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THE EPIDEMIOLOGY OF SHIGA-TOXIGENIC *ESCHERICHIA COLI* O157:H7 IN LOUISIANA DAIRY CATTLE, BEEF CATTLE, AND WHITE-TAILED DEER

A Dissertation
Submitted to the Graduate Faculty of
Louisiana State University and
Agriculture and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

In

The Interdepartmental Program in
Veterinary Medical Sciences
through the Department of
Pathobiological Sciences

By
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May 2003
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ABSTRACT

Shiga-toxigenic *Escherichia coli* (STEC) O157:H7 is an important human pathogen. Dairy cattle, beef cattle, and white-tailed deer (WTD) are involved in STEC O157:H7 transmission to humans. We conducted cross-sectional studies in Louisiana, using sensitive microbiological methods, in dairy cattle, beef cattle, and WTD to investigate the epidemiology of STEC O157:H7.

Results of the dairy studies are as follows. In the point prevalence study, summer-time prevalence in herds was 38.5%, with a cow-level prevalence of 6.5%. Among positive herds, cow-level prevalence ranged from 3%–34.6%. Three of five herds sampled in the longitudinal study were positive. Cow-level prevalence increased during spring and summer. Adult dairy cattle during the warm season had increased odds of STEC O157:H7 fecal shedding. Lactating dairy cows had increased odds of STEC O157:H7 fecal shedding compared to dry cows. In the mouth, hide, and fecal study, cow-level prevalence estimates of STEC O157:H7 in the mouth, on the dorsal hide, and from feces were 0%, 0.7%, and 25.2%, respectively.

We sampled weaned beef cattle at the beginning and end of a preconditioning program. Five of twenty-nine herds were shedding STEC O157:H7 or *Escherichia coli* (EC) O157:H7 initially. No cattle were shedding STEC O157:H7 or EC O157:H7 at the end. We found 0.7% of weaned beef cattle shedding STEC O157:H7. The animal-level prevalence of the O157:H7 serotype, including shiga toxin (stx)-deficient isolates, was 2.5%. We expected, but did not observe, increased shedding or the spread of STEC O157:H7 subtypes.

Two WTD field studies were conducted. We collected 338 fecals from hunter-harvested WTD and found one positive sample. The isolate was stx-deficient and sorbitol
positive. In the second field study, we isolated STEC O157:H7 in a captive WTD herd, but were unable to demonstrate seasonal trends in fecal shedding.

Louisiana reports relatively few human STEC O157:H7 cases. We detected STEC O157:H7 in each of our studies. We demonstrated high fecal prevalence, seasonal shedding, and hide contamination in dairy cattle. Epidemiologic studies in ruminant populations should be revisited using sensitive methods. Studies investigating the human incidence of STEC O157:H7 in relation to presumed ruminant reservoirs are warranted.
CHAPTER ONE
INTRODUCTION/LITERATURE REVIEW

1.1. Background Shiga-Toxigenic *Escherichia coli* (STEC)

*Escherichia coli* are ubiquitous intestinal bacterial flora of animals and humans. Although comprising a small proportion of the total fecal flora they are the predominant facultative anaerobe in the human colon and presumably exist there symbiotically (14,155). Infants and post-parturient animals are normally colonized shortly after parturition, acquiring their mothers’ intestinal flora (14,155). Pathogenic strains or clones of *E. coli* exist or have developed with the ability to cause a wide variety of diseases in humans (155,228,229). During the past twenty years, a clonal group of *E. coli* has emerged or been recognized that possess a unique disease-causing virulence factor armament, asymptomatically colonize food-producing ruminants, and are capable of causing significant morbidity and mortality in humans.

The emergence of or recognition of Shiga-toxigenic *Escherichia coli* (STEC) as a cause of diarrhea, hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (TTP) in humans is a significant public health concern worldwide. STEC isolated from humans with specific clinical signs are called Enterohemorrhagic *E. coli* (EHEC). Therefore, EHEC are considered to be a subset of STEC. Differences between EHEC and STEC are thought to be due to variability in virulence factor armament. Transmission of STEC to humans from animal reservoirs typically occurs by fecal contamination of food or water, direct or indirect contact with animals, or by person-to-person contact (231). Disease in animals is less commonly recognized, although many domestic and food animals are colonized by STEC. Apparently STEC do not commonly
colonize normal, healthy humans and are infrequently found in their feces (14,233). Healthy cattle and other ruminant species appear to be reservoirs from which STEC that are pathogenic to humans originate (18). Colitis in calves and edema disease in pigs are documented STEC related diseases of animals.

*E. coli* is classically differentiated based on the modified Kauffman serotyping scheme. Numerous O (somatic), H (flagellar), and K (capsular) surface antigens have been described (155). The “serotype” is defined by the O:H antigen combination. The unique and distinguishing feature of STEC serotypes, in comparison to other pathogenic *E. coli* is their ability to produce shiga toxins (stx) (95,113). Stx, also known as shiga-like toxin (SLT) or verotoxin (VT), derives its name from its similarity to shiga toxin that is produced by *Shigella dysenteriae* type one.

The term verotoxin refers to the ability of the toxin to cause lethal cytopathic effects in vero cell cultures (African green monkey kidney cells) and was introduced in 1977, around the same time as the term SLT (123,159,160). The current nomenclature is shiga toxin (stx / stx) (113,210). Table 1.1 lists current stx nomenclature and other abbreviations frequently used in STEC and STEC O157:H7 research. Comparative amino acid sequence homology of STEC stx to *S. dysenteriae* type one shiga toxin subdivides stxs into stx1 and stx2 subgroups. Stx1 differs by one amino acid and is antigenically indistinguishable from *S. dysenteriae* shiga toxin type one, while the stx2 group has 50-60% sequence homology to stx1 and *S. dysenteriae* type one shiga toxin (87,192). Stx1 is a highly conserved toxin, where stx2 is variable and includes an expanding list of variants that includes stx2, 2c, 2d, 2e, and 2f.

STEC toxin production is variable. STEC can produce stx1, stx2, both stx1 and stx2, or stx2 variants. Structurally, stxs are A-B toxins that inhibit protein synthesis. The A subunit
acts as a ribosomal RNA N-glycosidase, cleaving an adenine residue from ribosomal RNA (28s rRNA) at the site where elongation factor one-dependent attachment of aminoacyl tRNA occurs (95,193). This stops protein synthesis and causes apoptosis. Cleavage of the holotoxin (composed of the B pentamer and a single A subunit) results in the enzymatically active N-terminal A1 component and a C-terminal A2 component. The B subunit binds to a glycolipid receptor in mammalian cellular membranes called globotriaosylceramide (Gb3). Gb3 is also known as CD77 and is a differentiation antigen during human B cell development (138). Binding of the B subunit plays a crucial role in the entry of the A subunit into specific cells (136).

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Recent studies indicate that stx1 and stx2 may bind at different locations of the Gb3 moiety and that cellular messengers (tumor necrosis factor-alpha and IL-6) modulate the presence of the receptor and the sensitivity of the cell to stx (65,105). Clinically, elevated levels of IL-6 in the urine and blood of HUS patients correlate with more severe disease outcomes (155). Variability of stx2 may be a result of genetic recombination of the stx2 B
subunit genes rather than base substitutions (80,104). This provides a mechanism that could explain variable receptor affinity or tropisms demonstrated by different stxs.

Pathogenic mechanisms of stx are not completely understood, but it is thought that stx contributes to development of diarrhea, HC, TTP, and HUS. It is thought that HC occurs after the release of stx in the intestinal tract and subsequent apoptotic damage at the mucosal surface. HUS/TTP may occur following absorption of stx into the bloodstream with subsequent damage to vascular endothelial cells, particularly the renal vasculature (157,216). Translocation of stx from the intestinal lumen to the blood stream occurs (155). Stx has recently been detected in circulating blood of HUS patients. In those patients, stx2 was demonstrated in the systemic circulation and was exclusively bound to polymorphonuclear leukocytes (PMNs) (212). Understanding the pathogenic mechanisms of stx translocation, transport, and entry into target cells may lead to strategies that reduce harmful sequelae of human STEC infection.

All stxs are encoded on temperate lambdoid bacteriophages except stx2e, which is encoded chromosomally as is the shiga toxin of S. dysenteriae type one. Sequence homology between stx1 and shiga toxin has led researchers to propose that a progenitor E. coli acquired shiga toxin via bacteriophage from S. dysenteriae type one (71). Genetic sequence structure is identical for stxs. The operons are organized with the A and B subunits in tandem and an intervening sequence of 12 to 15 nucleotides (162). A and B subunits may be differentially regulated to produce the B pentamer. Transcriptional regulation occurs by binding of an iron-FUR protein co-repressor complex at the stx1 promoter that is located 5’ to the A subunit gene. Stx1 production is also repressed by reduced temperature. Stx2 production is not regulated by iron or temperature regulated (155,162).
Stx1 and stx2 show variable pathogenicity in animal models and *in vitro*. Stx2 causes HC in adult rabbits and is less toxic to Vero cells but more toxic to mice than stx1 (87,157). Differences in pathogenicity between stx1 and stx2 in humans are suspected based on the stx genotype of human isolates that can be *stx*1, *stx*2 or both (87).

It is thought that variation in virulence factor armament has led to the emergence of STEC. *Stx* acquisition by progenitor *E. coli* is thought to be responsible for the increased virulence of this important disease. This model of phage-mediated toxin transfer has caused speculation that more competitive gastrointestinal flora could acquire *stxs* by lateral transfer, creating new, more virulent human pathogens (192,194). Certainly, within *E. coli*, a large number of STEC serotypes have been documented in humans, ruminant food animals, other domestic animals, wild animals, and invertebrates.

Multiple STEC strains from over 100 serotypes are potentially able to cause human STEC infection, with serotype O157:H7 being the most notable (23). Common EHEC isolates include the serotypes O26, O103, O111, O145, and O157 (112). STEC O157:H7 is the most frequently isolated STEC in North America, Japan, and most of Europe. Elsewhere other STEC serotypes predominate. In Australia, O111 serotypes predominate and have been reported to cause severe sporadic disease as well as outbreaks, while O157:H7 isolations are rare (14,68,68,84,84). Reports from Argentina implicate non-O157 STEC more frequently than STEC O157:H7 in cases of HC and HUS. Most methods that specifically identify STEC O157:H7 will not effectively detect non-O157 serotypes. One study, which detected *stx* and stx, demonstrated that multiple STEC serotypes, but not STEC O157:H7, were present in Argentine boneless beef samples (3,139). Serotyping of STEC in another Argentine study produced 31 different O:H serotypes that included: O20:H19, O113:H21, O91:H21,
O116:H21, O117:H7, O171:H2 and OX3:H21, all of which were isolated from beef or cattle (165).

In U.S. retail meats, non-O157 STEC were isolated from 23% of ground beef, 18% of pork, and 48% of lamb tested (188). In 1999, it was estimated that 110,000 cases of disease were caused by STEC in the U.S., with more than 30% linked to non-O157 serotypes (150). Twenty to 25% of HUS cases in North America are estimated to be secondary to non-O157 STEC (88). Among non-O157 serotypes, the Centers for Disease Control (CDC) reported that O26 and O111 serotypes were first and second, respectively, in the numbers isolated among specimens submitted from 1983 to 1998 (37).

Farm residents have been shown to have serologic titers indicating exposure to STEC. In a study of dairy farm residents and their cattle, human non-O157 STEC infection was not associated with disease, indicating that previous exposure may be immunoprophylactic (233). Non-O157 strains possess a variable virulence factor armament (230). The variability in virulence factor armament may explain subclinical STEC colonization of humans. The number of STEC serotypes recognized in animals that can potentially cause disease in humans continues to expand. Newly described clinical STEC isolates continue to emerge that previously were unknown among humans. Some of these newly recognized serotypes might be more pathogenic than STEC O157:H7 (66,171).

Ruminants appear to be asymptomatic carriers of STEC except for HC in calves, which has been demonstrated experimentally. Reports of colitis and diarrhea in calves are sporadic and often occur in conjunction with experimental work and high infectious doses of STEC. E. coli O111:H-, O5:H-, and O26:H- are the most frequent serotypes isolated from calves with STEC-associated diarrhea (108,143,196). High doses (up to $10^{10}$ CFU) used in
experimental work and some natural infections may cause diarrhea via attaching and effacing (AE) lesions related to the outer membrane protein (OMP) intimin (60, 62). It appears that natural disease associated with either STEC O157:H7 or non-O157 STEC serotypes is rare in cattle. The presence of stx does not appear to have a significant role in cattle disease as in human disease (63). Stx is apparently not essential in the pathogenesis of disease in calves as is intimin, but may have a role in intestinal colonization and fecal shedding (63). Clinical disease surveillance among cattle is an impractical method for detection of STEC shedding because virtually all calves and adult cattle appear normal and healthy.

Multiple STEC serotypes can colonize beef and dairy cattle. In studies of fecal shedding in Australian dairy herds, two of the most common STEC serotypes were O157:H7 and O26:H11, with other serotypes reported for the first time in cattle (50, 51). A German slaughterhouse study found STEC isolates from 54 different serotypes in fecal cultures. All of the German isolates were non-O157 serotypes, some of which were previously reported as human pathogens (181). Ruminants in general appear to have the highest fecal shedding prevalence of STEC among animals studied (17, 169). Numerous serotypes of STEC have been isolated from cattle. Underestimation of the variety of serotypes that carry stx is a function of non-specific culture methodology and identification techniques.

Findings in sheep have also documented multiple STEC serotypes. Sheep are naturally colonized with STEC O157:H7 and non-O157 STEC (44, 45, 126). Non-O157 STEC serotypes isolated in sheep include O91:NM (non-motile), O128:NM, O88:NM, O6:H49, and O5:NM (127). Sheep have served as an experimental model for STEC in cattle (125, 128). The data from sheep suggested that STEC colonization of ruminants is more persistent than other pathotypes of E. coli. STEC colonization in sheep was longer in duration than
colonization by enteropathogenic *E. coli* (EPEC) or enterotoxigenic *E. coli* (ETEC). This suggests that STEC may either out-compete other *E. coli* or be more suited to the niche provided by the ruminant intestinal tract (54). Recolonization or intermittent shedding and horizontal transfer of STEC have been demonstrated in sheep (128). The presence of multiple STEC serotypes, including STEC O157, and studies that have demonstrated carcass contamination, indicate that sheep can be a vehicle for STEC into the human food chain (42,70).

In 1949, a toxin was recognized as the cause of Edema Disease (ED) in pigs. Initially called Edema Disease Principle (EDP), the toxin has been shown to be a stx termed stx2e (79,137). STEC serotypes that produce stx2e and cause ED do not produce stx1 and stx2. Three of the O serogroups implicated in ED are O141, O139, and O138 (77,203). The pathology caused by stx2e results from damage to small blood vessels leading to hemorrhage and edema in tissues of infected pigs (154).

STEC are a diverse group of organisms that are capable of colonizing vertebrate and invertebrate hosts (91). One survey of multiple species in Germany indicated that ruminants (sheep, goats, and cattle) are the species most frequently colonized. STEC were isolated less frequently in pigs, cats, and dogs. In this study, multiple serotypes were found that included forty-one different O:H serotypes and 23 untypeable O-groups. Serotypes O5:H-, O91:H-, O146:H21, O87:H16, and O82:H8 occurred in multiple animal species (17). STEC isolated from healthy animals in this survey were heterogeneous in serotype and virulence factors. Furthermore, the authors felt that some serotypes exhibited host specificity (19). STEC, specifically O157:H7, has also been found in equine fecal samples, avian samples, and fly samples (92). Studies indicate that STEC are rarely found in chickens (17,103,170). Wild
ruminants have been shown to shed STEC and have been implicated in foodborne outbreaks. STEC O157:H7 colonizes White-tailed deer (*Odocoileus virginianus*) (73,177).

By far the most notable STEC serotype is O157:H7. In fact, STEC are often broadly classified as O157:H7 or non-O157 (23). It is well documented that multiple STEC serotypes and strains cause disease in humans or are capable of causing disease based on their virulence factor armament (95). A position statement by the Council of State and Territorial Epidemiologists (CSTE) in 2000 recommended inclusion of non-O157:H7 STEC in the National Public Health Surveillance System (NPHSS) which includes surveillance for STEC O157:H7 (55). Infections with STEC O157:H7 are thought to be twice as common as non-O157 serotypes and thought by some to result in greater hospitalization rates and case-fatality rates (150).

**1.2. Shiga-Toxigenic *Escherichia coli* O157:H7 (STEC O157:H7)**

**1.2.1. Recognition and Emergence**

The expanding role of non-O157 STEC in diarrheal disease, HC, HUS, and TTP is documented, but less is known about the epidemiology of these serotypes compared to STEC O157:H7. Specific culture methods and biochemical properties have led to increased ability to detect and isolate STEC O157:H7 in clinical cases and from ruminant samples. Improvement in diagnostic techniques (such as detection of fecal stx) and changes in reporting guidelines for non-O157 STEC may alter the relative importance placed on differing STEC serotypes (55). Attention focused on STEC O157:H7 as an “emerging infectious disease” is warranted (10). It has been reported as the leading cause of HUS in the United States, Canada, and Europe and was estimated to cause 85–95% of HUS cases in North America (2,10,21,156,186). CDC investigators have estimated that 13-27 cases occur
for each confirmed case (150). The number of outbreaks reported has increased since recognition of STEC O157:H7 as a cause of foodborne illness. The number of sporadic cases attributed to the O157:H7 serotype increased as well. An increased emphasis and enhanced surveillance effort following widespread, highly-publicized outbreaks has complicated interpretation of the disease trends (10,64).

A triad of events, beginning in 1982 and culminating in 1992-1993, focused public health attention on STEC O157:H7. The emergence or recognition of the pathogen generated national surveillance programs and numerous research efforts. In 1982 a series of HC cases was classified as an outbreak and was associated with eating hamburger from the same fast-food restaurant chain in Michigan and Oregon. In all, forty-seven cases were recognized with similar clinical signs that included cramps, abdominal pain, watery diarrhea, and hemorrhagic diarrhea. *E. coli*, of the rare serotype O157:H7, was isolated from the patients and proposed as the causative agent. Previously, a single case of HC from 1975 was the only known isolation of this serotype (28,184). Subsequent laboratory investigation and case-control studies supported the assumption that *E. coli* serotype O157:H7, not previously reported to cause disease, was the likely causative agent. Concurrent investigations of outbreaks and sporadic cases of HC in Ottawa, Ontario, Canada, during 1982 and 1983, implicated the O157:H7 serotype and demonstrated an association with hamburger consumption.

An important outcome of the initial laboratory investigation was the recognition that the O157:H7 serotype was unable to ferment sorbitol or fermented it slowly (226). The authors suggested that screening for sorbitol fermentation, possibly with a sorbitol-based MacConkey agar (SMAC), could be a simple diagnostic aid in serotype identification.
Pathogenicity to vero cells was recognized, suggesting that an undescribed *E. coli* cytotoxin might be involved in the pathogenic mechanism (29,110). As mentioned, vero cell cytotoxin had previously been recognized in *E. coli* and termed VT (123). The similarity of *E. coli* toxin to shiga toxin led to the term stx. Stx was described around the same time as the initial outbreak (159). The VT and stx nomenclature used by different laboratories to recognize vero cell cytotoxicity and sequence homology to *Shigella dysenteriae* shiga toxin has caused confusion in classification. Serotypes described as verotoxin positive or stx positive are identical and the terms verotoxin-producing *E. coli* (VTEC) and STEC are synonymous (2).

A second key finding in the emergence of STEC O157:H7, following the initial outbreaks and recognition of cytotoxin, was the association of STEC with idiopathic HUS. HUS was first described in 1955 and is characterized by acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia following an acute diarrheal illness. Idiopathic HUS was recognized as a leading cause of acute renal failure in children. An infectious etiology was considered probable but had not been previously described (115).

A 1968 paper by Kibel and Barnard hypothesized that a strain of *E. coli* infected by bacteriophage could be responsible for HUS (155). Preliminary reports by Karmali *et al.* indicated that both STEC and stx were present in the stools of patients with idiopathic HUS, stimulating more rigorous case-control studies (116,117). A total of forty HUS cases were investigated for evidence of STEC infection. Controls were selected and matched by age, sex, and season of the year. Findings from this study and others indicated that STEC had a significant causal role in the etiology of childhood HUS (115).
The wide range of clinical signs, combined with the newly described etiology of HUS elevated STEC in importance as an emerging foodborne pathogen. In 1987, Washington State was first to institute a surveillance program for STEC O157:H7, making it a reportable disease. Results from the first year of surveillance demonstrated elevated age-specific rates in children, the elderly, and a summer pattern of infection (163). In the decade following the first outbreak, much of the epidemiology of STEC O157:H7 has been described. In the latter half of the decade, a series of epidemiologic reviews summarized the role of stx, the broad clinical picture of STEC infection, and the role of cattle in disease transmission (86,87,114,157,183).

