. coli* O157:H7 and the detection of genetically similar strains of *E. coli* O157:H7 in the feces of cattle and other species and environmental sources on cattle operations, suggest that instead of targeting specific cattle operations, an ecological approach may be necessary for control of this pathogen in the cattle industry. However, until such a time that more data is available about the factors that influence transmission and maintenance within and among cattle operations, it will not be possible to design more effective control measures for *E. coli* O157:H7 in cattle at the pre-harvest stage.

The work presented in the following chapters provides additional information as to the possible mechanisms of emergence and dissemination of this important threat to public health.

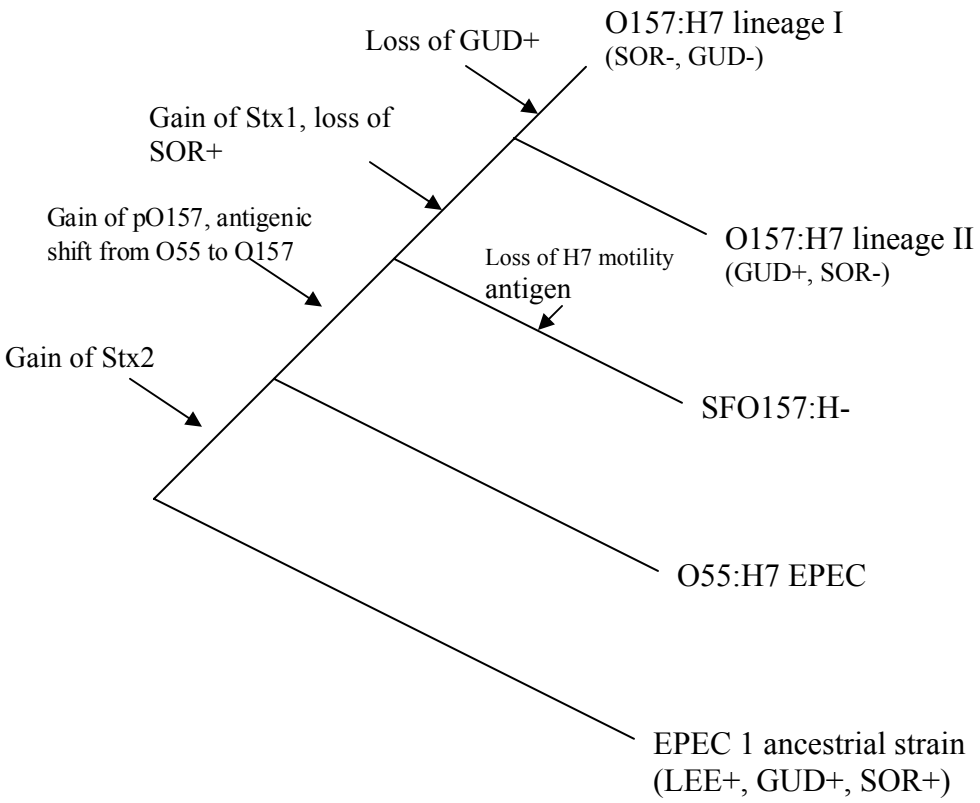

Figure 1.1. The following proposed steps for the evolution of the contemporary sorbitol nonfermenting, β-glucuronidase-negative, O157:H7 populations (EHEC lineage I) are based on models proposed by Feng et al. (1998) and Kim et al. (2001). In this model, the pathway proceeds from ancestral (left) to contemporary (right), with major steps including, the acquisition of the Stx2-converting prophage in a variant O55:H7 EPEC population; acquisition of the O157 *rfb* region and plasmid pO157, prior to the divergence of the sorbitol-fermenting (SF) O157:H- population; acquisition of the Stx1-converting phage and the TAI island (tellurite resistance acquisition island), and loss of the β-glucuronidase production (Gud-) and sorbitol fermentation (Sor-) characteristics.

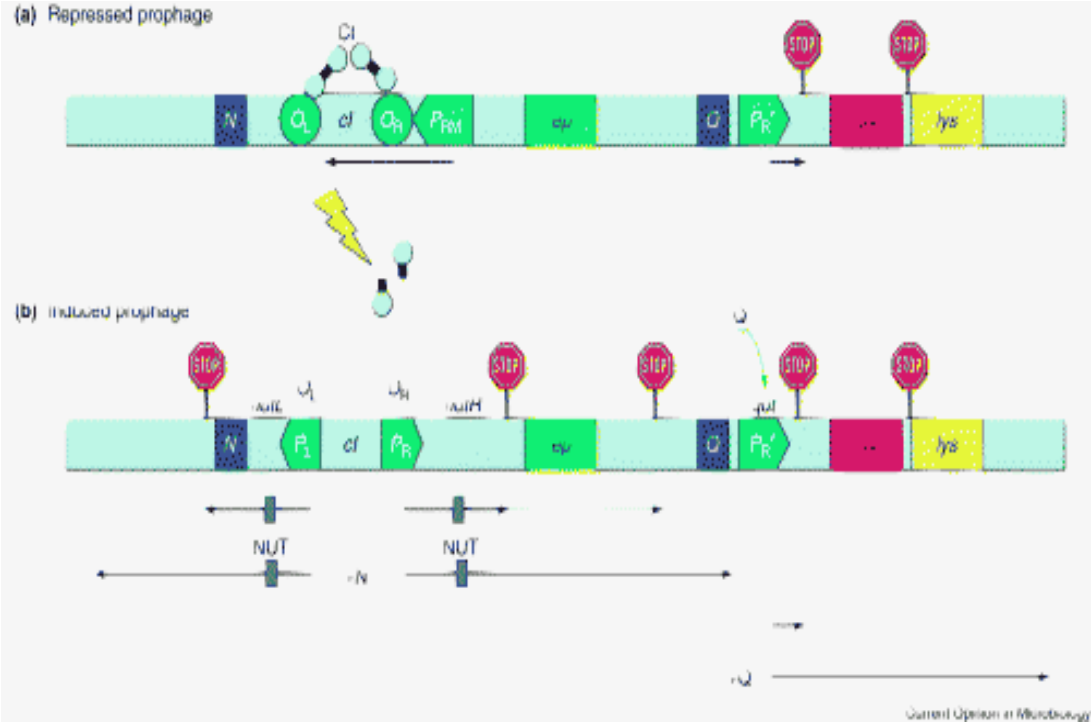

Figure 1.2: Early regulatory region of lambdoid phages showing location of relevant sites, genes, interactions, and patterns of transcription (not drawn to scale). **(a) Repressed prophage**: transcription that initiates the regulatory cascade (shown in [b]) is blocked by CI repressor binding at the operators O_L and O_R. The lightening strike indicates action of an agent that provokes the SOS response leading to increased production and activation of RecA. Activated RecA facilitates autocleavage of repressor. The single repressor molecules shown binding to operators is a simplification of the actual process. Transcription from P_{RM} directs synthesis of repressor in the lysogen. In the absence of *Q*, transcription initiating at $P_R{}'$ terminates at the immediate downstream terminator. Cleavage of repressor results in release of repression. **(b) Induced prophage**: the regulatory cascade begins with transcription initiating at P_L and P_R that terminates after synthesis of a short message. This early transcription allows expression of N, which acts at the NUT sites in the RNA to modify RNA Pol to a form that transcends terminators. Q, which is then expressed, acting at the *qut* site in the DNA, modifies transcription initiating at $P_R{}'$ to a termination resistant form that can transcribe downstream genes that include *stx* and *lys* as well as most of the genes required for production of viable phage. Shown below are transcription patterns in the absence and presence of N and Q. Genes and function of their products are as follows: *cI*, repressor; *lys*, lysis; *N* and *Q*, transcription anti-termination; *rep*, replication; *stx*, Shiga toxin. Regulatory signals are as follows: *nut* (DNA) and NUT (RNA), site for *N* modification of RNA Pol (works with several host factors at RNA); *O*, operators; *P*, promoters; *qut*, site for *Q* modification of RNA Pol; stop signs denotes sites or regions that contain transcription terminators (Waldor and Friedman, *Curr. Opin. Microbiol* 2003;8:459-465).