In 1993, a highly publicized, hamburger-associated outbreak of STEC O157:H7 focused public and governmental attention on the problem. In January of 1993, a multistate outbreak of STEC O157:H7 infection was first recognized in Washington State. During January, 230 culture-confirmed cases were reported. HC and HUS cases were reported following the consumption of hamburgers from several restaurants of one fast-food chain. The incriminated lots of ground beef had been distributed throughout several western states prompting an interstate recall of the ground beef following reports of bloody diarrhea in those states. (30). Processing errors and inadequate cooking were identified as probable causes for the outbreak. Surveillance implemented by the state of Washington was a key element in the early recognition and intervention to control the epidemic (8).

Subsequent case-control studies by public health officials found more than 500 laboratory confirmed cases and four deaths in Washington, Idaho, California, and Nevada from the 1993 outbreak. Difficulties encountered in the isolation of STEC O157:H7 during this outbreak investigation further emphasized the importance of using SMAC agar for
routine stool culture by laboratories when HC or HUS is reported (31). Comparison of the 1993 outbreak strain to EDL933 (American Type Culture Collection [ATCC], 43895), an isolate from the first hamburger-associated outbreak in 1982, revealed marked similarities in virulence factor armament (161). Stx1, stx2, and other putative virulence factors were present in both outbreak strains from food of animal origin. Traceback investigation into the origin of the meat determined that the contaminated lots most likely came from specific U.S. and Canadian slaughter plants. Farms and auctions in six western states were implicated but no single slaughter plant or farm was identified as the source of the outbreak strain (31,64).

The 1982 index outbreak, an association of HUS with STEC infection, and the 1993 multistate outbreak were key events that have led to the emphasis placed on the study and surveillance of STEC O157:H7. There have been numerous outbreak reports and research efforts that continue to recognize features related to this disease. Origins of STEC O157:H7 and the reasons for its sudden emergence in 1982 are unclear.

The factors that led to the emergence of STEC O157:H7 are difficult, if not impossible, to validate. It is possible that STEC O157:H7 existed and caused disease prior to 1982 but was unrecognized. The increasing number of cases and outbreaks reported could be an artifact of enhanced surveillance efforts and improved diagnostics. In 1993, the number of outbreaks and outbreak-related cases increased, which was the same year that the CSTE recommended surveillance (2,32). New Jersey Department of Health officials recognized an artifactual outbreak in 1994 that was caused by increased reporting of sporadic cases following initiation of a surveillance program. A case-control study failed to identify a source for the outbreak and subsequent analysis identified multiple strains associated with the cases (32).
As mentioned above, a case of STEC O157:H7 in a California woman was recognized during a retrospective analysis of serotyped strains, which were associated with HC (184). Similar retrospective analyses by the Canadian and the United Kingdom Public Health Laboratories found six isolates and one isolate, respectively (59,109). Although recognized previously, the STEC O157:H7 serotype appears to have been rare. Armstrong et al. offered three broad hypotheses for emergence in humans: 1) emergence of the bacteria in animal populations, 2) prior existence of the bacteria in animal populations with changes in slaughter and meat handling practices contributing to increased contamination of food products, and 3) prior existence of the O157:H7 serotype in the meat supply with changes in consumer eating habits responsible for increased infection rates (2). Certainly, changes in the cattle industry or changes in consumer habits could have impacted the emergence of a new pathogen occupying a newly created ecological niche.

Phenotypic and genotypic ancestry have been evaluated in an attempt to find evidence of a progenitor non shiga-toxigenic E. coli. Virulence factors of STEC O157:H7 and phenotypic variations appear to have been acquired over time making STEC O157:H7 distinct from other E. coli. Clonal relationships among isolates implicate a mechanism by which these organisms have been widely dispersed geographically, with eventual expansion of highly conserved, but genotypically distinct subpopulations. Some authors have suggested that the mechanism for clonal dispersion throughout most of the world could be via cattle or other ruminant populations that serve as a reservoir for STEC O157:H7 (228).

STEC that occur in ruminant populations can be epidemiologically related even if they are serologically diverse (18). This supports a mechanism for horizontal transfer of virulence factors between STEC that might have resulted in the more pathogenic STEC
O157:H7 serotype. Some have proposed that specific serotypes of STEC may exist preferentially in ruminant populations (18,19). Previously mentioned changes in population dynamics, production practices or consumer preferences could lead to dissemination of such a novel clonal pathogen into the human population.

Markers of genotypic and phenotypic variation, such as acquisition of \textit{stx}, loss of sorbitol fermentation, and loss of beta-D-glucuronidase (GUD) activity, are typical of the common clone of STEC O157:H7 found throughout the world (122). Molecular techniques used to detect genetic similarity among O157:H7 isolates from a variety of sources and geographic locations show marked homogeneity. Some authors have suggested that the serotype may have recently descended from an ancestral cell (228). Multilocus enzyme electrophoresis and nucleotide sequencing studies of genomic DNA support the concept of sequential emergence from a progenitor cell. These studies suggest that O55:H7 (an enteropathogenic \textit{E. coli}) and O157:H7 originated from a progenitor cell, then acquired \textit{stx2} prior to divergence with subsequent loss of GUD activity and sorbitol fermentation by STEC O157:H7 (71,122,211,229). The acquisition of \textit{stx1} and other virulence factors are hypothesized to have come after acquisition of \textit{stx2} (2,211).

Evidence for diversity within the STEC O157:H7 serotype group was demonstrated by the existence of phylogenetically distinct subpopulations. Pulsed-field Gel Electrophoretic patterns (PFGE) demonstrated genotypic variability among human outbreak strains, strains from ruminants, and strains from food (1,8,106). Stx encoding bacteriophages have been suggested as the mechanism for much of the genetic differences. Bacteriophage insertion, plasmid introduction, and transposon rearrangement may account for genotypic changes that
occurred during the temporal variation and emergence of STEC O157:H7 from STEC or non-STEC progenitors (18,121).

1.2.2. Pathogenesis

1.2.2.1. Putative Virulence Factors

Possession of stx1, stx2, stx1 and stx2, or other stx2 variants characterize STEC in general. Other putative virulence factors are also key components in producing the pathogenesis recognized in STEC O157:H7 infections. EHEC strains are characterized by expression of stx, the ability to cause AE lesions in intestinal epithelial cells, and the possession of a 60–Mda plasmid (pO157) encoding a hemolysin (155). Typically, EHEC are considered human clinical isolates, although some authors use the term to describe non-clinical or field isolates that possess the aforementioned virulence factors. Field isolates from ruminants in the following studies will be referred to as STEC with virulence factor characterization thus avoiding the clinical connotation of EHEC.

STEC O157:H7 has a 35-43 kb pathogenicity island called the Locus of Enterocyte Effacement (LEE) that encodes a type III secretion system, a translocated intimin receptor (TIR), and an outer membrane protein (OMP) termed intimin. All three are involved in attachment to intestinal epithelial cells (167,205). The eae gene, located in the LEE, encodes the 94-97 kDa intimin that promotes intimate attachment of the bacterium to the mucosal epithelial cells resulting in AE (155). AE is characterized by effacement of intestinal epithelial cell microvilli, intimate adherence of the bacterium to epithelial cells, and marked mucosal cell cytoskeletal changes forming a pedestal that cups the bacterium (167,204). The ability of STEC O157:H7 to produce AE lesions is probably sufficient to cause non-bloody
diarrhea (155). Deletion and complementation studies of eae have indicated that intimin is necessary for human intestinal colonization by STEC O157:H7 (74).

Hemolysin production is plasmid encoded in a highly conserved plasmid designated pO157 (27,155,195). Termed enterohemolysin, it is found in most EHEC O157:H7 strains associated with HUS and in STEC O157:H7 field isolates from ruminants (88,133,155). Enterohemolysin is encoded by a sequence that shares significant homology with hemolysin A that is found in uropathogenic strains of E. coli (155). It is hypothesized that a synergistic effect of enterohemolysin and stx may exist, although the mechanism is unclear (195). STEC O157:H7 is able to utilize heme and hemoglobin promoting bacterial growth in vitro. The role of enterohemolysin is unclear, although it lyses erythrocytes, which provides a mechanism for iron acquisition and subsequent bacterial growth (133,155,195). Enterohemolysin is one of several hemolysins encoded on pO157 and is classified as an RTX toxin that acts by a pore forming mechanism with specific tropisms for their target cells (155). RTX toxins may also play a role in cellular messaging that can affect thrombocyte numbers (155).

Stx, intimin, and enterohemolysin are considered the primary virulence factors because they are the most studied and well defined. Other putative virulence factors may be equally important in STEC O157:H7 pathogenesis, including other hemolysins, other intestinal adherence factors, and O157 lipopolysaccharide (LPS) (155).

1.2.2. Human Disease

As mentioned, the clinical spectrum of disease caused by STEC O157:H7 is broad. Infections in humans may be asymptomatic, present as watery diarrhea, HC, TTP, or HUS. Many cases of non-bloody or watery diarrhea are thought to be unreported. Patients
experiencing mild to moderate abdominal discomfort and watery diarrhea are frequently not recognized as cases because they do not present themselves for examination and therefore a stool sample is not submitted for culture. In 1993, the CSTE recommended that clinical laboratories screen at least all bloody stool samples for STEC O157:H7 with SMAC agar. To evaluate compliance with the recommendation, a national survey was conducted eighteen months later. Only 54% (70/129) of clinical laboratories were following the guidelines (24). Lack of compliance with new recommendations or a lag in implementing new diagnostic techniques will affect the quality of surveillance data and potentially result in underestimates of true disease incidence.

Clinically recognized manifestations of HC, TTP, and HUS are associated with and attributed to infection with STEC O157:H7 (10,115,184). The course of infection, following ingestion of STEC O157:H7, results in clinical symptoms from one to eight days post-infection. The average incubation period has been reported to be three days. STEC O157:H7 are thought to colonize the colon by intimin mediated AE. Patients initially develop abdominal cramping and watery diarrhea. A variable percentage of these patients’ diarrhea resolves without progression to HC. In mild disease, without bloody diarrhea, patients suffer less abdominal pain, vomiting, fever, and have a reduced chance of developing HUS (86).

The percentage of patients reported to develop bloody diarrhea varies and apparently depends on the ability to estimate the size of the exposed population. Estimates from sporadic cases and widespread outbreaks indicate that 70–95% of case patients have blood in their stools. The amount of blood can range from occult positive or a few visible streaks, to the entire stool specimen being composed of blood suggesting gastrointestinal hemorrhage (25,87,149). In confined outbreaks, where the numbers of people in the exposed population
were known, the percentage of case patients with bloody diarrhea was lower (87). This reduction probably reflected the recognition or diagnosis of cases with non-bloody diarrhea and asymptomatic cases that were not normally detected, thus increasing the size of the denominator in the case attack rate. Vomiting was reported in 30–50% of cases and fever, usually low grade, was detected in 30–40%. Blood in the feces becomes apparent two to three days after infection and resolution of HC generally requires six to eight days. Ninety-five percent of the HC case patients do not have significant sequelae (10,86,87,149).

Laboratory assays and physical examinations are often not helpful in making an early diagnosis. Clinical findings of abdominal pain, abdominal cramping, bloody stools, and the absence of fever are non-specific. Right-sided colonic inflammation has been documented by barium enema and colonoscopy. The observed clinical findings are similar to those seen with intussusception, inflammatory bowel disease, appendicitis or ischemic colitis. These non-specific findings have led to misdiagnosis and unnecessary surgery in some cases (86,201). Radiographic findings may show an ileus or a “thumbprinting pattern” in the ascending (right) and transverse colon consistent with submucosal hemorrhage and edema. Colonoscopy has documented pseudomembranes, ulcerations, and erythema/edema the entire length of the colon (10,25,149).

Histopathologic examination of biopsies and post-mortem tissues have demonstrated infectious or ischemic patterns of injury in the colon that are unevenly distributed and have occasional fibrin microthrombi (10,201). Initial lab work of case patients may show an elevated leukocyte count. Reports are conflicting regarding the presence of fecal leukocytes, some studies have found at least ten per high powered field while others describe fecal leukocytes as an infrequent finding (149,201).
One report evaluating STEC O157:H7 infection compared clinical signs or symptoms to *Salmonella*, *Campylobacter*, and *Shigella* (other commonly isolated enteric pathogens) in a logistic regression model (201). The goal of this hospital-based study was to describe clinical and epidemiological features of the infection. Significant clinical manifestations associated with STEC O157:H7 infection, when compared to the other enteric pathogens, included a history of bloody diarrhea (OR, 18.6 [CI, 7.4 to 48.6]), visibly bloody stool specimens (OR, 8.1 [CI, 3.6 to 18.3]), lack of fever (OR, 8.3 [CI, 1.6 to 50.0]), an elevated leukocyte count (OR, 4.0 [CI, 1.7 to 9.5]), and abdominal tenderness on physical examination (OR, 2.9 [CI, 1.2 to 7.2]) (201). These findings are consistent with other reports from sporadic cases and outbreaks.

Progression to post-diarrheal HUS occurs in a small percentage of STEC O157:H7 cases. Estimates of the rate of HUS development vary, but it is probably 2–7% among sporadic cases and possibly as high as 20% in outbreaks (11,25,87,149). Higher estimates observed during investigations of widespread outbreaks may be biased because undetected cases were not included in the rate denominator (11). Around 90% of HUS cases are associated with diarrhea prior to development of HUS (142). HUS has three classic components: microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure (10,115,149). The annual incidence of HUS in the U.S. and in Canada is approximately three cases per 100,000 in children less than five years old (142,186). HUS is considered the leading cause of acute renal failure in children, and there are few therapeutic options. Hemodialysis and supportive care are used to treat children with HUS. Other treatments have not been efficacious in reducing severity or in preventing complications in patients. One study found that no intervention actually decreased the risk of HUS (9). Mortality from HUS
is 3–5%. Approximately 5% of HUS patients develop chronic renal failure, and 30% experience other complications (25,142,149,198,199).

Other complications of HUS include acute neurological complications: stroke, seizure, lethargy, hemiparesis, decerebrate posturing, and coma (10,149,168). Pancreatitis, diabetes mellitus, pleural effusions, and pericardial effusions occur as rare complications. The long-term sequelae of HUS include cholelithiasis, colonic stricture, chronic pancreatitis, glucose intolerance, and cognitive impairment (149). Multiple systems can be affected and the range of symptoms and complications can be non-specific, complicating quick diagnosis.

TTP is a diagnosis sometimes given to patients with STEC O157:H7 infection. Clinically, it is characterized by hemolytic anemia, thrombocytopenia, neurologic disease, renal disease, and fever (87). TTP includes most of the clinical findings found in HUS with the exception of prodromal diarrhea and less pronounced renal impairment. Neurological findings are more pronounced in TTP case patients. Diagnosis of TTP occurs more frequently in adults and is probably the same syndrome as HUS when caused by STEC O157:H7 (10,25,149).

Risk factors for HUS were investigated in several studies with variable results. Factors associated with development or increased severity of HUS included presence of bloody diarrhea, fever, vomiting, elevated serum leukocyte count, absent or weak P1 antigen expression on erythrocytes, extremes of age (young and old), being female, bacterial toxin genotype, use of antimotility agents, and antimicrobial intervention (9,10,25,87,142,149). The increased risk of HUS associated with bloody diarrhea/HC is consistent with stx/AE mediated intestinal epithelial cell damage and subsequent translocation of stx into the bloodstream. Evaluating the potential for use of interventional pharmaco-therapeutics,
antimotility and antimicrobial drugs, is important because they are indicated in patients with diarrhea secondary to bacterial gastroenteritis.

Current opinion on the efficacy of antimicrobial agents is mixed. It is known from *in vitro* studies that exposure of STEC O157:H7 to antibiotics increases the release of stx. In fact, some antimicrobials, mitomycin for example, are used to enhance stx expression *in vitro* for diagnostic purposes. If sublethal or bacteriocidal antibiotic effects *in vivo* cause induction of *stx* prophages or lysogenic release of stx, it is plausible that toxin would be liberated in the intestine after antibiotic administration to patients. This could result in an increased risk of HUS. In a retrospective cohort of Washington State children, the use of antimotility agents was associated with development of HUS while antimicrobial use showed no association with HUS. The authors suggested that the data provided evidence against the use of antimotility agents to slow peristalsis (9). Neither agent had the desired effect of significantly reducing the duration of illness (9).

Another study in children indicated that use of antimicrobial agents was strongly associated with development of HUS, although this study did not find an association between antimotility agents and HUS. The authors concluded that antimicrobial use is contraindicated in STEC O157:H7 infection (234). Additionally, antimicrobial agents that inhibit DNA synthesis (new quinolones, trimethoprim and mitomycin) have been reported to increase toxin production *in vitro* and *in vivo*. Furthermore, treatment with mitomycin, a cancer chemotherapeutic, was associated with an increased incidence of HUS in cancer patients (146). More research utilizing prospective studies is necessary regarding clinical parameters and potential therapeutics that may be associated with or contribute to the development and severity of HUS.
As mentioned previously, some strains of STEC O157:H7 have stx1, stx2 or both. An association between the HUS/TTP complex and human clinical isolates that have stx2 only or stx1 and stx2 has been described in several studies (87,164). These studies suggested that stx2 has a more prominent role in HUS/TTP development than stx1. The previously mentioned data regarding stx2 in animal models is also consistent with stx2 having an essential and prominent role in the pathogenesis and clinical manifestations of disease caused by STEC O157:H7.

1.2.2.3. Ruminants

STEC O157:H7, like other STEC, is not a major cause of disease in ruminants. Ruminants, particularly cattle, are colonized by the O157:H7 serotype without clinical disease manifestations. It does not appear to be invasive or cause sepsis and is apparently a normal transient constituent of the colonic flora (57,216). As mentioned previously, research with non-O157 STEC indicate that calves may develop diarrhea that can be associated with AE lesions (108,143,196). Experimental work with cattle and sheep has been done and has demonstrated that STEC O157:H7 infection or colonization does not routinely result in clinical disease in healthy calves or adult cattle (57,216,235). STEC O157:H7 strains have been demonstrated to be pathogenic to gnotobiotic piglets, caesarian-derived colostrum-deprived (CDCD) piglets, and colostrum-deprived neonatal cattle (60). High doses administered to weaned three to four-month old cattle resulted in the development of AE lesions in the cecum and rectum (61). Experimental infection of six-day old sheep produced AE lesions in the cecum, colon, and rectum (221). The significance of the pathology caused by experimentally induced infections is unclear at this time.
A study comparing eae-positive and eae-negative strains of STEC O157:H7 supported the hypothesis that intimin is required for colonization, production of AE lesions, and pathogenicity in colostrum-deprived neonatal calves (62). In vitro and in vivo studies of colonic and rectal mucosa from eighteen-month old steers and piglets demonstrated AE lesions. The authors suggested that colonization of the large intestine mucosal epithelium might be critical in the colonization, carriage, and persistence of a carrier-shedder state in adult cattle (4).

Stx is the defining virulence trait in human disease, however, its role in ruminant colonization or carriage is unknown, and no known function in ruminant disease has been shown in experimental infections. The receptor for stx (Gb3) has been reported in colostrum-deprived neonatal calf kidneys, adult cattle kidneys, and brain tissue, but was not detected in the vasculature (174). A recent study reported that Gb3 receptors for stx are located in the bovine ileum, jejunum, cecum, and colon, independent of animal age (101). STEC O157:H7 did not cause fluid accumulation or infiltration of neutrophils in ligated mid-ileal loops of calves in one study, however, a non-O157 STEC with specific stx1, eae, and tir mutations did cause pathology (207). This suggests a mechanism that is not intimin or stx1 dependent in some STEC.

Intimin (AE lesion formation) has a role in the initial colonization of STEC O157:H7 in ruminants. Intimin’s role in the hypothesized carrier-shedder state is unclear. Intermittent shedding is documented in multiple studies; but a carrier state has not been proven at this point. Interaction or synergy between stx, other putative virulence factors, undescribed colonizing factors, and intimin could occur during intestinal colonization in adult cattle.
Bovine field isolates and human clinical isolates have been shown to differ genetically and in their expression of protein. An octamer-based genomic scanning method that used geographically and temporally diverse strains demonstrated two distinct lineages in the United States (121). Human and bovine isolates were described as non-randomly dispersed separate lineages. Significant differences in human clinical isolates and bovine field isolates have been demonstrated in the production and secretion of LEE-encoded virulence factors (148). The authors of these studies suggest that human and bovine isolates may be separate lineages and that only a few STEC O157:H7 bovine isolates are capable of causing human disease. More studies are needed to determine if separate lineages exist or if the differences observed were secondary to other factors associated with passage through a susceptible human host.