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CHAPTER 2

FOODBORNE PATHOGENS IN EUROPEAN STARLINGS ON OHIO DAIRY FARMS

INTRODUCTION

Human foodborne illnesses associated with animal originating products are largely caused by enteric bacteria such as *Campylobacter jejuni, Salmonella,* and *Escherichia coli* O157:H7 (*E. coli* O157:H7). Livestock, notably cattle, swine, and poultry play a central role in the epidemiology of these bacterial infections in humans. In the United States alone an estimated 76 million cases of human diseases occur each year due to consumption of contaminated food.

Prevalence of *Campylobacter jejuni, Salmonella* serotypes, and *E. coli* O157:H7 in diary cattle is respectively, 37.7% (Wesley et al., 2000), 5-10% (Wells et al., 2001), and 2-8% (Hussein and Sakuma, 2005). These bacteria are of a conern to public health because they can be transmitted to humans through bovine products and cause foodborne illnesses.

Therefore the reduction in the colonization and dissemination of *E. coli* O157:H7, *Campylobacter jejuni,* and *Salmonella* spp. via cattle at the production stage or at the farm level would potentially decrease theincidence of *E. coli* O157:H7, *Campylobacter jejuni,* and *Salmonella* spp.–associated disease in people.

However, it is unclear as to how these foodborne pathogens are disseminated to cattle on farms. European starlings (*Sturnus vulgaris)*, have an estimated population of 140-200 million birds (Johnson and Glahn, 1992), and in Ohio during the winter it is common to observe flocks of 500 to over 2,000 birds (Peterjohn, 2001) and large winter roosts containing 400,000 to 600,000 starlings (Sauer et al., 2004). Starlings congregate by the thousands to feed at cattle operations, and contaminate the farm environment with excrement that they produce. Although, *E. coli* O157:H7 and *Salmonella* spp. have been isolated from starlings as well as other species of wild birds, the extent to which starlings inhabiting Ohio dairy farms carry and disseminate these foodborne pathogens is unknown. Therefore, the purpose of this study was to determine the rate of carriage of *Camplyobacter jejuni, Salmonella* spp. and *E. coli* O157:H7 in starlings collected from Ohio dairy farms.

MATERIALS AND METHODS

Sample Collection. Thirty starlings per farm (n = 12) were either euthanized by the USDA Wildlife Services via poison during on-farm control programs or captured by mist nets on farms (n = 3) in NE Ohio during the winter of 2003 and 2004, and the months of August and September 2004.

All other bird species captured in the mist nets were immediately released. The protocol for the study was approved by the Ohio State University Animal Use and Care Committee (OSU ILACUC #2003A008), and the starlings were captured under an Ohio Department of Natural Resources, Division of Wildlife, Scientific Collection Permit (#383). The sample number for starlings caught via a mist net varied from 3 to 9 samples per farm. Starlings were euthanized by cervical dislocation and transported back to the lab in a chilled cooler within two days if collected by the USDA Wildlife Services, or within 1-2 h if caught via a mist net. Whole intestinal contents were surgically collected from each starling and placed in a sterile bag. Buffered Peptone Water (BPW) at the ratio of 1:10 was added to whole intestinal contents and homogenized in a Stomacher for 30 s at 5 strokes/min, and incubated at 37ºC for 24 h for enrichment of *Salmonella* and *E. coli* O157:H7, along with positive controls for *Salmonella* and *E. coli* O157:H7 (EDL933).

Isolation of *Campylobacter jejuni.* The stomached whole bird intestinal contents in BPW were streaked for isolation on Columbia blood agar containing vancomycin and amphotericin B (Remel) and incubated under microaerophilic conditions for 48 h at 42ºC. Suspect *Campylobacter* colonies (small, greyish colonies), were confirmed as *C. jejuni* by PCR (Stucki et al., 1995).

Isolation of *Salmonella* spp. Following pre-enrichment in BPW, 100 µl of 24 h culture was inoculated into 10 mls of Rappaport-Vassiliadis broth (RV) and incubated for 24 h at 37ºC. A loopful of selective enrichment broth was plated onto XLT-4 agar (xylose, lysine, lactose, and tergitol-4 containing media) and incubated for 24-48 h at 37ºC.

Up to 5 black suspect colonies (production of hydrogen sulfide) were picked from each plate and further biochemically characterizated by triple sugar iron agar (TSI) slants to look for the presence of glucose fermentation (yellow color) and production of the enzyme desulfhydrase (produces black precipitate), and urea agar to look for the inability to hydrolyze urea.

Isolation of *Escherichia coli* O157:H7. *Escherichia coli* O157:H7 present in any 1 ml aliquot of the overnight culture, along with a positive control was concentrated with anti-O157 immunomagnetic beads (Dynal, Oslo, Norway), and 75 µl of bead mixture was plated on sorbitol-MacConkey agar plates containing cefixime (50 ng/ml) and potassium tellurite (2.5 mg/ml) (Sigma Chemical Co., St. Louis, MO) and incubated at 37ºC for 24 hours. Up to five sorbitol-negative (clear/whitish) colonies were picked from each plate and further identified as *E. coli* O157:H7 based on lactose fermentation and the inability to cleave 4-methylumbelliferyl-β-glucuronide to a fluorescent product. Confirmation of *E. coli* O157:H7 was determined by a latex agglutination test for the presence of the O157 antigen (Oxoid, Basingstoke, Hampshire, United Kingdom), and multiplex PCR assays for the detection of *hylA, eaeA, rfbE, fliC,* stx_1, stx_2, and stx_2 variants (2, 2c, 2d, 2e, 2f) (Wang et al., 2002). Shiga toxin genes were detected in enrichments from starlings collected on farms 13, 14 and 15 by PCR (Karch and Meyer, 1998).

RESULTS

Of the European starlings tested for carriage of *Campylobacter jejuni* in their gastrointestinal tract, 38 of 150 (25.3%) tested positive, *Salmonella* was detected in 10 of 360 (2.8%), and 4 of 380 (1.1%) starlings tested positive for *Escherichia coli* O157:H7. No *E. coli* O157:H7 was found in starlings collected from the winter, and 20% (4 of 20) was found in starlings collected from the fall (Table 2.1). Two of the *E. coli* O157:H7 isolates, collected from Farm 13, were PCR positive for the stx_{2c} gene. PCR analysis of a subset of culture-negative *E. coli* O157:H7 fecal enrichments from starlings collected on farms 13, 14, and 15 using a set of primers to detect for the presence of *stx* showed that: 4 of the 6 enrichments from Farm 13 were positive for the presence of *stx*; 2 of 3 culture-negative *E. coli* O157:H7 samples collected from Farm 14 were positive for the presence of *stx*; and 4 of 7 fecal enrichments collected from Farm 15 were positive for the presence of *stx* (Figure 2.1).

DISCUSSION

From our findings, the overall prevalence of starlings inhabiting NE Ohio dairy farms harboring the foodborne pathogens *E. coli* O157:H7 (1.1%), *Salmonella* spp. (2.8%) and *Campylobacter jejuni* (25.3%) was comparable to other studies. However, the percentage of starlings harboring *E. coli* O157:H7 showed a seasonal effect, with a much higher carriage rate during the late summer months (20%) compared to winter (0%). This coincides with the peak prevalence period seen in cattle (Chapman et al., 1997; VanDonkersgoed et al., 1999; Wallace et al., 2000).

We were not able to determine whether a seasonal effect occurred in starlings for carriage of *Salmonella* spp. or *Campylobacter jejuni.* However, one study by Cravens et al., looking at the incidence of *Salmonella* spp. and *Campylobacter jejuni* in starlings and house sparrows inhabiting Broiler chicken houses in Georgia, USA, showed that on two different farms the percentage of positive birds for *Salmonella* spp. was the highest (14% of 24 samples) during the months of August through October compared to spring, summer, and winter months; whereas the percentage of positive birds for *Campylobacter jejuni* was the highest (50% of 14 samples) during the months of December through January on one farm compared to the other seasonal time periods (Cravens et al., 1999). Other studies have reported higher incidences of *Salmonella* spp. in starlings 1.3% (1 of 80 birds sampled) captured on dairy farms in Kings and Tulare counties between March 1998 and May 2000 (Kirk et al., 2002). The prevalence of *Campylobacter jejuni* in starlings has not been reported in the literature, but the isolation of this bacterium from starlings and other bird species including pigeons, blackbirds, sparrows, seagulls, ural owl, and some species of migratory waterfowl has been demonstrated (Kapperud and Rosef, 1983; Luechtefeld et al., 1980; Skirrow and Benjamin, 1980). One study showed that *Campylobacter jejuni* strains isolated from starlings were indistinguishable from isolates obtained from humans (Broman et al., 2004).

Salmonella spp. have also been detected in a wide variety of other bird species, including; house sparrows, cowbirds, house finches, red-winged black bird, pigeon, goldfinches, gulls, ducks, peregrine falcons, little owl, barn owl, buzzard, and etc. with an overall prevalence of ranging from 2.5%-4.2% (Kirk et al., 2002; Cizek et al., 1994; Palmgren et al., 1997; Reche et al., 2003).

Wild birds contributing to the epidemiology of foodborne disease, has been shown in a recent multi-state tomato-borne outbreak of *Salmonella* Newport infections in which the tomato processing plant was contaminated with bird droppings (Kretsinger et al., 2003). In yet another study, molecular comparisons of *Salmonella* strains isolated from wild birds on commercial poultry operations, showed that common strain types were shared between the wild birds and the laying hens (Liebana et al., 2003).

The prevalence of *E. coli* O157:H7 in wild birds is infrequently reported in the literature, and even less so for starlings. Based on low prevalence values observed in two previous studies, researchers have dismissed wild birds as a significant vector or source for *E. coli* O157:H7; 0.5% was found in wild birds inhabiting (1 of 200 pooled bird fecal samples) a feedlot setting (Hancock et al., 1998); undetectable levels were found in wild birds migrating into Sweden (Palmgren et al., 1997), and 0.02% was confirmed as *E. coli* O157 positive in gulls located at an urban landfill site (three positive gulls) and inter-tidal sediments in Morecambe Bay (10 positive gulls) (Wallace et al., 1997), and zero prevalence was found in 124 starlings sampled over a seven year period (Rice et al., 2003). However, a low prevalence in wild birds as indicated by these studies, can not yet rule out starlings as a prominent source for dissemination of *E. coli* O157:H7 to cattle because the intensity of sampling was low in the studies.

Starlings are very versatile and adaptive to multiple habitats. They may fly 24-48 km to feed daily, and will fly further distances (> 60 km) from roosting sites to feeding areas if a desirable source of food is plentiful in a distant location (Johnson and Glahn, 1992).

In fact, one study showed that indistinguishable *E. coli* O157 subytpes were isolated from two feedlots located approximately 100 km apart, and wild birds were determined to be the only potential common source shared between the two feedlots (VanDonkersgoed et al., 2001). Although, the serotype was not O157, another study showed that STEC isolates from a starling were idenitical to cattle and rat isolates, obtained from farms in close proximity, with respect to serotype (O26:H29), virulence profile, and pulsed-field gel electrophoresis type; indicating that wild birds may become infected from farm animals or vise versa and may play a role in STEC transmission (Nielsen et al., 2004).

On farms frequented by wild birds, feed contamination with bird excrement is likely to occur. Daniels et al. (2003) developed a method to estimate the frequency of livestock feed contamination with wildlife excreta and determined that on average cattle consume in their feed the droppings from 1,626 birds per year. Even if the prevalence of *E. coli* O157:H7 in starlings is 0.5%, each cow is estimated to consume at least eight *E. coli* O157:H7 contaminated bird droppings each year, and cattle can be infected with doses of *E. coli* O157:H7 as low as, 200 CFU (Besser et al., 2001). Experimentally, *E. coli* O157:H7 has been reported to replicate and persist in the gastrointestinal tract of laboratory pigeons for approximately 20 days when infected with 10^9 CFU, and 15 days when infected with 10^5 CFU (Cizek et al., 2000).

An interesting finding in this study was the high number of *E. coli* O157:H7 culture-negative starling fecal enrichments that tested positive for the presence of *stx* genes, indicating that these birds may be carrying other Shiga toxin-producing *E. coli* (STEC) serotypes.

A study by Morabito et al. (2001) showed that Shiga toxin (Stx) was detected in 10.8% of feral pigeons inhabiting three different squares of Rome (649 pigeons), with most of the isolates belonging to the serogroups O45, O18b, and O75. Although, further work is required to determine whether these STEC cause a potential health hazard for humans. The possibility exists for Stx toxin gene transfer to toxin-negative enteric bacteria in the bird's gastrointestinal tract and in the environment by phage transfection, allowing for non-pathogenic *E. coli* to become pathogenic and a health hazard to humans.

The low incidence of *E. coli* O157:H7 and *Salmonella* spp. could be attributed to the low sample number, and more starlings will need to be tested to determine the actual prevalence of these foodborne pathogens. However, this study demonstrates the presence of these foodborne pathogens in European starlings on dairy farms in NE Ohio, and may provide knowledge to: determine appropriate sample sizes for conducting a more in-depth prevalence study of *E. coli* O157:H7, *Salmonella* spp., and *Campylobacter jejuni*; test for seasonal effects on the prevalence of these organism in starlings; and look for other STEC serogroups harbored by starlings that may be a potential health hazard to humans.

Date	Farm	N [a]	Number Culture-Positive *E. coli* O157:H7	*Salmonella* sp.	*Campylobacter jejuni*
2/2003	1	30	0	2	NT [b]
	2	30	0	0	NT
	3	30	0	0	NT
	4	30	0	0	NT
	5	30	0	0	NT
	6	30	0	3	NT
	7	30	0	0	NT
2/2004	8	30	0	0	22
	9	30	0	0	12
	10	30	0	0	3
	11	30	0	1	0
	12	30	0	4	1
8/2004	13	8	2	NT [b]	NT
	14	3	0	NT	NT
9/2004	15	9	2	NT	NT

Table 2.1
Foodborne Pathogens in European Starlings on Ohio DairyFarms
[a]European starlings tested in the winter were baited with poison and killed by the USDA Wildlife Services, others were trapped live via a mist net.
[b] Not tested.