The importance of STEC O157:H7 infection or colonization in ruminants is well established. Pre-harvest epidemiology in ruminant populations is an area of great interest. In the following sections, methods for culture, presumptive identification, characterization, and sub-typing of STEC O157:H7 from ruminants will be reviewed. The pre-harvest epidemiology of STEC O157:H7 in dairy cattle, beef cattle and White-tailed deer will be described. Dairy cattle and beef cattle comprise the largest production systems for domestic ruminants in the U.S., while White-tailed deer have been implicated in foodborne outbreaks as well as being considered as a potential environmental reservoir for STEC O157:H7.
1.3. STEC O157:H7 Methods Utilized in Ruminant Field Studies

1.3.1. Culture Methods, Presumptive Identification, and Characterization

A variety of culture methods have been described and used in multiple field studies and epidemiological surveys of ruminants. Culture of ruminant feces for a specific serotype is challenging because an enormous amount of background flora exists. Excellent reviews have been written that summarize various culture methods used in different studies (2,153).

Combining several culture techniques has increased the sensitivity of culture methods to find the target organism (81). Culture methodology is continually changing in an effort to increase sensitivity and specificity of serotype identification, while reducing workload and expense. Detecting low levels of STEC O157:H7 is considered important because of the small infective dose required to cause human disease, thus qualitative rather than quantitative microbiological methods have been emphasized in field studies (153). The current methods described in the literature for microbial culture of bovine samples have evolved from direct plating techniques. As mentioned previously, STEC O157:H7 does not rapidly ferment sorbitol, and the substitution of sorbitol for lactose in MacConkey agar has enabled simple and reliable presumptive identification of STEC O157:H7 colonies in human clinical samples and bovine diagnostic samples (31,47,145,184).

Current culture protocols thought to be most sensitive in STEC O157:H7 identification use three components. First, feces or rectal/surface swabs are placed in selective enrichment broth that is supplemented with inhibitory antibiotics. Second, immunomagnetic separation (IMS) of the sample follows selective enrichment. Third, the post-IMS samples are plated on SMAC agar that is supplemented with selective antibiotics and biochemicals. Sorbitol-negative colonies are selected or “picked.” Sorbitol-fermenting
strains of STEC O157 from cattle are missed by this culture methodology. These strains are thought to be rare in the U.S., although they cause human disease in Germany and other parts of Europe and are described in cattle (20,208).

The broth enrichment of samples is intended to increase the number of target organisms while inhibiting the competing background flora. Protocols in the literature have reported the use of a variety of enrichment media (Trypticase soy broth (TSB), modified E. coli broth (mEC), and Buffered peptone water (BPW)) combined with one or more inhibitory antibiotics (novobiocin, cefixime, cefsulodin, vancomycin) (153). Results from the selective broth enrichment steps appear to be similar and increase the sensitivity of STEC O157 identification as compared to direct plating (127,147,189,237). Following broth enrichment, detection rates of STEC O157:H7 differ between studies using IMS and those not using the IMS method.

The IMS system (Dynabeads anti-\textit{E. coli} O157; Dynal Biotech, Lafayette Hill, Pa.) is a bacterial capture mechanism that was developed by covalently attaching O157-specific antibodies to magnetisable, superparamagnetic, uniform polystyrene beads. In the presence of a magnetic apparatus, the beads are magnetized allowing the bead-bacteria complex to be separated from the remainder of the sample (208). IMS has been demonstrated to be more sensitive in detecting STEC O157 than direct plating or molecular methods in studies of human clinical samples, food samples, and bovine samples (47,152,155,208). In addition to an increase in sensitivity, which is equal to or better than other highly sensitive methods (including PCR assays), the method is simple and leads to isolation of the bacteria that can be further characterized.
It is difficult to compare studies that did not use IMS to recent studies that have utilized the technique. Some authors suggested that the IMS procedure is four times as sensitive as direct plating (47). Others have stated that IMS was a more sensitive method than broth enrichment, although not at statistically significant levels (189). Furthermore, another study using IMS recognized a sevenfold increase in prevalence of positive samples compared to direct plating and estimated that the IMS method could detect one organism per gram of feces (99,153). A United States Department of Agriculture (USDA) culture protocol, using selective enrichment and IMS, was found to isolate STEC O157:H7 from fecal samples more than fivefold greater than a direct plating technique using split samples (118,130). The incorporation of improved detection methods, selective enrichment and IMS, have resulted in more frequent isolation and higher prevalence estimates than previously recognized in studies not using these techniques (67,81).

SMAC is considered the isolation medium of choice (152,208). SMAC containing cefixime and potassium tellurite was most often used to plate selectively enriched bovine samples with or without IMS (153). Cefixime targets *Proteus* spp., a sorbitol non-fermenter, which can result in false positive colony picks based on phenotype (46). Potassium tellurite is used to inhibit or eliminate growth of other *E. coli* and *Aeromonas* spp. in favor of STEC O157:H7, which demonstrates a higher mean inhibitory concentration than competing fecal flora (236).

The inability of STEC O157:H7 to produce GUD was used to discriminate between isolates and can be tested for by using the substrate 4-methylumbelliferyl-β-D-glucuronide (MUG) in plating media or as a separate assay (153,155,208). Selective differential plating media, which include fluorogenic and chromogenic agars, have not been extensively tested.
However, some comparisons indicated that they performed better than SMAC on food samples (15,16,144). Use of new plating media and modifications in culture protocols will occur that further optimize the selective isolation of STEC O157:H7.

Few studies have been done that directly compared the efficacies of the various published identification methods. Culture-related factors influence the sensitivity and specificity of the culture methods resulting in variable estimates of STEC O157:H7 prevalence in cattle. The amount of feces, handling of the fecal sample, the number of suspect colonies selected for presumptive identification, and the use of IMS have a significant influence on prevalence estimates (130,153). Protocols that culture larger fecal samples (>10g) have been associated with higher animal prevalence estimates. This observation is corroborated by a direct comparison of rectal swab sampling and 10 gram fecal samples (130). Direct comparison of the methods used to handle samples provided evidence that refrigeration or freezing of fecal samples prior to culture can cause microbial death and reduced prevalence estimates (130,147).

Determining the required number of colonies to select that will be used to identify isolates and subtypes is difficult (200). A larger number of colony picks (10-15 per sample) enhances detection when non-O157, non-sorbitol fermenting flora exists on plating media (153). The number of colonies selected for identification can strongly influence the correct identification of epidemiologic relationships in samples (173).

Selective enrichment, IMS, and plating media have been refined to increase detection of STEC O157:H7. Presumptive identification of suspect isolates from plating media, beyond phenotypic characteristics, was carried out by a variety of methods. Serologic and biochemical methods are used to confirm colonies as STEC O157:H7. Most of the currently
available immunological assays detect either O and H antigens or stx (155). Antisera to O157 LPS and H7 antigen are utilized in a variety of diagnostic tests that include enzyme-linked immunosorbent assays (ELISA), latex reagents, colloidal gold-labeled antibody assays, and others, most of which are available commercially (152,208). Production of specific monoclonal antibodies for use as diagnostic research and commercial reagents has been reported for O157 LPS, H7 antigen, stx1, and stx2 (97,141,227). Development and use of stx assays and diagnostics are becoming more important as non-O157 STEC disease is increasingly recognized.

Biochemical confirmation was performed as well. Strains of several bacterial species cross-react with O157 antiserum (152,227). Polyclonal antisera may cross-react with E. coli serotypes O7, O116, and other bacteria (Escherichia hermannii, Brucella abortus, Brucella melitensis, Yersinia enterocolitica serotype O9, Stenotrophomonas maltophilia, Citrobacter freundii, and group N Salmonella O30 antigen) (208,227). In fact, a subset of the O30 antigen group N Salmonella have the same O antigen structure as STEC O157:H7 and cross-react with O157-specific monoclonal antibodies (227). Previously mentioned discriminatory biochemical properties (sorbitol fermentation and GUD) have been incorporated into culture methods or tested by specific assays to confirm these unique properties of STEC O157:H7. Biochemical characterization should be performed to confirm isolates as E. coli by using commercially available test strips or automated systems (152).

Detection of cytotoxic activity, presumably by stx, was essential in the initial studies associating STEC infection to HUS (115). Testing cultures for cytotoxic activity involves cell culture techniques that use HeLa or Vero cells (155,208). Cytotoxin assays have largely been replaced by DNA-based methods that detect stx.
DNA-based methods for identification and further characterization include DNA specific probes used in colony hybridization assays and PCR assays. Genes and DNA sequences that have been studied to characterize presumptive STEC O157:H7 include eae (EA gene), stx (shiga toxin genes), uidA (O157 specific mutated GUD gene), a DNA sequence upstream of eae, the 60-Mda plasmid (pO157) and the hlyA that pO157 encodes (152,153,155,208). Studies on healthy cattle or carcass contamination have utilized different methods that incorporated DNA probes, PCR assays, or both on isolates derived from culture (153).

Using PCR for direct analysis of complex samples (e.g. fecal samples) can be problematic because of background flora and inhibitory factors (155). PCR assays, which have been described as multiplex reactions, can identify virulence factor genes and confirm serotype (76,78,166). PCR assays amplify genes encoding the O157 LPS (rfbO157) and H7 antigen (fliC) to detect or confirm serotype (72,166). Isolates sometimes are nonmotile (O157:NM) and require passage in motility media. Some exhibit motility but do not react serologically. One protocol using PCR and restriction fragment length polymorphisms (PCR-RFLP) has been described that characterizes fliC sequences that allow differentiation of flagellar antigen groups (72).

A DNA-based typing scheme has been suggested based on O157- and H7- specific primers that would allow sensitive and rapid detection of the O157:H7 serotype (224,225). As with culture methodology, characterization of isolates is constantly changing, particularly with molecular methods that identify homogeneous DNA sequences unique to STEC O157:H7.
1.3.2. Strain Typing Methods

STEC O157:H7 strain typing or sub-typing methods use phenotypic data, genentic data from DNA-based methods, and complex genotype analysis techniques (102). A strain is defined as an isolate or group of isolates that exhibit phenotypic or genotypic traits that are distinct from other isolates of the same species that have different traits (209). Strain typing is critical in identifying relationships among STEC O157:H7 isolates and in linking human cases to assist in identifying outbreaks (85). This is frequently referred to as “epidemiologic strain typing.” STEC O157:H7 is a target pathogen for molecular sub-typing in the National Standardized Surveillance and Cataloging Network maintained by the Centers for Disease Control. PulseNet utilizes pulsed-field gel electrophoresis to identify different strains and was established in 1998 (48). Studies in healthy cattle have used sub-typing techniques to evaluate the epidemiology of sub-types: within samples, from multiple samples acquired from an individual animal, within herds, and between herds (153).

Differentiation of sub-types is difficult due to the homogeneity and clonal nature of the O157 group (122,229). As mentioned, STEC O157:H7 strains appear to be a highly conserved group of clones with low genetic diversity (155). STEC O157:H7 sub-types do not show marked variation and may be considered “unrelated” based on a few genotypic or phenotypic criteria. To determine if isolates are indistinguishable or different, sub-typing methods must discriminate between related and unrelated isolates based on phenotypic or genotypic characteristics.

Phenotypic methods include phage typing, toxin phenotyping, and antibiotic resistance patterns. Genotypic methods use either whole genomic DNA or specific DNA components and include plasmid profiling, restriction fragment length polymorphism
analysis (RFLP), ribotyping, pulsed-field gel electrophoresis (PFGE), and random amplification of polymorphic DNA (RAPD) (102,155,208,209).

Phage typing is performed by applying lytic phages to isolates and interpreting the lysis pattern of the various phage stocks. The phage-typing scheme developed by Khakhria differentiates 82 sub-types (208). It is technically simple and has been standardized in reference laboratories. However, problems with phage typing preclude wide use of the method. These problems relate to the long-term stability, production, maintenance, and testing of the phage stocks. Additionally, the method is expensive, time-consuming, and some isolates are untypeable. A greater concern is that a majority of isolates fit into a few phage types limiting the discriminatory power of the typing system (155).

Toxin phenotyping also has limited discriminatory power because there are only 3 STEC O157:H7 toxin phenotypes. Isolates will possess stx1, stx2 or both. PCR to identify stx1 and stx2 is commonly used to characterize isolates and is sometimes used in conjunction with other typing schemes to enhance the discrimination of subtypes (155).

Antibiogram typing is a simple method to use, particularly in the clinical setting and for nosocomial infections (209). STEC O157:H7 isolates from the earliest outbreaks were pan-sensitive to antibiotic panels making discrimination of strains impossible (208). The recent emergence of antibiotic resistant strains has enabled the use of susceptibility testing for strain typing (102). Other phenotypic markers can be evaluated as well, including serotyping or biochemical profiling, but these are generally not as discriminatory as genotypic methods.

Genotypic strain typing methods are varied as well. It is important to note a distinction between DNA “fingerprinting” and strain sub-typing (208). Some authors have
proposed that DNA “fingerprinting” methods be considered as a comparative tool within a laboratory or a specific study. “Subtyping,” however indicates that the methods, reading, and interpretation are standardized between laboratories enabling comparison of sub-types. Despite these specific definitions, these terms are frequently used synonymously.

Plasmid profiling was utilized extensively for strain identification during the initial outbreaks of STEC O157:H7 in humans. Plasmids are separated electrophoretically on a gel and evaluated for differences in plasmid size and number. Strains can be further discriminated by use of restriction enzymes of particular plasmids (102). Recent comparative studies of discriminatory techniques indicate that plasmid profiling provides inadequate discrimination of strains (102,155,208,209).

RFLP methods cut the chromosome into hundreds of fragments using frequent-cutting restriction enzymes (209). Using a suitable DNA probe, labeled bacteriophage lambda DNA or stx genes for example, the restricted chromosome can be analyzed by Southern blotting techniques, thus enabling discrimination of genomic polymorphisms (102,208,209). RFLP is a sensitive and stable discriminatory method but requires several days and is labor intensive (208). Ribotyping is an RFLP method in which restricted DNA is probed with a plasmid containing the rRNA operon. This procedure uses Southern blotting to detect polymorphisms that depend on sequences that flank the rRNA operon (208). Ribotyping has not been demonstrated to be a successful discriminatory method, presumably because the rRNA genes are highly conserved (102,155,208).

RAPD, also called arbitrarily primed PCR, has been used successfully to distinguish between strains or DNA “fingerprints,” although reproducibility of results has limited the use of the technique for sub-typing (208). An 8-10 base primer of arbitrary sequence, which
anneals less stringently to the DNA, allows priming of imperfectly matched sequences. This technique is used to generate a hypothetically strain-specific array of amplicons (102). RAPD could become a useful sub-typing method if reproducibility is improved. Other PCR-amplification methods exist as well but none are generally accepted and commonly used.

PFGE is the method utilized for sub-typing by the CDC PulseNet members for selected foodborne bacteria (34,206). It is the most widely utilized sub-typing method for surveillance, outbreak investigation, and epidemiologic studies of bovine STEC O157:H7 field isolates (102,153,231). Other non-PulseNet protocols have been utilized in studies of healthy ruminants (7,22,153). In PFGE, macrorestricted DNA fragments, from bacteria embedded in agarose plugs, are separated in an agarose gel using a countour-clamped homogeneous field electrophoresis technique (CHEF) that applies a pulsed, alternating, orthogonal electrical field (102,208). In this technique less than 30 DNA fragments are generated. Ten to 20 fragments ranging in size from 20 to 700 kb result from cleavage with XbaI, a commonly used restriction enzyme in standardized protocols (102,209).

Comparative studies have suggested that PFGE is the most discriminatory sub-typing method in comparison to phage typing, plasmid profiling, and stx typing. PFGE is a reproducible technique and the results can be compared between laboratories (7,124,140). Its high discriminatory level detected differences between isolates that were considered to be clones by phage typing, RAPD, multilocus enzyme electrophoresis, and ribotyping (102). The number of variable bands that must be present to differentiate sub-types is unclear. Genetic events that can alter the PFGE banding profile include point mutations that create or destroy restriction enzyme sites, insertions or deletions of DNA, stx phage insertion, and gain or loss of plamids (22,213).
Tenover et al. suggested guidelines for interpretation of PFGE banding profiles used to determine strain relatedness with respect to outbreak investigations. These guidelines have been used for interpretation of non-outbreak strains as well (213). Differences exist in defining sub-types based on PFGE. Unlike Tenover et al., some authors have suggested that a single band difference may represent a different sub-type, particularly among epidemiologically unrelated isolates (102,208,232).

Differences in interpretation of PFGE do not diminish its role as a standardized and highly reproducible sub-typing method. Using multiple tests to determine sub-types has been suggested as a method to enhance discrimination (232). Performing PFGE with two restriction enzymes in parallel has been reported as a means to increase the discriminatory power when slight differences exist, but is more labor and time intensive (102,173). When applied to an epidemiological question, phenotypic characterization data and genotypic sub-typing methods (PFGE) are tools used in the epidemiological study rather than definitive stand-alone methods. A composite analysis should be utilized when possible to sub-type isolates in epidemiologic field studies.

1.4. Pre-Harvest Epidemiology in Ruminants

1.4.1. Dairy Cattle

The role of dairy cattle in the epidemiology of STEC O157:H7 disease in humans is multifaceted. Dairy cattle have been implicated as the contamination source for hamburger-related outbreaks because significant amounts of the ground beef produced in the United States originate from dairy cattle (69,87,147). Milk and milk by-products have also been implicated as vehicles responsible for foodborne outbreaks (87). Environmental contamination by fecal material and effluent run-off from dairy farms have been associated
with outbreaks, which were thought to be caused by drinking contaminated well water on a dairy farm and physical contact with contaminated pastures at a music festival (56,107).

STEC O157:H7 can be widespread in the dairy farm environment. Direct or indirect contact with dairy cattle during farm visits were associated with cases of disease (39,132,233). Retrospective analysis of case records in Wisconsin from 1992-1999 indicated that ground beef (5.8%) was associated with fewer infections than farm related exposure (13.4%), recreational water exposure (8.1%) or consumption of unpasteurized milk/dairy products (7.0%) (172). It is evident that STEC O157:H7 colonizing dairy cattle have multiple opportunities to infect humans. Furthermore, these organisms are at times ubiquitous in the dairy farm environment.

Pre-harvest studies evaluating STEC O157:H7 presence in dairy cattle have addressed the overall animal-level prevalence, herd-level prevalence, within-herd prevalence, and age-specific prevalence estimates within herds. Ecological assessment of isolates from within the dairy farm environment, evaluation of sub-types from within herds, and within animals has been performed. Longitudinal studies and risk factor analysis were conducted in an attempt to find temporal and management factors that are associated with fecal shedding. Differences in the sensitivity of culture methods make direct comparisons of these studies difficult.

Overall, cow-level prevalence estimates from studies using enrichment culture are low. In a review of STEC O157:H7 in healthy cattle, Meyer-Broseta et al. described on-farm studies in North America. Prevalence estimates in dairy cattle were commonly less that 1%, none being greater than 5%, and herd-level estimates range from 0-100% (153). Several studies documented an age-specific increase in prevalence among weaned calves and heifers (83,90,93,237). The age of weaned heifers in these studies was variable, but in general,
weaned heifers ranged from 2-3 months to breeding age (13-15 months). This same age group had the highest prevalence in studies that used more sensitive methods. Besser et al. stated that growing cattle (3-18 months of age) are more frequently colonized than pre-weaned calves or adults due to a less stable gastrointestinal flora in young animals (12,14). Because weaned calves or heifers are thought to have the highest prevalence among dairy cattle, they have been targeted in several studies to determine fecal shedding status. Prevalence estimates in this age group, using selective enrichment, were reported to be 1-10% (69,92,98).

Two national studies conducted by the United States Department of Agriculture-Animal and Plant Health Inspection Service (USDA-APHIS) and follow-up studies have focused on dairy cattle. Garber et al. and Zhao et al. reported results from case-control studies done as follow-up studies to the National Dairy Heifer Evaluation Project (NDHEP) (83,237). NDHEP tested fecals from 6,894 preweaned calves in 1,068 herds in 28 states and found 0.36% of preweaned calves and 1.8% of dairy herds shedding STEC O157:H7 (237). This survey was conducted from June 1991 to May 1992. The follow-up studies identified weaned calves and heifers as the most frequent shedders.