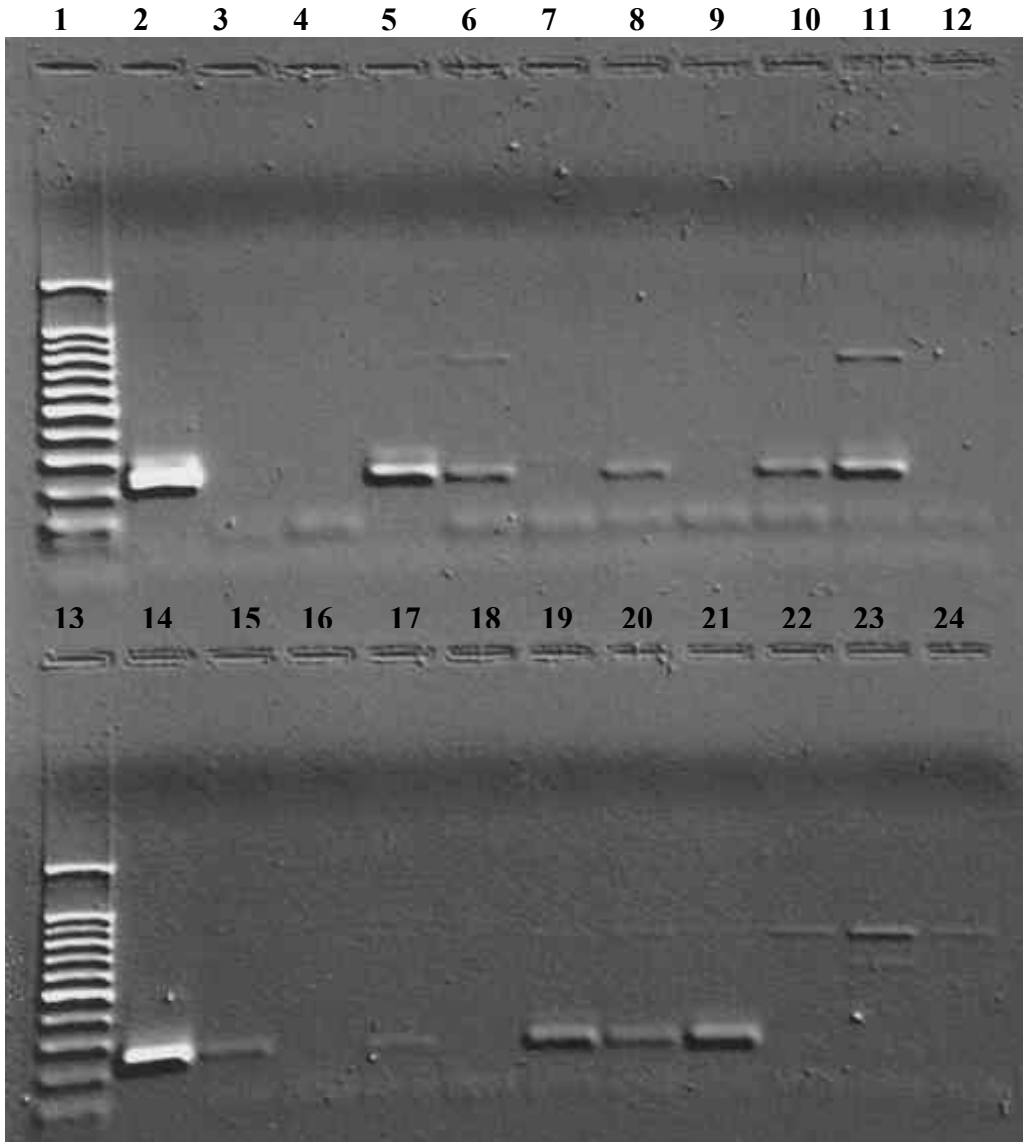

Figure 2.1 Agarose gel of PCR for amplification of Shiga toxin genes using MK1 and MK2 primers (Karch and Meyer, 1998) for the 19 starling *E. coli* O157:H7 in fecal culture enrichments from farms 13, 14, and 15. Lane 1: 100 bp ladder, lane 2: EDL933 positive control, lane 3: Negative control (water), lane 4: 13-1 sample, lane 5: 13-2 sample, lane 6: 13-3 sample, lane 7: 13-5 sample, lane 8: 13-6 sample, lane 9: 13-7 sample, lane10: 13-8 sample, lane 11: 14-1 sample, lane 12: 14-2 sample, lane 13: 100 bp ladder, lane 14: EDL933 positive control, lane 15: 14-3 sample, lane 16: 15-1 sample, lane 17: 15-2 sample, lane 18: 15-3 sample, lane 19: 15-8 sample, lane 20: 15-9 sample, lane 21: 15-10 sample, lane 22: 15-11 sample, lane 23: 15-12 sample, and lane 24: 15-13 sample.

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CHAPTER 3

CLONAL DISSEMINATION OF *ESCHERICHIA COLI* O157:H7 SUBTYPES AMONG NEIGHBORING DAIRY FARMS IN NE OHIO

INTRODUCTION

Shiga toxin-producing *Escherichia coli* O157:H7 (STEC), since its first recognition as a human pathogen in 1982, has remained a continuing source of human illness worldwide (1). The majority of human STEC infections have been traced to consumption of contaminated foods in the US (16), although other transmission routes to humans can occur via animal-to-person, person-to-person, water, and the environment (1). Cattle feces are considered the primary source from which both the food supply and the environment become contaminated with this pathogen (18, 27). Therefore, in an effort to enhance food/environmental safety, research efforts have been devoted to studying the ecology and epidemiology of *E. coli* O157:H7 in cattle production environments. If sampled repeatedly, *E. coli* O157:H7 can be isolated, at least intermittently, from most cattle farms (11). On-farm management practices, that include feed composition, probiotics, and environmental hygiene, can help to decrease the within farm prevalence of *E. coli* O157:H7 and thus decrease the number of infected cattle entering the food supply (2, 3, 13, 26).

However, the primary routes of transmission of *E. coli* O157:H7 between farms remains undetermined.

The results of genetic subtyping studies of *E. coli* O157:H7 have shown a complex and dynamic molecular epidemiology of this organism in cattle operations (8, 10, 11, 22). Indistinguishable subtypes of *E. coli* O157:H7 have been detected on multiple farms separated by hundreds of kilometers and lacking any identifiable common sources (8, 9, 20, 22). Research by Davis *et al.,* showed that *E. coli* O157:H7 isolates can also be transferred with considerable frequency over long global distances (7). How this process occurs is unknown and whether it occurs more often over shorter distances has not been explored. Therefore this study was conducted to determine the population genetic structure of *E. coli* O157:H7 isolated from distinct cattle farms within close geographic proximity in order to determine the extent, if any, that indistinguishable subtypes of *E. coli* O157:H7 are regionally distributed and shared among neighboring dairy cattle farms. Identification of genetically identical isolates from various sources on different farms would provide insight into the factors contributing to the dissemination of *E. coli* O157:H7 between farms, so we used pulsed-field gel electrophoresis (PFGE) to compare isolates of *E. coli* O157:H7 from various animal and environmental sources on different geographically close and distant farms. Understanding the routes of dissemination of foodborne pathogens between farms is a necessary step in being able to control pathogens at the pre-harvest stage.

MATERIALS AND METHODS

Sample Collection and Microbiological Analysis. *Escherichia coli* O157:H7 isolates (n=92) from dairy cattle, wild bird droppings, water trough sediment, and free-stall bedding material were obtained from a longitudinal study during the months of June through September 2003 from twenty dairy farms in NE Ohio. Sixteen of those farms sampled were located in a large primary geographic cluster (Wayne, Ashland, and Holmes counties) and four were located in two other geographically distant clusters (Ashtabula and Columbiana counties) (Fig 3.1). Of the 92 *E. coli* O157:H7 isolates obtained from the different dairy farms, 81 of those were isolated from cattle feces, five from free-stall bedding samples, three from water trough sediment, and three from wild bird excrement. Farms were visited at 2-week intervals for six visits for sample collection. Biosecurity measures, that included changing of coveralls and disinfection of boots with an iodine solution prior to sampling a farm, were performed to avoid transmission of *E. coli* O157:H7 subtypes between farms. Sample collection and microbiological analyses of the samples were performed as previously described for the bovine feces, water trough drinking water, water trough sediment, and free-stall bedding material (13). Wild bird excrement samples were collected from the farm environment using a sterile swab and pooled into sterile plastic bags. Within 6 h of collection, pooled wild bird excrement samples were diluted 1:20 in buffered peptone water (BPW), and incubated overnight at 42ºC. Detection of *E. coli* O157:H7 was performed using immunomagnetic separation methods, as previously described for the other collected samples.

Confirmation of *E. coli* O157:H7 was determined by a latex agglutination test for the presence of the O157 antigen (Oxoid, Basingstoke, Hampshire, United Kingdom), and multiplex PCR assays for the detection of *hylA*, *eaeA*, *rfbE, fliC, stx$_1$, stx$_2$,* and *stx$_2$* variants (2, 2c, 2d, 2e, 2f) (29).

A questionnaire regarding farm management practices was completed at the time of the first farm visit. Topics covered included feed, water, and waste management practices, employee and public biosecurity, animal health, classification of farm as open or closed herds, and on-farm inhabitation of wild animals (wild birds, rats, and raccoons). Management factors were screened for homogeneity between farms sharing and not sharing indistinguishable restriction endonuclease digestion profiles (REDPs) using chi-square tests with a Yates correction for continuity (25).

PFGE Typing. *Escherichia coli* O157:H7 isolates (n=92) were subtyped by PFGE of *Xba*I digested chromosomal DNA using standardized methods of the PulseNet National Molecular Subtyping Network for subtyping food-borne bacterial pathogens with minor changes (4). Briefly, isolates were grown overnight on Luria-Bertani (LB) agar, suspended in 5 ml cell suspension buffer (100 mM tris and 100 mM EDTA [pH 8.0]) and adjusted to an optical density of 1.3-1.4 at 610nm. The cell suspension (200 µl) was mixed with 10 µl Proteinase K (20 mg/ml) and an equal volume of 1% Seakem Gold agarose (Cambrex Bio Science Rockland, Inc). The mixture was dispensed into disposable agarose plug molds (Bio-Rad laboratories).

After solidification, the plugs were transferred to 2 ml round bottom tubes containing 3 ml cell lysis buffer (50 mM tris, 50 mM EDTA [pH 8.0], 1% sarcosine, 0.5 mg/ml proteinase K) and incubated for 2 h at 54°C. After lysis, the plugs were washed three times for 1 hour in TE buffer (10mM tris and 1mM EDTA [pH 8.0]) at 50°C with vigorous shaking, and stored in TE Buffer at 4°C. The agarose-embedded chromosomal DNA (~2mm plug slices) was digested with 30 U of *Xba*I for 4 hours at 37°C. The resulting fragments were resolved by CHEF-PFGE using a Chef Mapper system (BioRad) in 1% Seakem Gold agarose in 0.5x TBE (50mM tris base, 50 mM boric acid, 1mM EDTA [pH 8.0]) at 200V for 19 h with an initial switch time of 2.2 (s) and final switch time of 54.2 (s). When indistinguishable *Xba*I PFGE patterns were observed for isolates originating from different farms, isolate subtyping was repeated with *Bln*I (10 U for 16 h @ 37°C) and resolved by CHEF-PFGE for 21 h using same conditions as for *Xba*I (7). All gels were run with the Centers for Disease Control and Prevention reference strain *Salmonella* ser. Braenderup H9812. Gels were stained with ethidium bromide (10 mg/ml) for 30 min, de-stained in distilled water five times 20 min each, and photographed under UV transillumination using a digital gel documentation system (Chemilmager ^{TM} Alpha Innotech Corp, San Leondro, CA). REDPs were compared visually and aided with Bionumerics Fingerprint Cluster Analysis Software (Applied Maths, Belgium). Cluster analysis was performed using the unweighted pair group method with arithmetic means (UPGMA, position tolerance of 1.0% and optimization of 0.75%). Isolates generating distinguishable REDPs were assigned to a unique REDP cluster.

Distance between Farms. Latitude and longitude coordinates for farm locations in NE Ohio were obtained from the U.S. Census Bureau Mapping engine using 1998 Tiger/Line® data and 1990 Decennial Census data (http://tiger.census.gov). Latitude and longitude decimal degrees were used to calculate distance (km) between farms using a web-based latitude and longitude distance calculator powered by Pearl© 1997 (http://jan.ucc.nau.edu/`cvm/latlongdist.html).

RESULTS

Of the ninety-two *E. coli* O157:H7 isolates, 89 were subtypable by PFGE. Fifty distinguishable subtypes of *E. coli* O157:H7 were identified using PFGE of *Xba*I cleaved chromosomal DNA. Forty-six of the REDPs could only be isolated from an individual farm for at most on two consecutive 2-week intervals. However, four restriction REDPs (by PFGE of both *Xba*I and *Bln*I cleaved chromosomal DNA) were found on more than one farm (Table 3.1 and Figure 3.2). The geographical distance between farms which shared an indistinguishable *E. coli* O157:H7 REDP ranged from 8.7 km to 49.9 km with a moderate (7/16 farms) amount of isolate sharing within the farms located in the primary cluster but no sharing between the farms located in the two distant geographical clusters, as well as no sharing within those distally located clusters of farms (Figure 3.1). The sharing of indistinguishable subtypes between seven farms occurred in a temporal cluster during the months of August and September, and only a single farm (G) shared multiple subtypes with two different farms (Table 3.1).

REDP 2, 3 and 4 were found only in bovine or wild birds respectively, whereas the other REDP (1) was found in multiple samples of free-stall bedding (FAHRP374-1) and a cow (FAHRP 385-1) located on different farms. Three of the indistinguishable REDPs (REDP 2, 3, and 4) of *E. coli* O157:H7 isolates were detected on the same sampling date. One farm (G) shared indistinguishable REDPs (1 and 2) of *E. coli* O157:H7 isolates with two different farms (I and S), located 32.5 km and 8.7 km respectively from farm G. REDP 2 was shared between wild birds isolated from farms G and I on 9/8/2003. REDP 1 was detected in free-stall bedding on farm G on 9/8/2003 and in a cow on farm S on 9/15/2003. Indistinguishable subtypes of *E. coli* O157:H7 originating only from bovine sources occurred on four farms (K, L, B and T) which routinely bring live cattle onto their farms (open herds), whereas indistinguishable *E. coli* O157:H7 subtypes respectively originating from bovine, free-stall bedding, and wild birds were shared between farms (G, S and I) in which all replacement cattle were brought on the premises from outside sources (closed herds) (data not shown).

Sharing of indistinguishable REDPs was not associated with farm management practices addressed in this study ($P > 0.1$).

DISCUSSION

Within the study population indistinguishable subtypes of *E. coli* O157:H7 were disseminated among four groups of dairy farms located in close geographic proximity. Thirty-five percent (7/20) of the sampled dairy farms shared at least a single genetic PFGE fingerprint subtype with a geographically close dairy farm.

Within the 20 farms sampled in our study, the range of distance between farms was approximately 3 km to 174 km, however clonal dissemination of *E. coli* O157:H7 subtypes was shown to occur only between those farms located approximately 9 km and 50 km apart. Differences in farm prevalence levels of *E. coli* O157:H7 and farm management practices, such as exposure of stored feeds to the environment and wild animals, purchasing of feeds, and implementation of different biosecurity measures could contribute to the non-homogeneous dissemination of indistinguishable *E. coli* O157:H7 subtypes among all farms in a region. Farms located in close proximity might also share many common denominators, such as the bulk milk transport, human travel, natural water systems, feed sources, and non-bovine residing animals such as cats and dogs, insects, and wild animals. These common transmission mechanisms, or common exposures may explain the simultaneous detection of *E. coli* O157:H7 REDP(s) in the same sample source on the same sampling date on multiple geographically close farms. The sporadic isolation of *E. coli* O157:H7 from flies, horses, dogs, racoons (10, 24), wild birds (28), opossums (21), rats (6), pigs, sheep (5), and deer (23) may be contributing to the transmission of this organism among farms. The sharing of common REDPs has previously been shown to occur between opossum feces and cattle on different sampling dates from the same study location, confirming that *E. coli* O157:H7 is not specific to cattle and that wildlife may contribute to the maintenance or transmission of *E. coli* O157:H7 (21). The small number of farms in this study limited the statistical power to identify specific associations between particular farm management practices.

Nevertheless, the fact that many farms did share similar REDPs suggest frequent between-farm transmission of isolates and the identification of the mechanism by which this occurs could lead to important intervention strategies to control this pathogen at the pre-harvest stage of production.

Detection of the same REDP subtype in wild bird excrement on the same sampling date from two different farms located 32.5 km apart, supports the hypothesis that wild birds may be involved in the dissemination of *E. coli* O157:H7 between neighboring dairy farms, and reinforces the fact that *E. coli* O157:H7 strains are not restricted to cattle. Other studies have also isolated *E. coli* O157:H7 from wild birds, including pigeons, gulls, and starlings (17, 15, 10). One study showed the isolation of an *E. coli* O157:H7 strain from a pigeon that had an REDP indistinguishable from both cattle and drinking water on the same farm (24). Our study is the first report to identify indistinguishable REDP between wild birds sampled on two different cattle farms. Although the avian isolates were collected from the farm environment and not directly from wild birds, environmental contamination with bovine feces is unlikely to have accounted for this finding because the particular *Xba*I REDP genetic pattern of the avian isolate did not match any other genetic fingerprint patterns of *E. coli* O157:H7 isolates collected from the same farm or on other farms and their environments at the time of sampling. Six out of the seven farms that shared isolates, as well as nine out of the thirteen farms not sharing isolates, self-reported large populations of starlings (*Sturnus vulgaris)* on their farms. However, none of the bird excrement collected from non-isolate sharing farms tested positive for *E. coli* O157:H7.

It is plausible that starlings play an important role in the spatial dissemination of *E. coli* O157:H7 because of their extreme mobility, frequent movement among farms, and their behavior of congregating in areas where livestock are fed, watered, and housed. Starlings fly up to 60 km from roosting to feeding sites daily (12). Wild birds have also been implicated in the dissemination of other food-borne pathogens to farms, such as *Salmonella* and *Campylobacter* (14, 19).

Isolation of the same REDP subtype on the same sampling date from two different sources, one from free-stall bedding and the other from a wild bird indicates that transmission of *E. coli* O157:H7 isolates can occur between the farm environment and wild animal hosts, the direction of transmission which occurs more frequently is unknown.