These studies demonstrated a broad geographic distribution of STEC O157:H7 on U.S. dairy farms. Most studies in the U.S. have been conducted in states or regions in the northern part of the country. Case- and control-herds changed status with repeated testing, indicating that fecal shedding is endemically unstable within herds of dairy cattle and that the herd shedding status cannot be determined by a single test (83). The instability of colonization or infection status in animals within herds makes risk factor studies very difficult to interpret.
USDA-APHIS conducted a national survey in 1996, NAHMS Dairy ’96, to evaluate management practices and cattle health in adult dairy cattle (82). A follow-up study, which used a subset of herds from NAHMS Dairy ’96, clearly identified a seasonal increase in fecal shedding among cattle and in the number of farms considered positive. This result corroborated other published data and was consistent with a seasonal increase (summer) that has been observed in the incidence of human disease (82,90). Risk factors for fecal shedding that were significant included season (summer) and the management practice of flushing alleys (82).

Univariate analysis of feed ingredients found a significant association between feeding cottonseed and fecal shedding of STEC O157:H7. This observation was in contrast to that of Hancock et al. which described a negative association between fecal shedding and feeding of cottonseed (82,90).

Higher prevalence of STEC O157:H7 was observed in herds from southern states compared to herds in northern states. This is the opposite distribution of human clinical disease cases, where more cases occur or are recognized in northern states (10,25,82,201). Interestingly, Kabagambe et al., utilizing the NAHMS Dairy ’96 herds in a similar study of Salmonella fecal shedding, found the same associations between alley flushing, feeding of cottonseed and geographic region (south >north) (111). Salmonella and STEC O157:H7 are both gram-negative facultative anaerobes that are found among the fecal flora, so perhaps it is not surprising that management strategies and potentially intervention strategies would have similar effects with regard to fecal shedding.

Risk factor studies have identified significant associations between various environmental and management factors and STEC O157:H7 fecal shedding, however,
agreement between studies is lacking. Other than the lack of a herd size effect, few statistically significant associations have been found with respect to feeds, feeding strategies, manure management, production indices, cattle management, and farm management systems that are consistently reproduced (91).

Environmental sampling on dairy farms demonstrated that STEC O157:H7 could be found throughout the dairy farm environment. It has been isolated from birds, flies, water, feed, and a raccoon (176,197). The organism appears to be common in the farm environment, particularly during periods when cattle are shedding. It appears to persist more frequently in the dairy cattle themselves in comparison to the dairy farm environment (12,176).

Studies using more sensitive methods have demonstrated higher prevalence estimates in dairy cattle than found in earlier studies that did not use more sensitive IMS methods. The majority of these studies have been conducted outside of the United States. Mechie et al. followed a single UK dairy herd, which was associated with human disease, for 15 months. In this study, the overall prevalence of positive samples was 4.3% (n=3593) and the maximum prevalence estimate at any one visit was 68% in the group of heifers on the farm (151). Shedding in lactating cows was found to be highest following calving and at 7 months post-partum. Isolates were obtained from the bulk tank and all isolates were identical based on phage type, toxin genotype, and plasmid profile (151).

Investigation of an Ontario dairy farm with IMS culture techniques, following a human STEC O157:H7 case associated with the farm, found the prevalence among heifers to be 56% (n=45) and 68% (n=50) among adult cattle (107). Findings in studies using more sensitive methods (i.e., selective enrichment and IMS) have been consistent with other
studies that have stated that heifers or young dairy cattle were more frequently colonized, and also demonstrate a seasonal increase in fecal shedding (53,100,132,151).

Lahti et al. in Finland and Heuvelink et al. in the Netherlands reported high herd-level prevalence using IMS in longitudinal studies, 100% (n=5) and 90% (n=10) of herds, respectively (100,132). The proportion of cattle shedding STEC O157:H7 varied over the course of these studies and ranged from 0%-66.7%. In the United States, Besser et al. described a herd-level prevalence of 40% (n=10) using less sensitive methods (13). Besser et al. used IMS on a subset of preserved postenrichment broth samples and found additional isolates that were not previously recognized.

Cull dairy cow studies that used IMS have been performed. Chapman et al. sampled dairy cattle presented for slaughter in the UK and found a prevalence of 16.1% (n=1661) with a spring and summer seasonal peak (44). Heuvelink et al. sampled seasonally, in consecutive years, and found a prevalence of 10.6% (n=540) among cattle presented for slaughter that were predominantly cull dairy cattle (99). These cull cow studies were in contrast to other studies performed that used less sensitive methods and showed prevalence estimates in cull dairy cows of 3.9% (n=103) and 1.3% (147,178).

Clonal analysis, using PFGE on isolates obtained from dairy cattle, has been performed in multiple studies. Twenty-six STEC O157:H7 isolates obtained from the NDHEP were enzyme restricted using SfiI and XbaI yielding 14 and 18 subtypes, respectively. The isolates were obtained from calves on 20 farms in 16 states (134). Isolates from cattle on the same farms had indistinguishable pulsed-field patterns. Isolates from studies of dairy herds have typically been restricted with XbaI because it has been
demonstrated to be the most discriminatory restriction enzyme (197). Results have been consistent between studies (69,100,132,197).

Predominant subtypes were endemic in dairy herds and in dairy farm environments during periods of colonization. Isolates from the same farm were frequently identical or very similar while isolates from different farms were usually different subtypes (69,99,132). Some variability and diversity of subtypes occurred within farms and within animals although fewer subtypes occurred on dairy farms compared to beef feedlots, presumably because the population was stable with little cattle movement (69,180).

Temporal variability of subtypes has been demonstrated in longitudinal studies. Predominant subtypes can change over time within dairy herds, however in one study subtypes were shown to persist for 2 years (100,197). Determining the number of subtypes within an animal or on a farm is a function of the number of colonies selected for culture and the subsequent number of cultures to which PFGE is applied.

1.4.2. Beef Cattle

Evaluation of the literature regarding STEC O157:H7 in beef cattle was difficult because several methods were used to identify the bacteria. Pre-harvest studies have focused on feedlot cattle. Few studies have evaluated cow-calf farms or weaned calves prior to co-mingling of cattle in feedlots. Prevalence studies have described the farm- or feedlot-level prevalence, pen-level prevalence in feedlots, animal-level prevalence, and site-specific prevalence on individual feedlot cattle. Associations between risk factors and fecal shedding have been made. These studies have not been productive in identifying methods that could be broadly implemented in reducing the prevalence in beef cattle. Clonal analysis, as in studies of dairy cattle, has been performed primarily with PFGE.
Initial prevalence estimates in beef cattle, specifically fed cattle, were low. U.S. feedlot cattle have generally been thought to be colonized by STEC O157:H7 at low animal-level prevalence and widely distributed among feedlots. Hancock et al. estimated that the animal-level prevalence in Washington State was 0.33% (n=600) among feedlot cattle (90). In the USDA:APHIS:VS National Animal Health Monitoring System (NAHMS) study, Cattle on Feed Evaluation (COFE ’95), fecal samples from 11,881 cattle in 100 feedlots were tested from October through December 1994 (94). Animal-level prevalence was estimated to be 1.8 %, with the organism detected in 63 % of feedlots. From Pacific Northwest cattle feedlots (n=6), Hancock et al. reported an animal-level prevalence of 3.6 %, with at least one positive in each feedlot (92).

As Meyer-Broseta et al. pointed out, studies that used more sensitive methods had higher animal-level prevalence estimates (153). Recent estimates have been 5 to 12 times higher than earlier estimates. Studies conducted in Europe, Canada, and U.S. feedlots that used IMS methods have consistently demonstrated higher animal-level prevalence estimates that ranged from 9.5% to 28% (5,49,67,202,220).

The NAHMS Feedlot ’99 study that was conducted during the summer of 1999, in feedlots with 1000 head or greater capacity from the twelve top feeding states, found an animal-level prevalence of 11% (n=10,415). An information sheet provided by APHIS suggested that the increased prevalence level recognized in their study as compared to the COFE ’95 study was due to: 1) use of more sensitive methods during Feedlot ’99 and 2) the winter sampling period during the COFE ’95 study (218).

The previously cited commentary by Gansheroff and O’Brien (81) that discussed the higher prevalence estimates in cattle referred to a study of Midwestern feedlot cattle that
were presented for slaughter (67). Elder et al. sampled twenty-nine lots of cattle and found that 72% had at least one fecal positive animal in the lot and that the overall animal-level fecal prevalence was 28% (n=327) (67). Feedlot cattle sampled at an Alberta, Canada abattoir that used the more sensitive methods detected STEC O157:H7 in 12.4% (n=654) of yearling cattle, of which 91% were from feedlots throughout western Canada (220). Another study, conducted in Midwestern U.S. feedlots (n=5), found 23% of cattle (n=3162) shedding the organism in the summer months, with at least one positive fecal in each of the twenty-nine pens in the study (202). Pen-level prevalence ranged from 0.7% to 79.8%.

In a study of finished feedlot cattle (n=139) known to be shedding STEC O157:H7 at high prevalence (>20%), Keen and Elder sampled multiple sites on each animal including the oral cavity, five hide surfaces areas, and a fecal sample. Surprisingly, the prevalence was highest in the oral cavity (74.8%) followed by the back hide (73.4%), neck hide (62.6%), feces (60.4%), flank hide (54.0%), vetrum hide (51.1%) and hock hide (41.0%) (119). Additionally, fifty fecal negative cattle among the 130 cattle that were positive from one or more hide sites or the oral cavity site were detected. Keen and Elder indicated that the commonly utilized fecal sample may underestimate the prevalence of cattle that are colonized by STEC O157:H7. Other studies have isolated viable organisms from the hide (5,67). Barham et al. sampled feedlot cattle and detected a hide prevalence of 18% that was greater than the fecal prevalence of 9.5% (5).

Samples taken from feedlots have indicated that STEC O157:H7 was seasonally ubiquitous in the feedlot environment and not only colonized the digestive tract of feedlot cattle (oral cavity, rumen and large intestine/colon) but also colonized or contaminated the hide surfaces (119,220). As in the dairy farm environment, STEC O157:H7 was not restricted
to or unique to the bovine host. It has been isolated from rodents, water troughs and water trough sediment samples as well as feed samples from feedbunks (49,91,202,219).

The features recognized in studies of feedlot cattle include a clearly defined seasonality (warm season) in fecal shedding that is consistent with studies from dairy cattle (82,90,218-220). COFE ’95 and Feedlot '99 recognized a trend in fecal shedding from pens of feedlot cattle that were on feed for variable amounts of time. Cattle on feed for a shorter amount of time had a higher prevalence of fecal shedding compared to cattle that had been on feed for longer periods (94,218). Smith et al. also examined number of days in the feedyard, but found no correlation with fecal prevalence (202).

Other factors examined by Smith et al. included water quality parameters, feed quality parameters, and production indices that were not correlated with fecal prevalence (202). Van Donkersgoed et al. found the presence of STEC O157:H7 in water troughs to be associated with rainfall the week prior to sampling, numbers of coliform bacteria, and E. coli counts in the water troughs (219). Interestingly, Smith et al. evaluated pen condition and found a positive correlation between fecal prevalence and progressively wetter pen conditions (202).

Studies investigating the prevalence of STEC O157:H7 in cow-calf farms and weaned calves prior to entry into the feedlot are rare. Different management conditions exist in cow-calf herds relative to feedlots, with cattle being dispersed over large areas of rangeland. Animals are congregated intermittently for management purposes, whereas, feedlot cattle are in close contact in a highly intensive production situation.

Hancock et al., using less sensitive methods, reported that among pastured beef cattle the animal-level prevalence was 0.71% (n=1412) with a herd-level prevalence of 16% (n=25)
Sargeant et al. used enrichment and IMS in a study of ten cow-calf farms in Kansas. The sample-level prevalence was 1.3% (n=3152 fecal samples) and the animal-level prevalence was 1.9% (n=2058 animals sampled) (190). All ten farms had one or more positive fecal samples and significant differences in prevalence between farms were not observed. STEC O157:H7 was also isolated from a farm pond (one sample) and streams (two samples). Although longitudinal in nature, the study did not recognize an increased seasonal prevalence.

Laegreid et al., using selective enrichment and IMS, sampled weaned calves in October and November prior to the entry of the animals into the feedlot (131). Fifteen cow-calf herds were sampled in Missouri, Kansas, Montana, Nebraska and South Dakota (131). Herd-level prevalence was 87% (n=15) and within the positive herds, the animal-level prevalence estimates ranged from 1.7–20% with a mean of 7.4%. Serum anti-O157 antibody titers were also determined by blocking ELISA (129). All herds showed serologic evidence of exposure to O157 antigen with seroprevalence ranging from 63–100%. These studies demonstrated that a large proportion of calves entering the feedlot had already been exposed to STEC O157:H7 and that calves from cow-calf farms destined for feedlots were colonized and shedding the organism upon arrival. Clonal analysis or sub-typing of isolates from beef cattle has been performed. Laegreid et al. used PFGE to analyze isolates obtained from weanling cattle in geographically distinct locations. Most herds demonstrated one PFGE pattern, although some herds and individual animals had isolates with more than one PFGE pattern (131).

As mentioned above, increased diversity of subtypes may occur secondary to animal movement. Rice et al. surveyed forty-one cattle farms and found 81 subtypes among the 376
isolates (180). Sub-types were found among dairy herds and beef cattle feedlots that were indistinguishable. Several farms with indistinguishable sub-types were separated by greater than 600 km.

A greater diversity of subtypes was recognized in feedlots compared to dairy farms. Common subtypes have been isolated in the feedlot environment from feed troughs and water troughs (219). Hancock et al. found feedlot non-bovine isolates of STEC O157:H7 in dogs, water troughs (including water trough biofilm), a pooled bird fecal sample and a pooled fly sample. Several of these isolates had PFGE subtypes that were indistinguishable from cattle isolates (92). At one feedlot, water trough isolates and isolates from a dog were indistinguishable.

From a subset of isolates, Keen and Elder recognized five distinguishable subtypes among fifty isolates recovered from twelve randomly selected cattle, as reported in the previously mentioned study (119). Similar subtypes were found in cattle that were spatially and temporally clustered within the same feedlot, although in non-adjacent pens. Genetic diversity of isolates was evident within pens and from individual cows; however, a single subtype predominated within each pen.

The clonal analysis of isolates obtained from the study performed by Elder et al. was performed with PFGE and reported in a separate paper (6,67). The authors utilized Dice similarity coefficients in conjunction with the “unweighted pair group method to derive arithmetic averages” (UPGMA) for cluster analysis and generation of a dendrogram. Preharvest isolates, including hide isolates, showed a greater amount of diversity than only the fecal isolates. Predominate subtypes were present within clusters of preharvest isolates.
that were derived from fecal as well as hide samples. The study also reported that among all isolates, genetic subtype clustering corresponded to isolate motility and stx profile (6).

### 1.4.3. White-Tailed Deer (*Odocoileus virginianus*)

The existence of a wildlife reservoir for STEC O157:H7 has been a possibility, in light of the lack of host specificity exhibited in studies of cattle (91). The organism has been isolated from multiple animals and sources in the farm environment, which suggested that a reservoir might exist that is external to cattle (153). White-tailed deer (*Odocoileus virginianus*; WTD) are recognized as a potential source for dissemination of zoonotic organisms in the environment and in watersheds (182). A few researchers have investigated the role of WTD in the epidemiology of STEC O157:H7. WTD or other wild ruminants may serve as a potential reservoir from which transmission to cattle could occur. Indeed, cattle and WTD often share rangeland and indistinguishable PFGE subtypes have been identified in WTD and cattle from the same farm (179).

Wildlife diseases could emerge and amplify in domestic livestock following a “spill over” from wildlife that come into contact with livestock (58). Intensive agricultural production practices coupled with changes in technology, industry, and consumer habits have been suggested as mechanisms for STEC O157:H7 emergence in humans (2,58). Not only could WTD play a role in the epidemiology of the pathogen in cattle, a direct human health risk exists for those who handle and consume WTD, including meat from the farmed deer industry (41,75).

Outbreaks of disease have been traced back to deer by several authors. A sporadic case and an outbreak of STEC O157:H7 were attributed to the consumption of contaminated venison (120,175). Keene *et al.* reported in an Oregon disease outbreak investigation that
STEC O157:H7 was associated with the consumption of Black-tailed deer jerky (120). Six cases and five presumptive cases were identified in the outbreak. Isolates from the cases had indistinguishable PFGE patterns that were the same as isolates from the leftover jerky, uncooked meat from the same deer, a meat saw used to butcher the deer, and fragments of the deer hide. STEC O157:H7 was also isolated from 9% of fecal samples (n=32) collected in a nearby forest.

A sporadic case reported by the Connecticut Department of Health directly linked venison consumed by a seven-year-old boy following a hunting trip by his father, to the STEC O157:H7 isolated from his stool (175). In this case, the isolates recovered from the boy and frozen venison from the same deer had identical PFGE digestion patterns using XbaI and BlnI. Another larger outbreak involved unpasteurized apple juice and implicated deer as a potential source of the STEC O157:H7 contamination in the orchards where the apples originated (52). The source of the outbreak was not confirmed, although, investigators hypothesized that laborers might have picked up apples contaminated with deer feces from the ground. STEC O157:H7 was isolated from deer feces in a nearby wildlife refuge although the PFGE pattern was different from the outbreak PFGE pattern.

Studies of STEC O157:H7 in WTD indicated that free-ranging WTD are colonized at low prevalence proportion. Rice et al. found 1.8% of WTD (n=108) and 2.6% of beef cattle (n=191) shedding the organism using the methods of Sanderson et al. (179,189). Seven cattle and WTD isolates were obtained that had indistinguishable PFGE patterns and identical stx profiles (stx2 only), which according to the authors, indicated that strain sharing between wild and domestic ruminants could play a role in maintaining the organisms in bovine populations. Furthermore, a WTD prevalence study in Kansas that used an enrichment and
IMS protocol on cow-calf farms, found that 2.4% of fresh WTD fecal samples (n=212) picked up from the ground were culture positive (191). The authors acknowledge that they could not be certain, despite specified sampling criteria, that each fecal collected represented an individual deer. Thus, repeated sampling of positive or negative deer may have biased the prevalence estimates.

The repeated sampling of trails and bedding areas on the farm may have biased the prevalence estimate by increasing the probability of sampling the same animals during the study. Additionally, the sampling period, which included multiple visits to two cow-calf farms, was primarily during the winter months (late September through April). While this would be the period that hunters would be at greatest risk, studies in cattle have demonstrated that fecal shedding of STEC O157:H7 during winter periods occurs less frequently, therefore, sampling during the winter may bias prevalence estimates in WTD. Seasonal shedding patterns have not been demonstrated in WTD.

Renter et al. individually identified and sampled WTD at harvest during the 1998-hunting season in southeastern Nebraska. The study was designed to determine distribution patterns and estimate prevalence for STEC O157:H7 (177). Fecal samples were collected by hunters and sent to the laboratory for culture on ice, which is a potential cause of microbial death (130). Results did not demonstrate a spatial or geographic pattern that was statistically significant. The animal-level prevalence estimate was 0.25% (n=1608). Again, the study was conducted during the winter, which is the period that hunters would be at greatest risk for infection, if WTD were colonized and shedding. The authors mentioned that the cross-sectional nature of the study might have underestimated the true prevalence if WTD shed the organism transiently as cattle do.
Experimental studies and field studies indicated that WTD can be colonized and shed STEC O157:H7. Fischer et al. orally administered a $10^8$ CFU cocktail of nalidixic acid resistant STEC O157:H7 strains to six three-month-old, O157-negative WTD (73). One strain was a deer isolate from the outbreak in Oregon, two were human isolates, and two were from cattle (120). Results from the experimental studies were similar to the results from studies in other ruminant species and the authors stated that the similarity suggests that fecal shedding in WTD may be transient.

STEC O157:H7 was recovered at necropsy throughout the entire digestive tract of the deer following oral inoculation. The abomasums, cultured at necropsy, were positive for STEC O157:H7 four days-post-infection (DPI) but not at eleven, fourteen, twenty-five, and twenty-six DPI. Two deer that were necropsied at twenty-five DPI had bacteria recovered only from the cecum, spiral colon, and descending colon. Necropsy samples from the digestive tract contents consistently had a greater population of bacteria than the corresponding washed tissue samples. Gross pathological lesions and adherent bacteria on mucosal surfaces were absent in the deer. Furthermore, AE lesions were not detected on histopathologic examination of tissue sections.

Horizontal transmission was demonstrated when STEC O157:H7 was isolated from an uninoculated deer that was co-housed with a deer shedding STEC O157:H7. By day two post-contact, the uninoculated deer shed STEC O157:H7 in its feces. Interestingly, eight STEC O157:H7 strains were isolated from two deer at the end of the contact transmission trial that were genotypically the same as the deer strain used in the original mixture of isolates (120). The authors suggest that some level of host specificity may account for this observation.
In the study by Fischer et al., data from field studies reported that the prevalence was 0% (n=310) in fresh deer fecal samples collected from the ground. Fecal samples collected from the ground and from hunter-harvested WTD were positive for STEC O157:H7 at a prevalence proportion of 0.6% (n=469) in 1997. In 1998, fecal samples from hunter-harvested WTD were taken at a site that was positive in 1997 and STEC O157:H7 was not detected (n=140) (73). The prevalence proportion of STEC O157:H7 in fecal samples from beef and dairy cattle at the same location in 1998 was 4.3% (n=305). STEC O157:H7 isolates recovered from the WTD in 1997 and the cattle in 1998 had differing PFGE patterns and stx profiles. WTD isolates possessed both stx1 and stx2 genes while the bovine isolates predominantly had the stx2 gene.

1.5. Indistinct Epidemiological Factors

1.5.1. Reservoirs

It is commonly thought that cattle, both beef and dairy, are the primary reservoir for STEC O157:H7 that cause human disease (73,87,95,155). Little doubt exists that cattle play a significant role in the epidemiology of STEC O157:H7. Cattle, in experimental and field studies, have been shown to remain culture positive from weeks to several months. Furthermore, in experimental situations, the bacterium can remain viable in feces for eleven weeks (26,54,197,216,223). It is debatable whether cattle are the primary reservoir or one of many reservoirs in the farm environment.

STEC O157:H7 has been isolated from sheep, deer, dogs, birds, flies, horses, asymptomatic humans, feed samples, water samples, and others. It would seem that the detection of the organism in the farm environment, particularly when cattle are shedding the organism in the feces, is a matter of sufficient sample size. O’Brien and Kaper stated that
because the majority of research has been conducted on cattle populations, the investigation of other potential animal reservoirs, which may play an important role has been limited (158). Furthermore, Hancock et al. contend that there is little or no evidence that host specificity exists or that the ecology of the organism is different in cattle compared to other animals from which it has been isolated (91).

Two hypotheses have supporting evidence regarding reservoirs of STEC O157:H7. The first hypothesis states that cattle and other ruminants are the primary reservoir and responsible for positive isolations from feed, water, and other species that may be contaminated or transiently colonized. Furthermore, some cattle are thought to be colonized by the organism below detection limits or with the organism sequestered within the digestive tract when fecal shedding is not detected. Transient and seasonal shedding patterns observed in cattle would be explained by this “carrier state” in which the organism is retained within the ruminant host but is not being shed into the environment.

Brown et al. suggested that in calves, the primary location of STEC O157:H7 is the omasum and rumen when fecal shedding is absent. They further state that some cattle may be predisposed to a carrier state status from unidentified factors (26). Other authors have documented the persistence of STEC O157:H7 fecal shedding in comparison to other E. coli, and suggested that carriage of the organism in the rumen, omasum, and reticulum may be responsible for STEC O157:H7 persistence in ruminants (54,73,187).

Armstrong et al. described a “rumen hypothesis” that suggested the rumen as the principal location for colonization rather than the distal gastrointestinal tract (2). In addition, dietary factors affecting rumen and colonic volatile fatty acid concentrations and STEC O157:H7 acid resistance are thought to influence carriage and shedding of the organism in
the feces (96,215). A chronic carrier state or colonization of the digestive tract during extensive periods when fecal shedding is not detected has not been recognized (91).

Hancock et al. proposed a hypothetical ecological model that suggested that STEC O157:H7 is widely dispersed in nature and can sporadically colonize any number of animal hosts including non-ruminant hosts (91). In this model, STEC O157:H7 was one of many transient E. coli strains that exist within the complex microbiological farm environment. Cattle, therefore, are considered another incidental host and are not unequivocally designated as the reservoir species (92). Cattle and non-bovine animals can provide mechanisms for transport of the organism between farms; however, the authors suggested that contaminated cattle feeds might contribute to spread between farms. Additionally water troughs, particularly water trough sediments, have been implicated and are thought to contribute to the maintenance of the organism in the farm environment even when cattle are not shedding and thought to be free of the organism (89,91,135).

1.5.2. Temporality

Seasonality of STEC O157:H7 infection in humans, cattle populations, and retail meats is documented. The seasonal phenomenon is recognized in Northern countries with a temperate climate (10). Summer peaks of human cases have been recognized since Ostroff et al. reported a seasonal peak during the first year of statewide surveillance for the organism in Washington state during 1987 (163). Wallace et al. reported that 52% of cases reported to FoodNet during 1996 and 1997 occurred during June, July, and August (222). Seasonal increases were also noted for Campylobacter and Salmonella, although their increase was not as prominent as the increase for STEC O157:H7. Summer peaks in contamination of retail meats have been reported as well (43).
The reason for the increased incidence of human disease during the summer is unknown. Factors such as handling of food and cooking procedures for ground beef may play a role (10,25). Some authors have suggested that the increase in human incidence may be secondary to increased outdoor cooking activities, which might increase the risk of transmission from consumption of undercooked meat. The similarity of seasonal patterns observed in cattle, retail meats, and human incidence provides strong evidence for the existence of the relationship between colonization of cattle and human disease incidence (89).

Increased summer prevalence in cattle was documented in U.S. beef feedlots and in dairy herds in Europe and Canada. These studies used sensitive detection methods and reported that the peak in prevalence of fecal shedding was frequently epidemic within the affected herd (100,218,220). Presently, it is not known if the risk factors that caused this seasonal fecal shedding relate to climatic factors like temperature, rainfall, photoperiod, or management factors. Management factors, seasonal farming practices, stress or endogenous variation (hormonal, core body temperature etc.) within ruminants could all play a role in seasonal clustering in cattle. Hancock et al. suggested that variation in the intake of water or moisture content of cattle feeds, combined with increased bacterial growth during warmer months might account for the observed seasonal patterns (91).

Recognition that predominate subtypes were isolated on farms during periods of epidemic fecal shedding suggested that horizontal transmission occurred (95). Seasonal fecal shedding may occur secondary to other seasonal phenomena, such as increases in insect populations (flies) or migration patterns (birds) that could affect the prevalence of STEC O157:H7 in the farm environment (95,100,197). Meyer-Broseta et al. suggested that the
well-described seasonal pattern might not be associated with climatic factors typically considered in a seasonal phenomenon. Instead, season may be a confounding effect from undescribed or unstudied risk factors that were responsible for the summer peak of STEC O157:H7 (153).

1.5.3. Geographic Distribution

Infection of humans occurs worldwide and has been reported in at least thirty countries and on six continents (10). Geographic variation in human incidence is generally accepted, although not well described. In the U.S., outbreaks and sporadic cases are reported more frequently in northern states as compared to southern states (10,25,87,201). This gradient, in which more cases occur at northern latitudes, is recognized on the North American continent. More cases are reported in Canada, than in the northern U.S., followed by the southern U.S. (87).

A U.S. study reported that STEC O157:H7 was isolated from fecal specimens in northern sites four times as frequently as in southern sites (10). Wallace et al. presented sentinel site data that indicates that regional differences in the incidence of human disease are not entirely due to reporting bias (222). Incidence rates differed by state, ranging from 4.2 cases per 100,000 in a northern state, Minnesota, to 0.2 per 100,000 in Georgia, a southern state. Table 1.2 presents the number of cases from 1996 to 2000 in three northern and three southern states as recorded by the CDC from the National Electronic Telecommunications System for Surveillance (NETSS) (33,35,36,38,40). The southern states listed in Table 1.2 have consistently fewer reported cases over the 5-year period than the northern states. Differences in population-adjusted rates during 2000 are similar to those described by
Wallace et al. (222). Population data for 2000 was obtained from the U.S. Census Bureau Website (http://www.census.gov/). The factors that account for such variation are not known.

Disease reporting bias, differences in human susceptibility, variation in food processing procedures, disparity in human food consumption preferences, different farm husbandry practices, non-homogenous STEC O157:H7 prevalence, and disparate carriage among cattle or other reservoirs are all potential variables that could play a role in geographic variation and the north-south gradient observed in the distribution of STEC O157:H7 cases. European countries have reported regional differences in the incidence of STEC O157:H7 in humans (89). In some European studies, a hypothesis has been suggested that correlates low STEC O157:H7 incidence rates in humans with lower rates of STEC O157:H7 colonization in cattle herds (89).

Because of the increased incidence of human cases in the northern tier of states in the U.S., most studies in cattle have been conducted in those states. There are few studies designed to describe geographic variation of STEC O157:H7 levels in cattle populations and very few studies have been conducted in southern states, other than those included in national surveys.

**Table 1.2. Population-adjusted Shiga-Toxigenic *Escherichia coli* O157:H7 human cases for selected northern and southern states, based on 1996-2000 surveillance case reports**

<table>
<thead>
<tr>
<th>Year</th>
<th>MI</th>
<th>IA</th>
<th>MN</th>
<th>AL</th>
<th>MS</th>
<th>LA</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000 (cases/100,000)</td>
<td>1.4</td>
<td>6.1</td>
<td>4.2</td>
<td>0.2</td>
<td>1.3</td>
<td>0.3</td>
</tr>
<tr>
<td>2000</td>
<td>141</td>
<td>180</td>
<td>212</td>
<td>10</td>
<td>39</td>
<td>15</td>
</tr>
<tr>
<td>1999</td>
<td>127</td>
<td>114</td>
<td>175</td>
<td>28</td>
<td>9</td>
<td>14</td>
</tr>
<tr>
<td>1998</td>
<td>117</td>
<td>93</td>
<td>209</td>
<td>24</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td>1997</td>
<td>152</td>
<td>114</td>
<td>199</td>
<td>14</td>
<td>7</td>
<td>18</td>
</tr>
<tr>
<td>1996</td>
<td>100</td>
<td>123</td>
<td>239</td>
<td>15</td>
<td>13</td>
<td>9</td>
</tr>
</tbody>
</table>
Results from these surveys are conflicting. Published studies indicate that most, if not all U.S. cattle herds are likely to be identified as colonized, when repeated sampling is performed during the summer months. Some authors have noted geographic variation in the prevalence of STEC O157:H7, while others have found no differences (2).

COFE and Feedlot ’99 demonstrated regional differences in fecal shedding. Results from COFE suggested that feedlots from southern latitudes are more likely to be positive than northern feedlots and that the animal-level prevalence may be slightly higher as well (94,217). Preliminary data from Feedlot ’99 indicated that the percent of samples culture positive was slightly higher in southern regions in comparison to northern regions (218). Regional differences in prevalence among dairy herds were not demonstrated in the surveys that were conducted in association with the NDHEP (82,237). Garber et al. reported a higher prevalence among dairy herds in the south in the Dairy ’96 study.

Few hypotheses exist that account for variation in cattle populations. Differences in the Brucellosis vaccination status of cattle have been suggested as a possible explanation for geographic variability (83,87). It has been proposed that use of B. abortus vaccine on cattle may provide some cross-protective immunity because of the similarity of O157 LPS and B. abortus antigens. Cattle in northern states are less commonly vaccinated than cattle in the southern states because brucellosis has been reduced or eradicated in those areas and vaccination for B. abortus prohibited. In theory, northern cattle would be more predisposed to colonization by STEC O157:H7 than cattle in southern states, where brucellosis and brucellosis vaccination are more common.

The hypothesis assumes that STEC O157:H7 prevalence in cattle is correlated to the increased number of human cases reported in northern latitudes of the U.S. In fact, little or no
data support this hypothesis (83). Rather, the limited studies performed thus far demonstrate the opposite distribution of STEC O157:H7 prevalence in cattle herds. Additional studies are needed to determine if brucellosis vaccination affects STEC O157:H7 colonization of cattle. It appears that cattle in southern latitudes may be colonized at a higher prevalence in contrast to the distribution of human clinical cases.

Because most studies in U. S. cattle have been confined to relatively small geographic areas in the northwestern and northcentral U.S., it is difficult to assess geographic variability. Meyer-Broseta et al., in her review of cattle surveys, suggested that no firm conclusions could be drawn regarding geographical variation or localization of STEC O157:H7 in healthy cattle (153).

1.6. Research Objectives

1.6.1. Inherent Weaknesses of Cross-Sectional Studies

The majority of the STEC O157:H7 studies performed in beef cattle, dairy cattle, and WTD have been cross-sectional study designs (CSSD). This type of observational or descriptive study should include a representative sample of the individuals in a defined population (185). In CSSD, cases (fecal shedders) and risk factors are simultaneously recorded at a single point in time, thus determining prevalent cases rather than incident cases. These studies are often referred to as prevalence studies and allow some exploratory evaluation of risk factors. The limitations and disadvantages of cross-sectional studies are that they are not ideal for rare diseases or diseases with short or highly variable durations (185,214). Additional disadvantages include incomplete control of extraneous variables, inestimable disease incidence, and indeterminate temporal relationships between risk factors and disease (cause and effect) (214).
Associations of variables with the diseased state are estimable in a CSSD, however, the estimate can only be considered to represent that point in time, thereby, providing a “snapshot” of the association. Whether or not the association has any causal relationship to the diseased state cannot be determined by CSSD. Measures of association include the prevalence ratio, a measure of the risk of being “diseased” when possessing a particular risk factor, and the prevalence odds ratio, which gives an indirect estimate of the relative risk (214).

As discussed above, STEC O157:H7 in cattle is generally thought to be transient in nature with seasonal peaks during the summer, thus it may be considered rare if sampling does not occur simultaneously with fecal shedding. Additionally, fecal shedding may occur for a short or variable period of time. Thus, one-time sampling in a CSSD is not ideal for studying fecal shedding in ruminants with STEC O157:H7 and should be considered exploratory and utilized to generate hypotheses (91,153). A CSSD must therefore be designed, implemented, and interpreted with respect to temporal shedding patterns; otherwise misclassification bias will be likely to occur. Obtaining a valid prevalence estimate is a function of the diagnostic sensitivity and specificity of methods. Sample handling and sensitivity of methods in STEC O157:H7 culture and detection were discussed previously.

The CSSD has numerous advantages over analytical or etiologic studies. A randomly sampled population can produce reasonably valid estimates of prevalence proportion, and the proportion of subjects exposed and unexposed to specified risk factors within the target population. Compared to Case-control or Cohort designs, the CSSD is less expensive and time consuming to implement. Additionally multiple associations with the diseased state can be investigated with exploratory analysis. Furthermore, a CSSD offers a relatively fast and
inexpensive method to obtain preliminary data that can then be used formulate hypotheses for design of etiologic studies. The objectives of this study: estimating the prevalence, describing the distribution, and evaluating the potential associations with STEC O157:H7 fecal shedding in Louisiana dairy cattle, beef cattle, and WTD; are well suited to the CSSD because of the exploratory nature of the study (there are no previously published studies from Louisiana of which we are aware) and limited resources to obtain preliminary data.

1.6.2. Specific Research Objectives

1. Estimate the prevalence of STEC O157:H7 fecal shedding in Louisiana dairy herds.

2. Describe seasonal patterns of shedding in a longitudinal study by following five dairy herds during a one-year time period.

3. Estimate the STEC O157:H7 point prevalence for samples from the dorsal hide and mouth of fecal-positive dairy herds.

4. Describe the genetic relatedness of STEC O157:H7 dairy isolates based on PFGE.

5. Estimate the point prevalence of fecal shedding in a cohort of weaned beef calves (n=450 calves) at the entry point into a back-grounding program and a time point following a forty-five-day feeding period prior to entry into the feedlot.

6. Describe the genetic relatedness of isolates from the weaned beef cattle using PFGE.

7. Estimate the point prevalence of STEC O157:H7 fecal shedding in wild Louisiana hunter-harvested WTD during the at-risk period for venison contamination by hunting and processing practices.

8. Estimate the point prevalence of fecal shedding of STEC O157:H7 in a captive herd of WTD by serial fecal culture for one year and describe trends in seasonal shedding patterns observed in that herd.
1.7. References


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CHAPTER TWO

SHIGA-TOXIGENIC *ESCHERICHIA COLI O157:H7* (STEC O157:H7) IN LOUISIANA DAIRY CATTLE

2.1. Introduction

Shiga-toxigenic *Escherichia coli* O157:H7 (STEC O157:H7) is an important human pathogen that can be transmitted by a variety of routes. Clinical case presentation is variable with patients exhibiting clinical signs that may include non-bloody diarrhea, hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (TTP) (20). Ruminants, particularly cattle, have been described as the primary reservoir of shiga-toxigenic *E. coli* including STEC O157:H7, from which humans become infected (4,39). Dairy cattle have been implicated as vehicles of foodborne, waterborne and direct contact transmission to humans. Hamburger-associated outbreaks have implicated dairy cattle as the STEC O157:H7 source because large amounts of ground beef produced in the United States originate from dairy cattle (15,21,36). Milk and milk by-products have also been implicated as vehicles responsible for foodborne outbreaks (21). Environmental contamination of fields with fecal material or effluent run-off from dairy farms has also been associated with outbreaks (11,29). Epidemiologic investigations have demonstrated subtypes of STEC O157:H7 from dairy cattle that were indistinguishable from human clinical enterohemorrhagic *E. coli* O157:H7 isolates (EHEC) (34,35).

Enigmatic questions regarding the epidemiology of human infection in the U.S. include 1) a seasonal increased incidence associated with the summer months and 2) an increased population-adjusted incidence in northern states compared to southern states (geographic variation) (7,21). Seasonal increases or epidemics of fecal shedding among cattle, including dairy cattle, have been demonstrated (23,25,37).
In cattle, the summer increase in fecal shedding appears to positively correlate with the peak incidence of STEC O157:H7 in people (23,40). This suggests a biologically plausible hypothesis for the increase in human incidence, namely, that increased prevalence in cattle provides greater exposure risk for humans during the summer months. Reasons for the geographic variation are unclear, and little effort has been spent investigating the difference in disease incidence. Bias in reporting, differential levels of immunity in the human population, and differential prevalence proportions in animal populations could contribute to the variability.

Geographic variation in prevalence among dairy cattle has not been extensively studied. Understandably, most studies in dairy cattle have been conducted in regions where human cases are commonly diagnosed. A similar hypothesis to the one proposed for seasonal variation might explain the geographic differences in human disease incidence, namely, that prevalence among cattle populations varies between northern states and southern states and positively correlates to the geographic distribution observed in the human incidence distribution. Studies have shown that cattle in all regions of the U.S. are colonized with STEC O157:H7 (8). However, Garber et al. indicated that southern herds were more frequently found to be shedding STEC O157:H7 than herds in northern states (18).

The prevalence proportion of dairy cattle, especially adults, colonized by STEC O157:H7 is thought to be low. Typically, cow-level prevalence estimates have been from 1-5% for heifers and <1% for adult dairy cattle. In a review of STEC O157:H7 in healthy cattle, Meyer-Broseta et al. described on-farm studies in North America. In the studies reviewed, prevalence estimates were commonly less that 1%, none being greater than 5%, and herd-level estimates range from 0–100% (38).
Less sensitive detection methods were suggested as a factor contributing to the low prevalence estimates. Meyer-Broseta et al. recommend the use of immunomagnetic separation (IMS) isolation techniques to enhance sensitivity. Using more sensitive methods (selective enrichment and IMS), recent studies in beef feedlot cattle have reported higher prevalence estimates than previously thought (17). Using selective enrichment and IMS, beef feedlot cattle have recently been shown to be colonized with STEC O157:H7 at a higher prevalence in the oral cavity and on various hide surfaces than in the feces (30). In addition, studies using more sensitive methods conducted in Canada and in Europe indicated that dairy cattle may also be colonized at higher prevalence than previously reported (25,29,34,37).

Mechie et al. followed a UK dairy herd for 15 months that was associated with human STEC O157:H7 clinical cases (37). The proportion of positive samples was 4.3% (n=3593), and the maximum prevalence estimate at any one visit was 68% in the group of heifers on the farm. Shedding in lactating cows was found to be highest following calving and at 7 months post-partum. All isolates were identical based on phage type, toxin genotype, and plasmid profile. Further evidence from an investigation of an Ontario dairy farm with IMS culture techniques, following a human STEC O157:H7 case that was associated with the farm, found the prevalence proportion to be 56% (n=45) among heifers and 68% (n=50) among adult cattle (29). Studies of dairy cattle in the U.S. have primarily used less sensitive detection methods (38).

Epidemiologic studies have identified factors associated with STEC O157:H7 fecal shedding in cattle, but agreement between studies is lacking. The studies determined that there was not an association with herd size and fecal shedding of STEC O157:H7. Other than the lack of a herd size effect, few statistically significant associations have been consistently
reproduced among studies between STEC O157:H7 fecal shedding and the types of feeds, feeding strategies, manure management, production indices, cattle management or farm management systems (22).