Indistinguishable subtypes of *E. coli* O157:H7 originating only from bovine sources on farms practicing open herd management, whereas indistinguishable subtypes of *E. coli* O157:H7 respectively originating from bovine as well as other sources were shared between farms practicing closed herd management. Although this does not preclude the possibility that new *E. coli* O157:H7 may be introduced with incoming cattle, our data would suggest that maintaining a closed herd will not prevent the introduction of *E. coli* O157:H7 into the herd. Instead non-bovine sources, such as human or vehicle movement, and wild animals, as previously mentioned, may be acting as more important vehicles in the transmission of *E. coli* O157:H7. These findings are in accord with those of a study by Rice et al., in which no significant difference in the effect of open verses closed herd management practices on the number of subtypes present between dairy farms was found to occur (22).

The temporal clustering of non-shared *E. coli* O157:H7 subtypes observed in this study are consistent with the findings of Rice et al. (22), in which some *E. coli* subtypes introduced onto a farm colonize cattle, but fail to be maintained within the herd.

In this study, the sharing of indistinguishable *E. coli* O157:H7 subtypes were found to occur in the late summer months, and only a single farm shared multiple subtypes. Whether the duration of sampling and number of samples tested impact the detectable *E. coli* O157:H7 diversity is uncertain. Renter et al. found both the number of samples collected and duration of time for collecting the samples, as well as the source from which the samples were collected to impact the detection of diverse *E. coli* O157:H7 subtypes between farms. In contrast, Rice et al. found no correlation between the diversity of subtypes detected and the sample size or duration of time in collecting samples (22).

Three out of the four REDPs were identified on the same sampling date. Although laboratory cross-contamination is possible, the likelihood that laboratory contamination alone is responsible for these findings is low. First, during the isolation and detection of *E. coli* O157:H7 using immunomagnetic separation methods, samples from each farm were run separately in the assay along with a negative control (water), each which invariably tested negative. In addition, three of the shared REDPs were unique to each farm on a particular sampling date, indicating that it was unlikely that the sample was contaminated with other samples from the same farm that were analyzed simultaneously.

In this study we have shown that farms in close geographic proximity do share indistinguishable *Xba*I/*Bln*I REDP *E. coli* O157:H7 subtypes, and at a moderate frequency (7/20 farms).

The on-farm sources contributing to the dissemination of this organism may include cattle, wild birds and free-stall bedding. The mechanisms of regional *E. coli* O157:H7 transmission between farms may include cattle movement between open-herd farms, and wild bird movement among farms, as indicated by the isolation of indistinguishable REDP subtypes between cattle on farms practicing open herd management and between wild birds on closed farms separated up to 50 km apart. Given the distribution of *E. coli* O157:H7 and REDP subtypes in cattle environments eradication of this pathogen on farms is unlikely due to the organism's lack of host specificity, asymptomatic carriage by adult cattle, and protracted environmental survival. However, the control of *E. coli* O157:H7 on a farm by limiting the introduction of the organism from bovine and non-bovine sources, and impacting the within farm prevalence of *E. coli* O157:H7 via specific farm management practices may help to control this foodborne pathogen at the pre-harvest stage.

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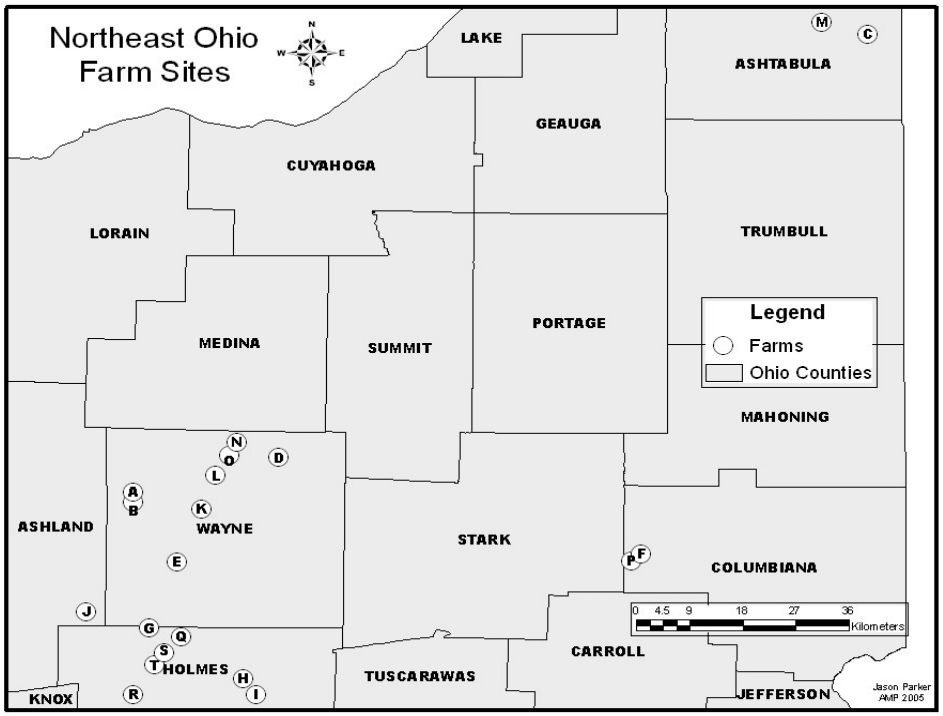

Figure 3.1. Location of the twenty Northeast Ohio dairy farms tested in the study.

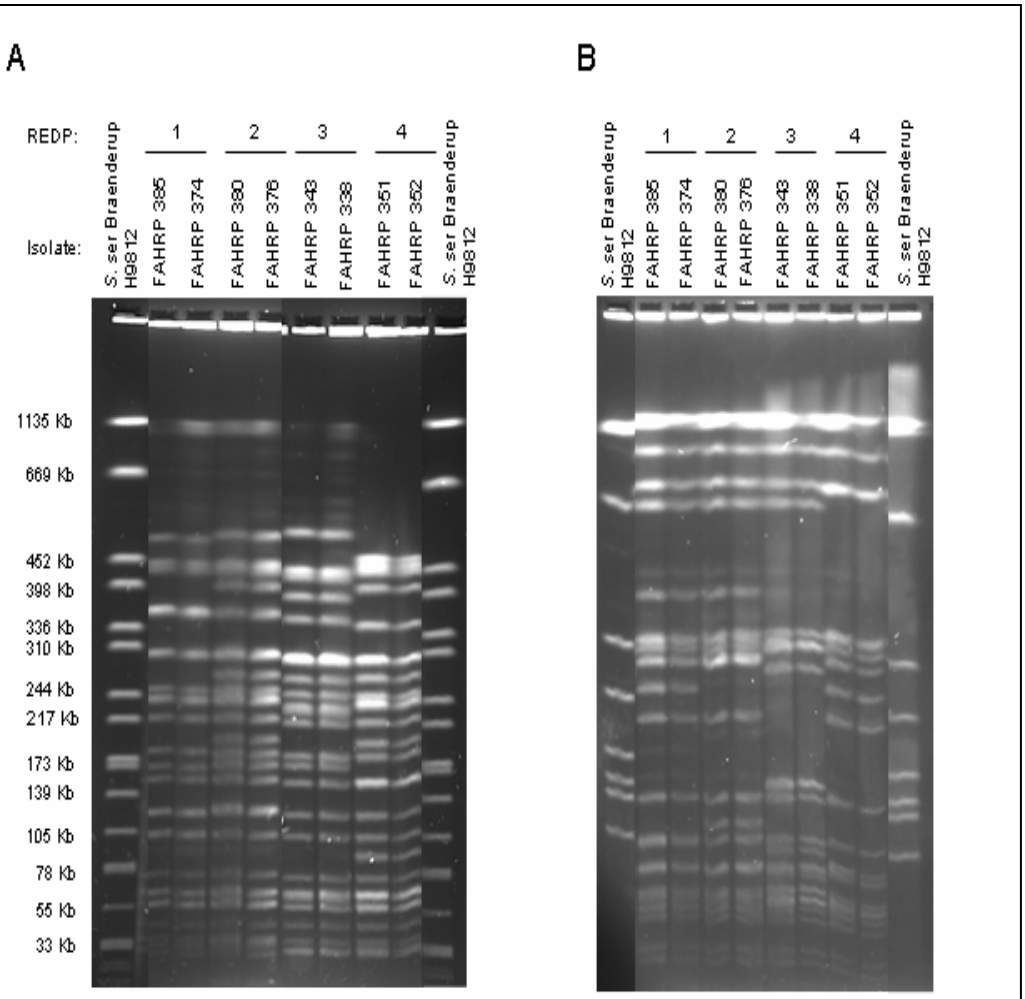

Figure 3.2. Composite pulsed field gels of the following FAHRP strains 385, 374, 380, 376, 343, and 338 afte
*Xba*I digestion, and (B) *Bln*I digestion and designated REDP using both visual and Bionumerics Fingerprint Clu
Analysis software (data not shown). Electrophoresis conditions used were according to the standard Pulse-Ne
Protocol with switch times from 2.2 to 54.2s over a (A) 19 hour run time and (B) a 21 hour run time.