Pulsed-field gel electrophoresis (PFGE) of STEC O157:H7 XbaI restricted DNA has been used to evaluate genetic variability of isolates from dairy cattle. XbaI appears to be the most discriminatory restriction enzyme for STEC O157:H7 (26). Results have been consistent between studies. Predominant subtypes of STEC O157:H7 were endemic within dairy herds and in dairy farm environments during periods of cattle colonization. Isolates from the same farm were frequently identical or very similar, while isolates from other farms were usually different subtypes (15,25,34,44). Limited variability and diversity of subtypes occurred within farms and within animals (15,43). Temporal variability of subtypes has been demonstrated in longitudinal studies. Predominant subtypes can change over time within dairy herds, although in one study, the same subtypes persisted for two years (25,44).

Louisiana’s dairy industry is relatively small by national standards, ranking thirtieth in the number of dairy cows in 1998 (50). However, concerns have been raised regarding surface water contamination by dairy cattle because the cattle are concentrated in areas where the level of human activities and water tables are high. One study suggests that dairy cattle may be a significant source of fecal coliforms, particularly *E. coli*, in contaminated surface water from watersheds associated with dairy farms (12).

Louisiana, like most southeastern states, has not reported a large number of human cases of STEC O157:H7 relative to northern states, and STEC O157:H7 in Louisiana dairy cattle has not previously been described. To describe STEC O157:H7 epidemiology in Louisiana, cross-sectional studies, using sensitive methods were designed to: 1) estimate
cow-level and herd-level point prevalence (PP) of STEC O157:H7 fecal shedding in Louisiana dairy cattle (PP study), 2) describe seasonal shedding patterns in a longitudinal dairy (LD) study of 5 herds sampled quarterly during 2001 (LD study) and 3) estimate the site-specific point prevalence in the oral cavity, on the dorsal hide surface and in the feces of Louisiana dairy cattle (MHF study). Isolates from these studies were confirmed, and putative virulence factors were characterized. Pulsed-field gel electrophoresis (PFGE) patterns were compared visually to discriminate between subtypes within and between herds.

2.2. Materials and Methods

2.2.1. Dairy Herds and Sampling Protocol

Dairy farmers enrolled in the Dairy Herd Improvement Association (DHIA) were contacted by Louisiana Cooperative Extension Services personnel to identify dairy farms willing to participate in the study. From the group willing to participate, thirteen herds were identified and enrolled. All thirteen dairy herds were sampled during June, July, and August of 2001. Sampling for the PP study was conducted during the summer months to increase the probability of detecting herds that were shedding STEC O157:H7. In the LD study, five herds were sampled quarterly from February to October (2001). Two positive herds that had relatively high fecal prevalence in the PP study were sampled to estimate fecal, dorsal hide and oral cavity prevalence of STEC O157:H7 in the MHF study. Data regarding fecal shedding did not exist in twelve of the study herds. One herd had been sampled once during the previous year and was positive (one positive animal).

Adult cattle in the lactation string were sampled to determine point prevalence estimates in the PP study. The number sampled per herd was determined based on the number of lactating cattle in the herd. Assuming randomization, a number sufficient to call
the group of lactating cattle negative at the 95% confidence level, assuming 3% prevalence, were sampled. Similar sampling criteria were used for the longitudinal study and sampling in the MHF study.

Adult cattle from four lactation strata were targeted in the longitudinal study: dry cows, early lactation (<100 days in milk (DIM)), mid-lactation (100-200 DIM), and late lactation (>200 DIM). We assumed a 305-day standard lactation period. Cattle were chosen in order to obtain a cross-sectional estimate of prevalence among all adult cattle on the farm and explore differences in fecal shedding between the groups. Depending on the dairyman’s preference and facilities for restraining cattle, the samples were collected prior to or shortly after milking.

Approximately 20 g of feces were collected per rectum with a new palpation sleeve for each cow and maintained at ambient temperatures during transport to the laboratory. The sampling protocol for the MHF study was based on previously described methods (30). Dorsal hide samples and oral cavity samples were collected from restrained cattle with two sterile gauze swabs (7.6 x 7.6 cm swabs) placed together and moistened with approximately 5 ml of sterile water. Approximately 500 cm² was uniformly sampled across the dorsal midline at the base of the neck. Oral cavity samples were obtained using long-handled obstetric forceps that were flame sterilized with alcohol. Buccal surfaces, buccal and lingual gingival surfaces and, the tongue were uniformly sampled. Swab samples were immediately placed in media.

2.2.2. Culture Methodology

Because the sensitivity of methods significantly influences the magnitude of prevalence estimates, culture techniques were selected that have demonstrated higher
sensitivity than conventional enrichment and direct plating techniques (10,17,32,38,48). The culture techniques have been described previously (13,30).

Ten g of fresh bovine feces were incubated at 37° C for 6 hours in 90 ml of gram negative broth (Difco, Sparks, MD) supplemented with cefsulodin (10 mg/l, Sigma, St. Louis, MO), vancomycin (8mg/ml, Sigma, St. Louis, MO) and cefixime (0.05 mg/ml, Lederle, Pearl River, NY). Swab samples were placed in 20 ml of 1.5x (60g/l) brilliant green 2% bile broth (Becton Dickinson, Cockeysville, MD) and incubated at 37° C for 6 hours. Immunomagnetic separation (IMS) was performed on a 1 ml aliquot using Dynabeads anti-\textit{E. coli} O157 uniform, paramagnetic, polystyrene microscopic beads that have adsorbed and affinity purified antibodies against \textit{E. coli} O157 covalently bound to the surface (Dynal Inc., Lake Success, NY).

The immunomagnetic bead/broth suspension was then washed three times with 1 ml of phosphate buffered saline containing 0.05% Tween 20 (Sigma, St. Louis, MO) (PBS-tween20) following capture of the paramagnetic beads using a magnetic particle concentrating separation rack (Dynal Inc., Lake Success, NY). Following the final wash the bead/bacteria complex was resuspended in 100 \( \mu \)l of PBS-Tween20.

An aliquot of 50 \( \mu \)l of the bead/bacteria complex was spread plated on sorbitol MacConkey agar (ctSMAC) containing cefixime (0.05mg/l) and potassium tellurite (2.5mg/l) and incubated at 37° C for 18-24 hours. A maximum of three colonies with typical STEC O157:H7 phenotypic characteristics were selected as suspects and placed into 5 ml of MacConkey broth and 2 ml of trypticase soy broth (TSB) for 18-24 hours at 37° C.
2.2.3. Confirmation by Enzyme-Linked Immunosorbent Assay (ELISA)

Indirect enzyme-linked immunosorbent assay (ELISA) was used for identification of isolate serotype (13,30). The ELISA was performed using murine monoclonal antibodies (MAb) to H7 antigen, anti-H7 MAb 2B7, and O157 antigen, anti-O157 MAb 13B3 (24,51). Briefly, 1 ml aliquots of the TSB and MacConkey broth cultures were heat killed by placing them in boiling water for 10 minutes. Positive control (stx-deficient \textit{E. coli} O157:H7; ATCC 43888), negative control (\textit{E. coli} O78:H11; ATCC 35401), and samples were transferred to 96-well plates and incubated at 37° C for one to two hours. Following incubation, the plates were washed with ELISA wash buffer. MAb 13B3 or MAb 2B7 was then added, followed by incubation at 37° C for 10 minutes, and wash steps. A horseradish peroxidase labeled goat anti-mouse antibody was added followed by incubation and wash steps. One component ABTS (2,2’-azino-di (3-ethyl-benzthiazoline-6-sulfonate); KPL, Guilford, UK) substrate was then added and allowed to react for ten to twenty minutes at room temperature before stopping the reaction with 1% SDS (sodium dodecyl sulfate). Absorbance was read at dual wavelengths of 405/490. Reactions were considered positive at 0.2 + the OD of the negative control.

Isolates reactive with MAb 13B3 (O157 positive ELISA) were inoculated in TSB and evaluated for motility using phase-contrast microscopy. Isolates that had the correct phenotype (1-2 mm, sorbitol-negative colonies) on ctSMAC, fermented lactose in MacConkey broth (yellow color change), positively reacted with anti-O157 MAb 13B3, and positively reacted with anti-H7 MAb 2B7 or were non-motile (NM) were considered to be \textit{E. coli} O157:H7 or \textit{E. coli} O157:NM.
Additionally, a subset of isolates were tested by Sensititre gram-negative (AP80) autoidentification plates (Accumed International, Westlake, OH) and confirmed as *E. coli* by reactions to thirty-two substrates. Sensititre identification of these isolates indicated that they were MUG-negative and confirmed them as sorbitol-negative (biochemical properties characteristic of STEC O157:H7). A single isolate from each positive sample was archived in a brain heart infusion (BHI) broth-glycerol suspension at –80° C for future characterization.

### 2.2.4. Characterization by Polymerase Chain Reaction (PCR)

Isolates were characterized by polymerase chain reaction (PCR) for *rfb*O157 and *fliC*H7, and the putative virulence factors *stx*1, *stx*2, *eae*A, and *hly*A. Somatic (O157), flagellar (H7), and virulence factor gene sequences were amplified using previously published primer pair sequences (16,41). Duplex reactions were run for *stx*1 and *stx*2, as well as *eae*A and *rfb*O157. PCR for *hly*A and *fliC*H7 were run as uniplex reactions. PCR cycling conditions were as previously described (16,41).

Amplified gene products were subjected to electrophoresis on a 2% agarose gel and then stained with ethidium bromide. Gel images were captured digitally, photographed and scored. Clear, well-defined bands of the correct size for *rfb*O157 (259 base pairs (bp)), *fliC*H7 (625 bp), *stx*1 (180 bp), *stx*2 (255 bp), *eae*A (384 bp) and *hly*A (534 bp) that were consistent with the positive control were considered positive PCR reactions. A well-characterized STEC O157:H7 strain was used as the positive control (ATCC 43895). A 100 bp DNA ladder was used as the standard. E coli O157:H7 or O157:NM isolates were considered to be STEC O157:H7 or STEC O157:NM if PCR reactions for *stx*1, *stx*2 or both were positive.
2.2.5. Pulsed-Field Gel Electrophoresis (PFGE)

Each STEC O157:H7 isolate was analyzed with enzyme restriction and PFGE. Previously described methods were used (33). Summarizing, isolates were grown in Luria-Bertani (LB) broth to an approximate optical density (OD) of 0.8. Chloramphenicol was added to the cells for approximately one hour followed by centrifugation and resuspension in cell suspension buffer. Pulse-field quality agarose was used to make a 2% agarose solution. Equal volumes of the cell suspension and agarose were gently mixed and plugs (agarose embedded DNA) were cast. Plugs were allowed to solidify, placed in buffer and sequentially subjected to lysozyme and proteinase K digestion. Plugs were washed (4x) and phenylmethylsulphonyl fluoride (PMSF) was used to inactivate residual proteinase K.

For restriction enzyme digestion, XbaI was selected. However, following digestion of the plugs with 50 units of XbaI in XbaI buffer, electrophoresis revealed that enzyme digestion did not occur or was incomplete. XbaI enzyme digestion failed on two subsequent attempts as well. A second enzyme, SpeI (New England Biolabs, Beverly, MA), a six base cutter, was used to digest duplicate plugs in NEBuffer 2 (New England Biolabs, Beverly, MA). Following digestion with 50 units of SpeI, plugs were rinsed and loaded on a 1% agarose gel along with DNA size standards and a standard STEC O157:H7 strain (ATCC 43895). Following PFGE separation of SpeI-restricted DNA, gels were stained with ethidium bromide.

The SpeI-restricted DNA fragment patterns were analyzed visually. DNA size standards, MidRange I/II and lambda ladder PFG markers (New England Biolabs, Beverly, MA) were used to compare fragments from gel to gel. Genetic relatedness of isolates within herds was determined for epidemiologically related isolates according to the criteria of
Tenover et al. (49). Isolates differing by more than 2 bands in epidemiologically unlinked herds (between herds) were considered separate subtypes. Several authors have suggested strict criteria for determining subtypes in epidemiologically unlinked isolates because STEC O157:H7 is thought to be a highly conserved clone (5,6,26,48).

2.2.6. Statistical Analysis

Prevalence estimates with 95% confidence intervals (CI) were calculated by dividing the number of positive samples by the total number of samples. (PEPI 4.0) Fisher’s exact statistics were used to evaluate the contingency tables because of the sparse data in some cells (StatExact3). The longitudinal study data were analyzed using exact chi-square statistics and a logistic regression model (SAS v8.2 PROC GENMOD) to estimate the effect of herd, sample date (season), and lactation status on the probability of STEC O157:H7 fecal shedding. Likelihood ratio statistics were computed as tests of the effects for inclusion in the model. Data were entered and stored in Excel 2000.

A logistic regression model using the generalized estimating equations (GEE) was used to adjust for correlation between observations (cattle) within specific herds that were repeatedly measured. Correlation in the longitudinal panel data implies that animals sampled from the same herd over time are correlated or more alike than animals from different herds (31). This correlation within herds over time may result in instability of parameter estimates. GEEs are used in generalized linear models (GLM) to account for correlated or repeated measures data in a longitudinal or clustered study design (27). The working correlation matrices used were the independent and exchangeable models (28). SAS v8.2 was used with the REPEATED option in PROC GENMOD. Wald statistics were evaluated to estimate the effect of lactation and season in the model. Proposed analogs for the score statistic that are
available in SAS v8.2 were also requested (2). Data were collapsed into fewer categories and analyzed based on the results of the initial models. Parameters for lactation and season were dichotomized (lactation: yes or no, season: warm or cold) in the analysis.

2.3. Results

2.3.1. Point Prevalence Study

STEC O157:H7/NM were isolated in each of the studies conducted in Louisiana during 2001. Cow-level and herd-level point prevalence estimates and the exact 95% CI for STEC O157:H7 fecal shedding in the PP study are shown in Table 2.1. Confidence intervals were estimated using exact methods to generate CIs for negative herds. Contingency table analysis using the exact methods indicated that STEC O157:H7 fecal shedding status varied significantly by herd (Fishers exact=93.3, df=12, p<0.05).

Table 2.1. Point prevalence of Shiga-Toxigenic Escherichia coli O157:H7 in Louisiana adult dairy cattle sampled from the lactation string in thirteen herds (summer 2001)

<table>
<thead>
<tr>
<th>Herd</th>
<th>Date</th>
<th>Herd size</th>
<th>Target n</th>
<th>Prevalence</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6/4/01</td>
<td>80-90</td>
<td>55</td>
<td>0/55=0%</td>
<td>0.0 to 6.5</td>
</tr>
<tr>
<td>2</td>
<td>6/4/01</td>
<td>80-90</td>
<td>55</td>
<td>0/54=0%</td>
<td>0.0 to 6.6</td>
</tr>
<tr>
<td>3</td>
<td>6/13/01</td>
<td>80-90</td>
<td>55</td>
<td>18/52=34.6%</td>
<td>22.0 to 49.1</td>
</tr>
<tr>
<td>4</td>
<td>6/25/01</td>
<td>240-250</td>
<td>90</td>
<td>11/100=11%</td>
<td>5.6 to 18.8</td>
</tr>
<tr>
<td>5</td>
<td>6/25/01</td>
<td>27 Census</td>
<td></td>
<td>0/26=0%</td>
<td>0.0 to 13.2</td>
</tr>
<tr>
<td>6</td>
<td>6/25/01</td>
<td>130-140</td>
<td>60</td>
<td>0/63=0%</td>
<td>0.0 to 5.7</td>
</tr>
<tr>
<td>7</td>
<td>7/3/01</td>
<td>130-140</td>
<td>60</td>
<td>10/63=15.8%</td>
<td>7.9 to 27.3</td>
</tr>
<tr>
<td>8</td>
<td>7/9/01</td>
<td>130-140</td>
<td>60</td>
<td>0/60=0%</td>
<td>0.0 to 6.0</td>
</tr>
<tr>
<td>9</td>
<td>7/9/01</td>
<td>130-140</td>
<td>60</td>
<td>11/61=18.0%</td>
<td>9.4 to 30.0</td>
</tr>
<tr>
<td>10</td>
<td>7/17/01</td>
<td>130-140</td>
<td>60</td>
<td>2/66=3.0%</td>
<td>0.4 to 10.5</td>
</tr>
<tr>
<td>11</td>
<td>7/23/01</td>
<td>80-90</td>
<td>55</td>
<td>0/55=0%</td>
<td>0.0 to 6.5</td>
</tr>
<tr>
<td>12</td>
<td>7/23/01</td>
<td>130-140</td>
<td>60</td>
<td>0/61=0%</td>
<td>0.0 to 5.9</td>
</tr>
<tr>
<td>13</td>
<td>8/20/01</td>
<td>130-140</td>
<td>60</td>
<td>0/75=0%</td>
<td>0.0 to 4.8</td>
</tr>
</tbody>
</table>

Cow level prevalence estimate   52/791= 6.6%    4.9 to 8.5
Herd level prevalence estimate  5/13=38.5%    13.9 to 68.4
Cow level prev. (among + herds) 52/342=15.2%   11.6 to 19.5
2.3.2. Mouth, Hide, and Fecal Study

Results of the MHF study are summarized in Table 2.2. The mouth, hide, and fecal site-specific estimates and 95% CIs are shown. One animal was hide-positive, and no isolates were obtained from the oral cavity swabs. The PFGE pattern from the hide isolate could not be distinguished from the STEC O157:H7 isolate from that cow’s feces or other isolates from the same herd.

Table 2.2. Mouth, hide and fecal site-specific prevalence estimates and the associated exact 95% confidence interval for the mean of Herd A and Herd B

<table>
<thead>
<tr>
<th>Site</th>
<th>Herd A</th>
<th>Herd B</th>
<th>Total</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouth</td>
<td>0/58</td>
<td>0/77</td>
<td>0/135=0%</td>
<td>0.0 to 2.7</td>
</tr>
<tr>
<td>Hide</td>
<td>1/58=1.7%</td>
<td>0/77</td>
<td>1/135=0.7%</td>
<td>0.0 to 4.1</td>
</tr>
<tr>
<td>Feces</td>
<td>12/58=20.6%</td>
<td>22/77=28.6%</td>
<td>34/135=25.2%</td>
<td>18.1 to 33.4</td>
</tr>
</tbody>
</table>

2.3.3. Longitudinal Dairy Study

Longitudinal study data consisted of four cross-sectional samples in the adults (milking cows and dry cows) from five study herds. Three of the five herds (60%) in the longitudinal study had at least one positive sample at multiple time-points, while two herds remained negative throughout the year. Chi-square analysis, using exact chi-square statistics, was performed for fecal shedding status and compared shedding rates by herd, sample date (February, May, July, October), and lactation status (dry, <100 dim, 100-200 dim, >200 dim).
Figure 2.1. Prevalence of fecal shedding during the 2001 longitudinal study, five dairy herds were sampled quarterly. Error bars indicate the Exact 95% confidence intervals for the proportion of cattle shedding Shiga-Toxigenic *Escherichia coli* O157:H7 in their feces.