Farm	Collection Date	Source	Representative Strain No.	*Xba*I/ *Bln*I REDP[a]	*Xba*I REDP n [b]	*stx*$_1$ [c]	*stx*$_2$ [c]	Distance Between Farms (Km)
S	9/15/2003	Cow	FAHRP 385	1	2	-	+	8.7
G	9/8/2003	Bedding	FAHRP 374	1	1	-	+	
I	9/8/2003	Wild Bird	FAHRP 380	2	1	-	+	32.5
G	9/8/2003	Wild Bird	FAHRP 376	2	1	-	+	
K	8/18/2003	Cow	FAHRP 338	3	1	+	+	23.6
L	8/18/2003	Cow	FAHRP 343	3	1	+	+	
B	8/19/2003	Cow	FAHRP 351	4	1	-	-	49.9
T	8/19/2003	Cow	FAHRP 352	4	2	-	-	
T	9/15/2003	Cow	FAHRP 352	4	16	-	-	
T	9/15/2003	Wild Bird	FAHRP 402	4	16	-	-	
T	9/15/2003	Bedding	FAHRP 401	4	16	-	-	
T	8/19/2003	Water	FAHRP 356	4	16	-	-	
T	9/15/2003	Water	FAHRP 400	4	16	-	-	

Table 3.1. Indistinguishable *E. coli* O157:H7 strains displaying a given REDP shared between geographically close dairy farms in NE Ohio

[a] REDP was generated using both *Xba*I and *Bln*I restriction endonuclease enzymes.

[b] Number of *E. coli* O157:H7 isolates out of the 89 subtypable isolates from each farm with the *Xba*I generated REDP.

[c] Presence of gene sequences of *stx*$_1$ and *stx*$_2$.

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CHAPTER 4

ISOLATION OF SHIGATOXIN-NEGATIVE *ESCHERICHIA COLI* O157:H7 STRAINS FROM CATTLE, BIRDS, AND THE FARM ENVIRONMENT

INTRODUCTION

Shiga toxin-producing *E. coli* (STEC) strains have emerged as foodborne pathogens responsible for outbreaks of human intestinal diseases, and the potentially fatal hemolytic-uremic syndrome (HUS), worldwide (6). Cattle have been shown to be a major reservoir for STEC, with the most common virulent serotype being *E. coli* O157:H7. Transmission of STEC from cattle to humans can occur via food, direct contact and waterborne routes.

Shiga toxins (Stx), produced by *E. coli* O157:H7 and other STEC, are considered to be a principal virulence factor in the pathophysiology of HUS (1). The stx genes, encoded on temperate bacteriophage, are stably transferred to the chromosomal DNA of host *E. coli* by lysogeny (13). Hence, bacteriophages can be efficient vectors for lateral transmission of stx_1 and stx_2, and thus may play an important role in the emergence of shigatoxigenic strains.

MATERIALS & METHODS/RESULTS/DISCUSSION

The isolation of *Escherichia coli* O157:H7 strains that do not encode known *stx* variants are being reported more often in the literature (3, 4, 11, 15, 16, 17, 18). Whether these isolates contain DNA sequence variations that preclude gene target detection with the PCR, or whether they represent an infrequent or sporadic loss of the *stx* gene in the natural reservoir or during culture has not been fully explored. In either case, the role of *stx*-negative *E. coli* O157:H7 in the ecology and epidemiology of human disease causing STEC is unknown. Although Stx are considered to be the essential virulence factor of STEC, it has been suggested that *stx*-negative O157 can cause diarrhea and HUS (11). In our study we have not only detected sorbitol non-fermenting, *stx*-negative *E. coli* O157 isolates in cattle, as other studies have previously reported, but herein report for the first time the isolation from the farm environment and other inhabiting animal sources on the farm.

During the months of June through September 2003, 3,600 fecal samples from lactating cows, 120 bedding material, water trough and sediment samples, and 67 composite samples of wild bird excreta were collected from the farm environment of twenty commercial dairy farms located in NE Ohio (8). Ninety-two samples (81 cow fecal samples, 5 bedding samples, 3 water trough sediment samples, and 3 wild bird pooled excreta samples) tested positive for *E. coli* O157:H7 using enrichment and immunomagnetic separation methodology. Typical of most *E. coli* O157:H7 isolates from the US, none of the isolates fermented sorbitol.

PCR analysis was used for detection of eight *E. coli* O157:H7 virulence genes including the major *stx* variants (2, 2c, 2d, 2e, 2f, 1), and *eae, ehxA*, and two genes that define the serotype O157:H7 (14). Twenty-two of 92 confirmed *E. coli* O157:H7 isolates were positive for *ehxA, eae, rfbE,* and *fliC*, but negative by PCR for all Shiga toxin variants assayed (Figure 4.1 Lanes 1-12). Overnight supernatant from mitomycin C-induced cultures of these PCR *stx*-negative isolates also tested negative for the production of Stx via the Premier EHEC Shiga toxin ELISA (Miridian Biosciences, Inc.), and showed no cytopathogenicity for Vero cells (5, 9). These *stx*-negative isolates represented 17/81 (21%) of the total cow fecal isolates, 2/5 (40%) of the bedding samples, 2/3 (67%) of water trough sediment samples and 1/3 (33%) of the total pools of wild bird excreta that tested positive for *E. coli* O157:H7 in this study. Nineteen of 22 *stx*-negative isolates recovered were indistinguishable by PFGE analysis following *Xba*I digestion (100% Dice Similarity Coefficient, optimization 0.75%, tolerance 2-4%) (Figure 4.2). Although many of the *stx*-negative *E. coli* O157:H7 isolates exhibiting the predominate RFLP originated from bovine fecal samples were collected from one farm on one occasion, additional *stx*-negative isolates were obtained from three other farms and collected on at least three other sampling dates. Furthermore, stx_2 positive *E. coli* O157:H7 isolates were obtained from a bovine fecal sample on farm T on a previous date, and from two other farms sampled on September 15, 2003 (8), demonstrating that the isolation of *stx*-negative isolates was neither common to all samples analyzed on a specific date, nor the exclusive strain type of *E. coli* O157:H7 isolated from each farm.

Among the three *stx*-negative *E. coli* isolates that did not exhibit the predominant RFLP, one isolate was not typable by PFGE (FAHRP 312), and the other two isolates (FAHRP 387 & 261) differed by 2 to 4 bands from the predominant RFLP (Figure 4.2). Although only minor differences in banding patterns were observed among the distinguishable isolates, it is not possible to determine the genetic relatedness of the isolates obtained from this study using a single-enzyme PFGE method (2).

The possibility of spontaneous loss of the *stx* gene by these *E. coli* O157:H7 strains during enrichment, as reported by Karch et al. for non-O157 STEC strains can not be ruled out (7); however, due to the large number of *E. coli* O157:H7 isolates collected which lack the *stx* gene (22/92), it seems unlikely that spontaneous loss of *stx* would have occurred in so many enrichment cultures in this study, and on multiple occasions. A study by Neilsen et al. (16) suggests that phage loss occurrs in the intestinal tract of the animal instead of outside the host or during routine laboratory culturing.