Fecal shedding differed significantly by herd (Fishers exact=32.0, df=4, p<0.0001) and sample date (Fishers exact=18.9, df=3, p<0.001). Fecal shedding did not differ significantly by lactation status (Fishers exact=3.2, df=3, p=0.36) or when lactation was dichotomized as dry cow or lactating cow (Fishers exact=2.7, df=1, p=0.13). Figure 2.1 is a graphical depiction of fecal shedding prevalence among adult dairy cattle sampled during 2001. Point estimates for cattle fecal shedding in all herds are shown as well as the estimate from positive herds.
Table 2.3. Logistic regression models for estimation of sampling month effect and lactation days in milk (LactDIM, four categories) effect on the dichotomous outcome of Shiga-Toxigenic *Escherichia coli* O157:H7 fecal shedding in five Louisiana dairies

<table>
<thead>
<tr>
<th>Variable (p val a)</th>
<th>Parameter</th>
<th>OR 1</th>
<th>SE 2</th>
<th>OR 95% CI 3</th>
<th>p value c</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Model A: Categorized variables</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herd (&lt;0.0001)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Month (&lt;0.0001)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>October (ref)</td>
<td>1.00</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>February</td>
<td>1.00</td>
<td>1.43</td>
<td>0.06</td>
<td>0.06 - 16.7</td>
<td>0.9919</td>
</tr>
<tr>
<td>May</td>
<td>15.42</td>
<td>1.06</td>
<td>1.9</td>
<td>1.9 - 124.4</td>
<td>0.0102</td>
</tr>
<tr>
<td>July</td>
<td>13.35</td>
<td>1.08</td>
<td>1.6</td>
<td>1.6 - 111.1</td>
<td>0.0165</td>
</tr>
<tr>
<td>Lactation DIM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0.2121)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry cow (ref)</td>
<td>1.00</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>&lt;100 DIM</td>
<td>5.31</td>
<td>0.87</td>
<td>0.96</td>
<td>0.96 - 29.4</td>
<td>0.0556</td>
</tr>
<tr>
<td>100 to 200 DIM</td>
<td>2.96</td>
<td>0.85</td>
<td>0.6</td>
<td>0.6 - 15.5</td>
<td>0.1998</td>
</tr>
<tr>
<td>&gt;200 DIM</td>
<td>3.26</td>
<td>0.84</td>
<td>0.6</td>
<td>0.6 - 16.7</td>
<td>0.1560</td>
</tr>
<tr>
<td><strong>Model B: Dichotomized variables b</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herd (&lt;0.0001)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Season (&lt;0.0001)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cold (O/ F) (ref)</td>
<td>1.00</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Warm (M/J)</td>
<td>13.25</td>
<td>0.75</td>
<td>3.0</td>
<td>3.0 - 58.0</td>
<td>0.0006</td>
</tr>
<tr>
<td>Lactating (Y/N)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0.0566)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry cow (ref)</td>
<td>1.00</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Lactating cow</td>
<td>3.60</td>
<td>0.77</td>
<td>0.8</td>
<td>0.8 - 16.3</td>
<td>0.0981</td>
</tr>
</tbody>
</table>

a Likelihood ratio (Type 3 analysis) statistics for inclusion of variable in the model
b Dichotomization of Month from model A based on test of significance for May and July betas, May and July not significantly different from each other
c p value for Wald test
1 OR=odds ratio; 2 SE=standard error; 3 CI=confidence interval

Tables 2.3 and 2.4 present the results of the logistic regression model. Table 2.3 shows the logistic regression results with herd, date (four categories), and lactation status (four categories) in the model (Model A) and a collapsed model (Model B) with dichotomized date (warm or cold season), and dichotomized lactation status (dry or lactating). The herd effect is controlled for by its inclusion in the logistic regression model, although the models ignore potential correlation due to repeated sampling of the same herds. The confidence intervals for the odds ratios for the herd effect were extremely large because of the herds that were negative throughout the sampling period.
Table 2.4. Generalized estimating equations logistic regression for estimation of sampling month effect and lactation days in milk effect on the dichotomous outcome of Shiga-Toxigenic *Escherichia coli* O157:H7 fecal shedding in five Louisiana dairies

<table>
<thead>
<tr>
<th>Variable</th>
<th>Parameter</th>
<th>OR&lt;sup&gt;1&lt;/sup&gt;</th>
<th>SE&lt;sup&gt;2&lt;/sup&gt;</th>
<th>95% CI&lt;sup&gt;3&lt;/sup&gt;</th>
<th>p val&lt;sup&gt;b,c&lt;/sup&gt;</th>
<th>OR&lt;sup&gt;1&lt;/sup&gt;</th>
<th>SE&lt;sup&gt;2&lt;/sup&gt;</th>
<th>95% CI&lt;sup&gt;3&lt;/sup&gt;</th>
<th>p val&lt;sup&gt;b,c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Model A: Categorized variables</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Month</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>October (ref)</td>
<td>1.00</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>1.00</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>1.00</td>
</tr>
<tr>
<td>February</td>
<td>0.90</td>
<td>1.42</td>
<td>0.1-15.4</td>
<td>0.9730</td>
<td>1.00</td>
<td>1.27</td>
<td>0.1-12.7</td>
<td>0.9704</td>
<td></td>
</tr>
<tr>
<td>May</td>
<td>12.40</td>
<td>1.23</td>
<td>2.8-138.1</td>
<td>0.0411</td>
<td>12.10</td>
<td>1.16</td>
<td>1.2-117.2</td>
<td>0.0316</td>
<td></td>
</tr>
<tr>
<td>July</td>
<td>11.50</td>
<td>1.28</td>
<td>0.9-138.2</td>
<td>0.0592</td>
<td>10.70</td>
<td>1.20</td>
<td>1.0-113.1</td>
<td>0.0494</td>
<td></td>
</tr>
<tr>
<td><strong>Model B: Dichotomized variables</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Season</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cold (O/F) (ref)</td>
<td>1.00</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>1.00</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>1.00</td>
</tr>
<tr>
<td>Warm (M/J)</td>
<td>11.90</td>
<td>0.93</td>
<td>1.9-74.3</td>
<td>0.0079</td>
<td>10.90</td>
<td>0.87</td>
<td>2.0-60.2</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>Lact (Y/N)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry cow (ref)</td>
<td>1.00</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>1.00</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>1.00</td>
</tr>
<tr>
<td>&lt;100 DIM</td>
<td>3.6</td>
<td>0.94</td>
<td>0.6-22.8</td>
<td>0.1679</td>
<td>3.80</td>
<td>0.82</td>
<td>0.7-18.8</td>
<td>0.1081</td>
<td></td>
</tr>
<tr>
<td>100 to 200 DIM</td>
<td>2.70</td>
<td>0.20</td>
<td>1.9-4.1</td>
<td>&lt;0.0001</td>
<td>2.60</td>
<td>0.17</td>
<td>1.9-3.7</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>&gt;200 DIM</td>
<td>3.10</td>
<td>0.32</td>
<td>1.6-5.7</td>
<td>0.0004</td>
<td>2.90</td>
<td>0.28</td>
<td>1.7-5.0</td>
<td>0.0001</td>
<td></td>
</tr>
</tbody>
</table>

* Working correlation structure: <sup>a</sup>
  - Independent: working correlation matrix not estimated
  - Exchangeable: matrix estimated with constant correlation between any two observation times

<sup>b</sup> P value of Wald statistics shown in table

<sup>c</sup> P values of score statistics for Type 3 GEE analysis are not shown, score tests for inclusion of the variable in the model were not significant (p>0.09) constituting a reversal in conclusion in the LR GEE analysis, see discussion.

<sup>1</sup> OR=odds ratio; <sup>2</sup> SE=standard error; <sup>3</sup> CI=confidence interval

Likelihood ratio statistics for inclusion of herd in the logistic regression model were highly significant, indicative of the strong herd effect (positive herds v. negative herds during the sampling period). Parameters of interest, including the effect of season and lactation status, are reported in the table. The odds ratios and 95% CIs are shown along with the associated p-values (Wald statistics) and likelihood ratio statistics for inclusion of the effect in the model.
Table 2.4 shows the results of the GEE approach to the logistic regression (GEE-LR). Results described in the table include odds ratios, associated 95% CIs, Wald tests and score tests for the independent and exchangeable correlation matrices.

2.3.4. Composite characterization and PFGE

Isolates were classified as *E. coli* O157:H7 or *E. coli* O157:NM. Further characterization as STEC O157:H7/NM was performed as described in the materials and methods section. All isolates were PCR positive for *rfb* O157, *fliC* H7, *eae* A, *hly* A, and *stx* 2, and none of the isolates were positive for *stx* 1.

Table 2.5. Composite polymerase chain reaction characterization and pulsed-field gel electrophoresis subtype data from Shiga-Toxigenic *Escherichia coli* O157:H7 field isolates in Louisiana dairy studies (2001)

<table>
<thead>
<tr>
<th>Herd</th>
<th>Study</th>
<th>n(pos)</th>
<th>n(tot)</th>
<th><em>rfb</em> O157</th>
<th><em>fliC</em> H7</th>
<th><em>stx</em> 1</th>
<th><em>stx</em> 2</th>
<th><em>eae</em> A</th>
<th><em>hly</em> A</th>
<th>PFGE</th>
<th>Int</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>PP,LD,MHF</td>
<td>42</td>
<td>326</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>a*</td>
<td>12</td>
</tr>
<tr>
<td>B</td>
<td>PP,MHF</td>
<td>33</td>
<td>331</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>b**</td>
<td>6</td>
</tr>
<tr>
<td>C</td>
<td>PP,LD</td>
<td>12</td>
<td>159</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>c*</td>
<td>17</td>
</tr>
<tr>
<td>D</td>
<td>PP</td>
<td>10</td>
<td>63</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>d*</td>
<td>NA</td>
</tr>
<tr>
<td>E</td>
<td>PP,LD</td>
<td>3</td>
<td>184</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>e**</td>
<td>22</td>
</tr>
<tr>
<td>F</td>
<td>PP</td>
<td>2</td>
<td>66</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>f,g***</td>
<td>NA</td>
</tr>
<tr>
<td>Totals</td>
<td></td>
<td>102</td>
<td>1129</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* PFGE subtype based on the classification scheme by Tenover *et al.* (49) for within herd classification, PFGE subtypes for isolates between herds (epidemiologically unlinked) based on a difference of 2 or more bands (5,6,26,48)

*+ Isolates considered indistinguishable within a herd

**Isolates considered indistinguishable or closely related within herd

***Isolates from herd f considered different

2 Interval indicates the maximum time in weeks between isolates obtained from herds sampled at multiple points during 2001 (involved in multiple studies), herds D and F in PP study only

3 Studies include Point Prevalence (PP), Longitudinal Dairy (LD), and Mouth, Hide, and Fecal (MHF); Herds A and B: n(tot) includes fecal cultures and swab cultures from MHF study

Table 2.5 summarizes the PCR characterization within positive herds participating in the PP, LD, and MHF studies during 2001. Table 2.5 demonstrates that isolates from
individual herds had indistinguishable or closely related PFGE types (clones), except in herd F, where the two isolates obtained had distinct PFGE patterns and were classified as “different” subtypes according to the criterion of Tenover et al. The isolates from herds D and F were obtained on the same sampling date during the PP study. Time intervals represent the greatest number of weeks between isolation of STEC O157:H7 within herds.

2.4. Discussion

Because peak fecal shedding has been described during the summer months, sampling in the PP study was concentrated during June, July, and August to increase the probability of detection and estimate maximal STEC O157:H7 fecal shedding. Results from the PP study indicated that STEC O157:H7 commonly colonized adult lactating dairy cattle in Louisiana and that fecal shedding occurred at high prevalence proportions in some herds relative to other U.S. studies (38). Prevalence proportion point estimates in positive herds ranged from 3% to 34.6%.

STEC O157:H7/NM fecal shedding has been described as episodic and endemically unstable. One of the study herds, which was negative at the time it was sampled for the PP study, was positive during the Longitudinal study, thus illustrating the point that one-time sampling of herds will underestimate the herd-level prevalence of fecal shedding (19,22). We attribute the higher prevalence estimates in adult lactating dairy cattle during the PP study (Table 2.1) to the increased sensitivity of the detection methods used (Selective enrichment and IMS). Most studies have found adult dairy cattle to be colonized by STEC O157:H7 at low prevalence proportions (~1%) (38). Our findings are consistent with the previously mentioned studies that also used selective enrichment and IMS and found higher prevalence in dairy cattle than non-IMS based studies.
Results of the MHF study indicate that viable STEC O157:H7 can be cultured from the dorsal hide surface of adult lactating dairy cattle. Fecal prevalence, at the time that the oral cavity swabs and dorsal hide swabs were taken, was 20.6% in herd A and 28.6% in herd B. The lack of a positive oral cavity swabs and only one positive hide swab was not consistent with the reported data in feedlot cattle. Differences in the bacterial microflora of the oral cavity and on hide surfaces may account for the differences between our study and the previous work by Keen and Elder (30). For example, differences in the bacterial microflora may competitively exclude or reduce STEC O157:H7 from the oral cavity/hide niche in dairy cattle or reduce the sensitivity of the culture methods optimized for culture of feedlot cattle. Differences in management (feeding methods, exposure to manure) that promote increased hide and oral cavity contamination or colonization in feedlot cattle compared to dairy cattle may also contribute to the differences we observed.

As mentioned above, the PFGE pattern of the hide isolate and the fecal isolate from the same animal were indistinguishable from the herd subtype. Finding a viable STEC O157:H7 on the hide is an important finding because of recent outbreaks associated with direct contact at farms, fairs and petting zoos. The Centers for Disease Control and Prevention (CDC) have recently issued recommendations regarding public safety and direct contact with livestock at open farms, fairs, and petting zoos (9,34,52). From a public health perspective it is important to note that these results show that hide contamination/colonization occurs and that proper hygiene should be emphasized when people, particularly children, have direct contact (petting, feeding) with dairy cattle.

The longitudinal study results indicate that Louisiana dairy cattle seasonally shed STEC O157:H7. Figure 2.1 graphically depicts the cow-level prevalence for all five herds.
and for the subset of herds that were positive. The May and July cross-sectional samples had the highest prevalence. This is consistent with other studies that have shown high prevalence during warm months (summer) and winter nadirs in STEC O157:H7 fecal shedding.

Logistic regression models were used to examine the effect of sampling month (four category and dichotomized) and stage of lactation (four category and dichotomized) but controlled for the herd effect. Each cow (n=635) sampled represented a binomial outcome in the model. The likelihood ratio (LR) statistic for herd was <0.0001, indicating that the herd variable was significant in the model. Odds ratios for herd varied widely because two of the herds sampled were not positive during the study. The odds ratios for the individual herds are not reported since they are not meaningful. Variables of interest including Month, Lact DIM (number of days in lactation), season (dichotomized Month), and lactating (dichotomized LactDIM) are shown in Table 2.3.

In Model A the season category had 4 levels: The October cross-sectional sample functioned as the reference category. The May and July samples were significantly higher than the October sampling period. The February cross-sectional sample was not significantly different than October. Interpretation of the LR test (type 3 analysis) indicates that sampling Month (p<0.0001) contributes significantly to the model. The LR statistic (p=0.2121) for Lact DIM in Model A indicates that the 4-category parameterization of lactation does not contribute significantly to the model.

Based on parameter estimates and the Wald tests in Model A for month, the sampling month variable was dichotomized to reflect warm season and cold season. Dichotomization of Lact DIM was performed to explore potential differences in lactating cows and dry cows and was not based on the results in Model A. Results from Model A indicate that the <100
DIM category had an odds ratio of 5.31 and approached statistical significance (p=0.0556) in comparison to the dry cow reference category. Future studies incorporating more herds and a greater sample size may be able to discern a <100 DIM or early lactation effect on STEC O157:H7 fecal shedding. We dichotomized stage of lactation based on management differences for dry cows and lactating cows, as well as the raw data, which indicated that fewer fecal shedders occurred in the dry cow category.

Table 2.3 presents Model B results of the dichotomized model. Dichotomized month (season) was highly significant in the model based on the LR statistic. Fecal shedding of STEC O157:H7 during the warm season was significantly higher than the cold season reference category. The interpretation of the season effect in Model B is that the odds of STEC O157:H7 fecal shedding during the warm season is 13.25 times the odds during the cold season for cows sampled in our study. Absence of unity (1.0) from the 95% CI and the associated p-value (<0.05) indicate statistical significance.

Lactating cows had increased odds (3.6) of being fecal shedders compared to dry cows, although not at statistically significant levels based on the p-value of 0.05. Dichotomization in model B reinforced the conclusion reached in Model A that the warm season increased the odds of fecal shedding among the sampled dairy cows. Dichotomization of lactation status into a dry cow category compared to the lactating cow category did not show significant differences in the two groups although statistical significance was approached by the Wald test (p=0.0981). The LR statistic for inclusion of the dichotomized lactation variable in the model also changed and approached statistical significance (p=0.0566).
The GEE approach to LR accounts for clustering by herd of the observations and was used to control for correlation within herds (n=5) that were repeatedly sampled over time. Independent and exchangeable correlation structures were modeled. The independence working correlation model assumes that repeated observations for a subject (herd) are independent, similar to normal logistic regression models. Consistent parameter estimates and covariances are provided for the repeated measures data given that the regression model is correctly specified (47).

The exchangeable correlation structure assumes constant correlation between any two observation times. In other words, the subjects (herds) are assumed to have constant correlation between pairs of observations from different sampling dates (28,47). Intuitively, this can be thought of as controlling for herd-to-herd variation; GEE’s account for the fact that animals from the same herd are correlated when sampled repeatedly, thus observations from a specified herd are more alike than observations from differing herds.

The effect estimates are robust in both models as shown in Table 2.4 and are similar to the normal LR shown in Table 2.3. They decrease in magnitude from the normal LR to the Independent GEE LR and from the Independent GEE LR to the Exchangeable GEE LR. Inference regarding the parameter effects differs between the adjusted models. Model A and Model B conclusions are consistent with respect to the four-category Month variable in model A and the dichotomized season variable. Warm season increases the odds of STEC O157:H7 fecal shedding in adult dairy cattle in our study relative to the cold season.

Lact DIM (4-category) and the dichotomized lactation in Model B differ from the normal logistic regression model. In Model A, cows in early lactation (<100 DIM) are not statistically different from the dry cow category, where, the cows in the 100-200 DIM and
>200 DIM categories are significantly different compared to the reference category (dry cow). This result would suggest dichotomization by combining dry cows and cows <100 DIM in one group and placing the cows 100-200 DIM with cows > 200 DIM into the second group.

Model B is dichotomized based on the management differences, as was Model B in Table 2.3. Here, lactating cows appear to have significantly increased odds of fecal shedding compared to dry cows. As mentioned in Table 2.4, score statistics for type 3 GEE analyses were requested using SAS v. 8.2. The score test available is analogous to the score test in LR that is based on the likelihood function. With GEE there is no likelihood function. Agresti indicates that the proposed score test for GEE may be trustworthier than Wald tests, although it has not been widely accepted (2). In each case (Model A: Independent and Exchangeable, Model B: Independent and Exchangeable) the score test was not significant (p>0.09), indicating that month/season and lact DIM/ lact Y/N variables do not contribute to the model. This constitutes a reversal in conclusion compared to the Wald tests.

Interpretation of the GEE models is problematic because of the divergence in results between the Wald tests and score tests. Agresti indicates that divergence of significance tests for GLM’s may indicate violation of assumptions used in large-sample methods (1). Further, Fahrmeir and Tutz state that consistency and asymptotic normality results improve with a fixed number of independent observations as the number of clusters (herds) goes to infinity (14). Thus, while there is not a clear answer for the number of clusters required to obtain consistent results, the number of data clusters in our study (five clusters or herds) is not sufficiently large enough to provide asymptotic normality. When the number of clusters is small, the model-based (i.e. normal logistic regression) variance estimators may have better
properties for inference; the empirical ("robust" or "sandwich") variance estimator used to perform inference in the GEE analysis is asymptotically unbiased when the number of clusters is large (>20) (27). Therefore, while the parameter estimates are consistent, the data may be biased by the small number of clusters resulting in divergence of inference in statistical significance tests.

The differences observed in parameter estimates between the models relate to the method used for estimation. Logistic regression (Table 2.3) uses a maximum likelihood (ML) approach. GEE-LR (Table 2.4) are marginal models that report estimates that are the solutions of quasi-likelihood equations (generalized estimating equations) (2). Thus, the independent LR in Table 2.3 and independent GEE-LR differ in parameter estimates, although the estimates are similar or robust. Standard errors (SE) in the GEE-LR result from adjustments made using the empirical dependence that exists in the data. Model-based SEs are adjusted based on the data dependence structure to give more appropriate (robust) SEs (Table 2.4: Independent) that affect inference (2).

Regarding the logistic regression analysis, the parameter estimates indicate consistent results in both the normal (model based) analysis and the GEE (empirical) analysis. Warm season increases the odds of STEC O157:H7 fecal shedding in cattle sampled in our study compared to the cold season. Lactating cows show increased odds of fecal shedding compared to dry cows. Inference based on variance estimators should only be considered reliable in Table 2.3 because of the problematic divergence of statistical tests in the GEE analysis, although Agresti indicated that Wald tests are primarily used for inference in GEE LR (3). Study design incorporating a larger number of herds would be advantageous in providing asymptotically unbiased variance estimators for inference in the GEE LR analysis.
An appropriate design might include multiple herds (n>20), with greater consistency of results as the number increased.

Differences in fecal shedding between dry cows and lactating cows, although not statistically significant in the model, might be attributed to the different management procedures used in the two groups of cows. Differences that could be investigated include the effects of dry cow vs. lactating cow rations, physiologic stress caused by lactation, fecal pH secondary to rumen by-pass grain, amount of long stemmed fiber in the diet, the amount of grain fed per animal and animal density/housing. Further investigation of these differences is warranted and inference may indicate a clear association if power is enhanced by inclusion of more herds in the study design.

The characterization data of STEC O157:H7 isolates obtained during the studies demonstrated that the isolates possessed identical virulence factor complements (Table 2.5). The presence of stx2 was detected in each isolate. Stx2 is thought to be the more significant virulence factor in human disease than stx1 (21,46). Vero cell assays or other diagnostic tests that detect functional stx were not performed. Assuming that the PCR product represents a functional gene that would enable *E. coli* O157:H7 to produce stx2, the isolates obtained in our studies appear to have the virulence factors necessary to cause disease (HC, HUS, TTP) in susceptible humans (potential enterohemorrhagic *E. coli* or EHEC).