To assess the ability of these *stx*-negative *E. coli* O157:H7 isolates to become pathogenic, we investigated the capability of a detoxified derivative of a stx_2- encoding bacteriophage to infect and lysogenize the *stx*-negative *E. coli* O157:H7 isolate FAHRP 402. Briefly, the chloramphenicol acetyl transferase (CAT)-encoding vector, pHS3, was used to replace the stx_2 gene (Δ*stx2::cat*) of a toxigenic strain of *E. coli* O157:H7 of bovine origin (FAHRP 62) (9,12). An overnight culture of the detoxified FAHRP 62 Δ*stx2::cat* isolate was then induced to lysis using mitomycin, and the infectious Δ*stx2::cat* phage particles were subsequently used to infect an *E. coli* O157:H7 *stx*-negative isolate of avian origin recovered in this study (FAHRP 402).

Successful lysogenic conversion of the *stx*-negative isolate (FAHRP 402) by the Δ*stx2::cat*–encoding bacteriophage was confirmed via bacterial growth on chloramphenicol (30µg/ml) containing media and by PCR using primers specific for the inserted cat gene (12) (Figure 4.1).

Some researchers have shown minor changes in PFGE patterns upon lysogenization of *E. coli* by the same lambdoid phage (18). This variability may be attributed to the preferred insertion sites for *stx*–encoding phages being occupied or absent in the *E. coli* strains used in the study. In contrast, in our study, twelve out of twelve of the Δ*stx2::cat* containing lysogens were indistinguishable by PFGE of *Xba*I digested chromosomal DNA (data not shown), suggesting insertion of the phage DNA into the same site in each lysogen. Lysogenization into different chromosomal locations may have occurred if the preferential integration site had been occupied by other prophages.

This study reports the isolation of *stx*-negative *E. coli* O157:H7 strains from multiple cattle, as well as the natural occurrence of *E. coli* O157:H7 that do not produce Stx in environmental and avian sources collected on farms. This study also demonstrates that *stx*-negative *E. coli* O157:H7 that possess the capability to acquire *stx* through transfection, may colonize cattle, wild birds, and contaminate the environment. Given that stx_2-encoding *E. coli* O157:H7 and other STEC occur sporadically on farms and that bacteriophages, in general, persist under environmental conditions longer than bacteria, these results suggest that *stx*-negative *E. coli* O157:H7 strains that harbor other important virulence genes may acquire stx_2 from the dairy farm environment, and thus be a potential source of new STEC strains.

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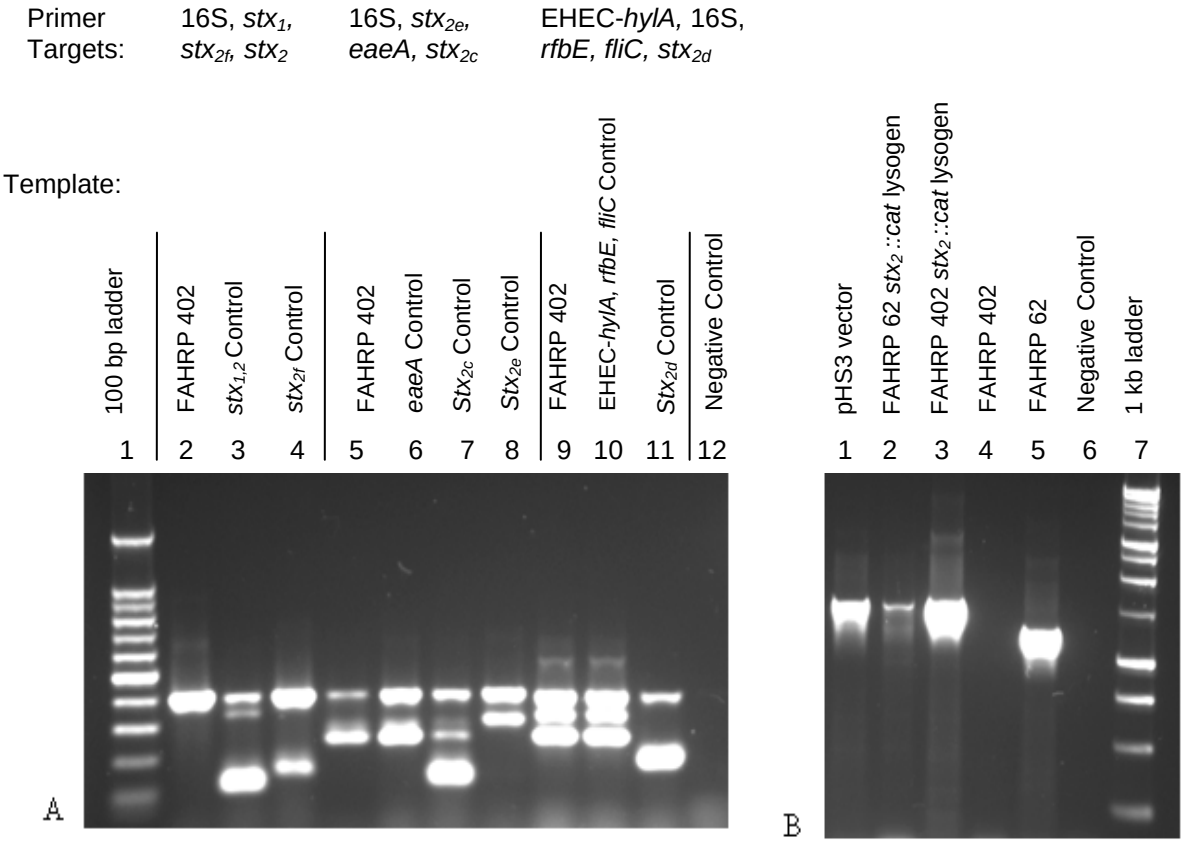

Figure 4.1 Ethidium, bromide-stained gel of PCR amplification products for A. E. coli O157:H7 virulence genes of FAHRP 402 (lanes 2, 5, 9). Human EDL 933 isolate control for *stx*$_1$ (338 bp) & *stx*$_2$ (115bp) (lane 3), *eaeA* (248 bp) (lane 6), EHEC-*hylA* (569 bp) (lane 10), *rfbE* O157 (327 bp) (lane 10), *fliC* (247 bp) (lane 10), human HI8 isolate control for *stx*$_{2f}$ (150 bp) (lane 4), human E32511 isolate control for *stx2c* (124 bp) (lane 7), ED53 isolate control for *stx*$_{2e}$ (303 bp) (lane 8), human EH250 isolate control for *stx*$_{2d}$ (175 bp) (lane 11), and 5µl pools of water from each PCR reaction mixture was used as the negative control (lane 12); B. PCR amplification of the *cat* (1,567 bp) and *stx*$_2$ (1,259 bp) genes using HSB1 and HSB3 primers (lanes 1-5), water was used as a negative control (lane 12).

ID	Source	Collection Date	Farm	REDP Pattern
.E. coli standard.				Control
.FAHRP 312	..Cow	7/28/2003	P	Non-typable
.FAHRP 351	..Cow	8/19/2003	B	1
.FAHRP 352	..Cow	8/19/2003	T	1
.FAHRP 388	..Cow	9/15/2003	T	1
.FAHRP 389	..Cow	9/15/2003	T	1
.FAHRP 393	..Cow	9/15/2003	T	1
.FAHRP 394	..Cow	9/15/2003	T	1
.FAHRP 395	..Cow	9/15/2003	T	1
.FAHRP 399	..Cow	9/15/2003	T	1
.FAHRP 386	..Cow	9/15/2003	T	1
.FAHRP 390	..Cow	9/15/2003	T	1
.FAHRP 391	..Cow	9/15/2003	T	1
.FAHRP 392	..Cow	9/15/2003	T	1
.FAHRP 396	..Cow	9/15/2003	T	1
.FAHRP 397	..Cow	9/15/2003	T	1
.FAHRP 398	..Cow	9/15/2003	T	1
.FAHRP 402	..Wild Bird	9/15/2003	T	1
.FAHRP 356	..Water trough sediment	8/19/2003	T	1
.FAHRP 400	..Water trough sediment	9/15/2003	T	1
.FAHRP 401	..Bedding	9/15/2003	T	1
.FAHRP 261	..Bedding	6/10/2003	L	2
.FAHRP 387	..Cow	9/15/2003	T	3
.E. coli standard .				Control

Figure 4.2. REDP, farm, collection date, and source of XbaI-digested bacterial DNA from stx-negative E. coli O157:H7 isolates. Isolate FAHRP 312 was not typable by PFGE.

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