The PFGE results for STEC O157:H7 isolates obtained in our studies are summarized in Table 2.5. The PFGE patterns were compared visually. Failure to digest the chromosomal DNA with *Xba*I prompted the use of *Spe*I for macrorestriction of the isolate DNA. As mentioned, *Xba*I has been reported as the most discriminatory enzyme used for PFGE of STEC O157:H7. Based on PFGE of *Spe*I macrorestricted DNA and visual interpretation,
seven subtypes were recognized. Two isolates were obtained in herd F, each being a distinct PFGE subtype (subtypes f and g). Herd F was enrolled in the PP study and was sampled only once. Subtypes f and g also differed in their motility characteristics (data not shown).

With the exception of herd F, isolates from within a specified herd (A, B, C, D, and E) had indistinguishable or closely related PFGE patterns and were classified accordingly (subtypes a, b, c, d, and e). Herds A, B, C and D were involved in multiple studies and thus isolates were obtained at various sampling dates. PFGE subtypes remained indistinguishable or closely related across sampling dates. Across all studies, the shortest interval between obtaining the first and last isolate from a given herd was 6 weeks (herd B) and the longest interval was 22 weeks (herd E).

Our PFGE findings are consistent with other studies that have shown that STEC O157:H7 are often clonal within a herd and that particular subtypes can persist for months or years (15,25,34,44). Rice et al. suggested that dairy herds have fewer subtypes than feedlot cattle and that the number of subtypes isolated in each herd is influenced by the movement of animals into and out of the herd (43). Using XbaI macrorestricted DNA for PFGE could potentially demonstrate more subtypes within study herds than what we found with SpeI. A second factor that may have influenced the number of subtypes recognized within a given herd was the number of confirmed isolates that we selected and tested from a given sample (42,45). We only archived one isolate per positive sample and could have missed other PFGE subtypes present in the sample. Current plans are to repeat the PFGE with XbaI and compare banding patterns both visually and with available gel band analysis software.

In conclusion, these findings have implications for understanding and controlling foodborne, waterborne, and direct contact transmission of STEC O157:H7/NM. More
sensitive methods should be used to evaluate STEC O157:H7/NM in dairy cattle. Future large-scale epidemiologic studies will benefit from enhanced detection techniques that may make identification of significant cow-level and herd-level associations with STEC O157:H7/NM fecal shedding evident. Additionally, the findings demonstrate that adult cattle from dairy herds in a southern state with low human disease incidence may frequently be shedding STEC O157:H7/NM. Potential relationships in seasonal patterns and geographic variation of STEC O157:H7/NM prevalence in cattle and human disease incidence deserve further investigation.

2.5. References


Ref Type: Conference Proceeding


Escherichia coli O157:H7 and other Shiga toxin-producing E. coli strains. ASM, Washington, DC.


111
Ref Type: Conference Proceeding


CHAPTER THREE

STEC O157:H7 IN A COHORT OF WEANED, PRECONDITIONED RANGE BEEF CALVES

3.1. Introduction

Shiga-toxigenic *Escherichia coli* O157:H7 (STEC O157:H7) is responsible for foodborne outbreaks of human diarrheal disease. Complications related to infection include hemorrhagic colitis (HC), hemolytic-uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (TTP) (22,40). Ground beef has been identified as a common vehicle since the earliest, widespread outbreaks of STEC O157:H7 and continues to have a significant role in the epidemiology of the disease (1,7,43). Feedlot cattle supply a considerable amount of ground beef to U.S. markets (56).

The possibility of controlling the pathogen in livestock, particularly in beef cattle, is important not only from a public health standpoint, but also from the perspective of livestock producers, slaughter houses, meat packers, and retailers. Not only does contaminated ground beef cause human illness and associated economic costs, detection of STEC O157:H7 in ground beef has a tremendous economic impact from product recalls by processors and distributors. A 1997 outbreak, associated with a single processing plant, prompted a national recall of twenty-five million pounds of ground beef (14). Recently, a large voluntary recall, secondary to positive tests for STEC O157:H7 in lots of hamburger, was expanded after human cases were associated with the contaminated ground beef. In total, nineteen million pounds of fresh and frozen ground beef products were recalled (55).

Hazard analysis and critical control point (HAACP) strategies aimed at reducing STEC O157:H7 contamination can be implemented at various points in the farm-to-fork continuum. HAACP programs are designed to identify and intervene at high-risk points for
carriage of STEC O157:H7 through the food chain (46,56). Pre-harvest research indicated that beef cattle are intestinally colonized at higher prevalence levels than previously thought and that slaughter-ready cattle in feedlots may have higher contamination/colonization levels in the oral cavity and on their hides than in their feces (20,30).

The concentration of cattle in feedlots concurrent with intensive economic pressure to quickly maximize weight gains has been suggested as a factor in the emergence of STEC O157:H7 (1). Commingling of calves can provide a mechanism for the transfer of microbial flora between animals (horizontal transmission) and might contribute to episodic or epidemic fecal shedding (6,34). Several studies have suggested that newly arrived cattle at feedlots and those “on feed” for the shortest amount of time have a higher prevalence of STEC O157:H7 than animals acclimated to the feedlot environment (25,58,59). This observation has been attributed to changes in the gastrointestinal flora that might occur secondary to dietary stress associated with adjustment to feedlot rations, which have a high percent of grain or concentrates (25,34,45). In contrast, Smith et al., in a study of Midwestern U.S. feedlots, found that prevalence of STEC O157:H7 was not correlated with mean body weight or number of days on feed (50).

The first step in extending HAACP strategies to the farm level should be the identification of risks and potential intervention points in the farm production system. For example, the STEC O157:H7 feedlot prevalence levels may be affected by different farm production decisions associated with the calf crop. Weaned calves may go directly into feedlots or be enrolled in preconditioning programs prior to entry into the feedlot (56). Most feedlot calves originate from cow-calf farms that are widely dispersed throughout the United States. Few researchers have evaluated STEC O157:H7 prevalence levels in weaned calves.
prior to entry into feedlots. Laegreid et al. found that weaned range calves from thirteen of fifteen herds (87%), in five states, were shedding STEC O157:H7 prior to entry into the feedlots (34). Within the positive herds, the prevalence proportion ranged from 1.7–20% with a mean of 7.4%. Additional serologic evidence, based on blocking ELISA serum antibody titers, indicated that most calves (83%) and all herds (100%) had been exposed to STEC O157:H7 (32). In another study of ten cow-calf farms in Kansas, the authors isolated STEC O157:H7 from cattle on all ten farms. Forty of 3,152 (1.3%) fecal samples from all cattle tested were positive and in the subpopulation of calves on the farms, eleven of 804 (1.4%) fecal samples were positive for STEC O157:H7 (46). Both studies utilized immunomagnetic separation (IMS), which is a selective method shown to be more sensitive than conventional enrichment or direct plating detection techniques (9,20,33,38,51).

Information on the farm-level prevalence of STEC O157:H7 will contribute to the decision making process required for pre-harvest HACCP strategies. If critical control points can be identified prior to arrival of calves at the feedlot, then actions to reduce transmission could be implemented. While feedlots are concentrated in specific regions, steers and heifers originate from farms and ranches throughout the U.S. (56). These steers and heifers comprise the largest portion of animals used for meat in U.S. beef markets, 82% (56). The aggregation and commingling of calves whose destination is centralized feedlots can be complex, and typically begins with local sales, cooperatives, and contact buyers (56). Preconditioning programs are a way for producers to market a value-added product to feedlot buyers. Feedlot buyers purchase these preconditioned animals in their attempt to maximize gain and reduce losses that are associated with disease and death. Most preconditioning programs involve vaccination against common respiratory diseases, anthelminthic treatment for external and
internal parasites, and introducing the weaned beef calves to grain rations to prepare them for the transition to high carbohydrate feeding/finishing rations used in the feedlots.

Louisiana is predominantly a cow-calf production state with a spring calving season (53). Cow-calf pairs are kept on pasture until fall when the calves are weaned and moved for sale, transported to feedlots, or shipped to preconditioning operations. The Louisiana Calf to Carcass Project (CTC) is an annual program designed by LSU Agricultural Center (LSU AgCenter) to help cattle producers maximize profits through a preconditioning program (13). Cattle are marketed on a grid (value based) system that fits the quality and yield grades for Louisiana cattle. Producers receive production data and carcass trait data through the program (13). In conjunction with Louisiana CTC program, a census sampling of calves enrolled in the 2001 program was performed. Fecal samples were taken at the beginning and at the end of the forty-five-day preconditioning period.

Our goal was to estimate the point prevalence of STEC O157:H7 fecal shedding in this cohort of calves and to determine if significant differences occurred in the fecal prevalence of STEC O157:H7 at the time of enrollment into the forty-five-day preconditioning period and at the end of the program. STEC O157:H7 isolates that were obtained in the study were confirmed and characterized. Pulsed-field gel electrophoresis (PFGE) was performed to evaluate genetic diversity of the isolates and to determine if horizontal transmission of STEC O157:H7 subtypes occurred during commingling of the calves.
3.2. Materials and Methods

3.2.1. Calf to Carcass Program and Sampling Protocol

Criteria for enrollment of steers and non-pregnant heifers in the CTC program included dehorning, vaccination with an 8-way clostridial vaccine and bovine respiratory disease (BRD complex: IBR-PI3-BVD-BRSV) vaccine, and identification of each animal individually and by owner (brand, ear tag, etc.) (13). For participation in the CTC program, each producer was required to supply a minimum of three animals weighing at least 500 pounds. Producers delivered the consigned calves on September 6, 2001 to one of three preconditioning sites (PC site) designated as PC sites A, B, and C.

Upon arrival, cattle were weighed, vaccinated (BRD complex and 8-way clostridial vaccines), given a prophylactic antibiotic injection (Micotil, Elanco), and treated for internal and external parasites. Calves were commingled and pastured in one area (PC site B) or in two or more adjacent pastures (PC sites A and C). Animals were revaccinated with BRD complex vaccine and 8-way clostridial vaccine on September 24, 2001. Cattle were kept on pasture and fed hay, minerals, and water. Cattle were also placed on a medicated transition ration containing oxytetracycline at 200 grams/ton (Nutrena Doin’ Fine Transition Ration Oxy200). The ration fed to these animals was labeled for feeding weaned beef cattle on pasture or in the feedlot for prevention and treatment shipping fever complex. Following the forty-five-day preconditioning period, the cattle were weighed, given a body condition score, loaded and shipped to a feedlot in the Midwestern United States.

To determine the prevalence of STEC O157:H7, fecal samples from all CTC cattle (n=408) at the PC sites were obtained upon arrival directly from the farms of origin (designated as time one; (T1)) and following the forty-five-day preconditioning period.
(n=453) prior to loading for transport to the feedlot (designated as time two (T2)). Approximately twenty grams of feces were obtained per rectum using a new glove for each animal. Fecal samples were maintained at ambient temperature during transport to the laboratory at the Louisiana State University School of Veterinary Medicine and placed in media within twenty-four hours.

3.2.2. Culture Methodology

Because the sensitivity of methods significantly influences the magnitude of prevalence estimates, culture techniques were selected that have demonstrated higher sensitivity than conventional enrichment and direct plating techniques (9,20,33,38,51). The culture techniques have been described previously (15,30).

Briefly, ten grams of fresh bovine feces were incubated at 37 °C for 6 hours in 90 ml of gram negative broth (Difco, Sparks, MD) supplemented with cefsulodin (10 mg/l, Sigma, St. Louis, MO), vancomycin (8mg/ml, Sigma, St. Louis, MO) and cefixime (0.05 mg/ml, Lederle, Pearl River, NY). Immunomagnetic separation (IMS) was performed on a 1 ml aliquot using Dynabeads anti-\textit{E. coli} O157 uniform, paramagnetic, polystyrene microscopic beads. The anti-O157 beads have adsorbed and affinity purified antibodies against \textit{E. coli} O157 covalently bound to the surface (Dynal Inc., Lake Success, NY).

The immunomagnetic bead/broth suspension was then washed three times with 1 ml of phosphate buffered saline containing 0.05% Tween 20 (Sigma, St. Louis, MO) (PBS-Tween20) following capture of the paramagnetic beads using a magnetic particle concentrating separation rack (Dynal Inc., Lake Success, NY). Following the final wash the bead/bacteria complex was resuspended in 100 µl of PBS-tween20.
An aliquot of 50 µl of the bead/bacteria complex was spread plated on sorbitol MacConkey agar (ctSMAC) containing cefixime (0.05mg/l) and potassium tellurite (2.5mg/l) and incubated at 37° C for 18-24 hours. A maximum of three colonies with typical STEC O157:H7 phenotypic characteristics were selected as suspects and placed into 5 ml of MacConkey broth and 2 ml of trypticase soy broth (TSB) for 18-24 hours at 37° C.

3.2.3. Confirmation by Enzyme-Linked Immunosorbent Assay (ELISA)

Indirect enzyme-linked immunosorbent assay (ELISA) was performed for identification of isolate serotype (15,30). The ELISA was performed using murine monoclonal antibodies (MAb) to H7 antigen, anti-H7 MAb 2B7, and O157 antigen, anti-O157 MAb 13B3 (26,61). Briefly, 1 ml aliquots of the TSB and MacConkey cultures were heat killed by placing them in boiling water for 10 minutes. A positive control (E. coli O157:H7; American Type Culture Collection (ATCC) 43888), negative control (E. coli O78:H11; ATCC 35401) and the samples were transferred to 96-well plates and incubated at 37° C for one to two hours. Following incubation, the plates were washed with ELISA wash buffer. MAb 13B3 or MAb 2B7 was then added, followed by incubation at 37° C for 10 minutes, and wash steps. A horseradish peroxidase labeled goat anti-mouse antibody was added followed by incubation and wash steps. One component ABTS (2,2’-azino-di (3-ethyl-benzthiazoline-6-sulfonate); KPL, Guilford, UK) substrate was then added and allowed to react for ten to twenty minutes at room temperature before stopping the reaction with 1% SDS (sodium dodecyl sulfate). Absorbance was read at dual wavelengths of 405/490. Reactions were considered positive at 0.2 + the optical density (OD) of the negative control.

Isolates reactive with MAb 13B3 (O157 positive ELISA) were inoculated in TSB and evaluated for motility using phase-contrast microscopy. Isolates that had the correct
phenotype (1-2 mm, sorbitol-negative colonies) on ctSMAC, fermented lactose in MacConkey broth, positively reacted with anti-O157 MAb 13B3, and positively reacted with anti-H7 MAb 2B7 or were non-motile (NM) were considered to be *E. coli* O157:H7 or *E. coli* O157:NM.

Additionally, a subset of isolates were tested by Sensititre gram-negative (AP80) autoidentification plates (Accumed International, Westlake, OH) and confirmed as *E. coli* by reactions to thirty-two substrates. A single isolate from each positive sample was archived in a brain heart infusion (BHI) broth-glycerol suspension at –80° C for future characterization.

### 3.2.4. Characterization by Polymerase Chain Reaction (PCR)

Isolates were characterized by polymerase chain reaction (PCR) for *rfb*<sub>O157</sub> and *fliC*<sub>H7</sub>, and the putative virulence factors *stx*<sub>1</sub>, *stx*<sub>2</sub>, *eae*<sub>A</sub> and *hly*<sub>A</sub>. Somatic (O157), flagellar (H7) and virulence factor genes were amplified using previously published primer pair sequences (19,41). Duplex reactions were run for *stx*<sub>1</sub> and *stx*<sub>2</sub>, as well as *eae*<sub>A</sub> and *rfb*<sub>O157</sub>. PCR for *hly*<sub>A</sub> and *fliC*<sub>H7</sub> were run as uniplex reactions. PCR cycling conditions were as previously described (19,41).

Amplified gene products were subjected to electrophoresis on a 2% agarose gel and then stained with ethidium bromide. Gel images were captured digitally, photographed and scored. Clear, well-defined bands of the correct size for *rfb*<sub>O157</sub> (259 base pairs (bp)), *fliC*<sub>H7</sub> (625 bp), *stx*<sub>1</sub> (180 bp), *stx*<sub>2</sub> (255 bp), *eae*<sub>A</sub> (384 bp) and *hly*<sub>A</sub> (534 bp) that were consistent with the positive control were considered positive PCR reactions. A well-characterized STEC O157:H7 strain was used as the positive control (ATCC 43895). A 100 bp DNA ladder was used as the standard. *E. coli* O157:H7 or O157:NM isolates were considered to be STEC O157:H7 or STEC O157:NM if PCR reactions for *stx*<sub>1</sub>, *stx*<sub>2</sub> or both were positive.
3.2.5. Pulsed Field Gel Electrophoresis (PFGE)

Each STEC O157:H7 isolate was subjected to restriction enzyme digestion and PFGE. Previously described methods were used (34). Summarizing, isolates were grown in Luria-Bertani (LB) broth to an approximate OD of 0.8. Chloramphenicol was added to the cells for approximately one hour followed by centrifugation and resuspension in cell suspension buffer. Pulse-field quality agarose was used to make a 2% agarose solution. Equal volumes of the cell suspension and agarose were gently mixed and plugs (agarose embedded DNA) were cast. Plugs were allowed to solidify, placed in buffer and sequentially subjected to lysozyme and proteinase K digestion. Plugs were washed (4x) and phenylmethylsulphonyl fluoride (PMSF) was used to inactivate residual proteinase K.

For restriction enzyme digestion, \textit{XbaI} was selected. However, following digestion of the plugs with 50 units of \textit{XbaI} in \textit{XbaI} buffer, electrophoresis revealed that enzyme digestion did not occur or was incomplete. \textit{XbaI} enzyme digestion failed on two subsequent attempts as well. A second enzyme, \textit{SpeI} (New England Biolabs, Beverly, MA), a six base cutter, was used to digest duplicate plugs in NEBuffer 2 (New England Biolabs, Beverly, MA). Following digestion with 50 units of \textit{SpeI}, plugs were rinsed and loaded on a 1% agarose gel along with DNA size standards and a standard STEC O157:H7 strain (ATCC 43895). Following PFGE separation of \textit{SpeI}-restricted DNA, gels were stained with ethidium bromide.

The \textit{SpeI}-restricted DNA fragment patterns were analyzed visually. DNA size standards, MidRange I/II and lambda ladder PFG markers (New England Biolabs, Beverly, MA) were used to compare fragments from gel to gel. Genetic relatedness of isolates within herds was determined for epidemiologically related isolates according to the criteria of
Tenover et al. (52). Isolates differing by more than 2 bands in epidemiologically unlinked herds (between herds) were considered separate subtypes. Several authors have suggested strict criteria for determining subtypes in epidemiologically unlinked isolates because STEC O157:H7 is thought to be a highly conserved clone (2,3,27,51).

3.2.6. Statistical Analysis

Prevalence estimates with 95% confidence intervals (CI) were calculated as the number of positive samples divided by the total number of samples, (PEPI 4.0). Fisher’s exact statistics were utilized to estimate CIs for null values. Prevalence proportions were compared statistically at the 95% confidence level using exact methods. Data were entered and stored in Excel 2000.

3.3 Results

3.3.1. Calf to Carcass (CTC) Cohort

Twenty-nine cow-calf farms enrolled calves in the 2001 CTC program and were preconditioned at one of the three sites. The PC sites were located in southeastern (Idlewild Experiment Station), southwestern (McNeese State University), and north Louisiana (Louisiana Tech University). Calves originated from twenty-one different parishes (counties) and were primarily of Brahman breed influence, which is representative of the Louisiana and Gulf South cow-calf industry. Table 3.1 reports the number of animals enrolled from individual herds at each preconditioning location. Four herds (n=47 calves) were sampled at T2 that were not sampled initially. These calves participated in the program, but were preconditioned at home and not available for sampling at T1. At T1, 408 calves were sampled. Following the preconditioning period, 453 calves were sampled. Of the 408 calves sampled initially, 406 were resampled at T2 along with the calves preconditioned at home.
Table 3.1: Number of calves sampled in the Louisiana Calf-to-carcass program upon arrival at preconditioning sites and following a forty-five-day preconditioning period

<table>
<thead>
<tr>
<th>Preconditioning Site</th>
<th>A # Sampled</th>
<th>B # Sampled</th>
<th>C # Sampled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herd</td>
<td>T1 / T2a</td>
<td>T1 / T2a</td>
<td>T1 / T2a</td>
</tr>
<tr>
<td>1</td>
<td>6 / 6</td>
<td>6 / 6</td>
<td>6 / 6</td>
</tr>
<tr>
<td>2</td>
<td>6 / 6</td>
<td>16 / 16</td>
<td>3 / 3</td>
</tr>
<tr>
<td>3</td>
<td>10 / 10</td>
<td>6 / 6</td>
<td>50 / 50</td>
</tr>
<tr>
<td>4</td>
<td>20 / 20</td>
<td>3 / 2</td>
<td>70 / 69</td>
</tr>
<tr>
<td>5</td>
<td>3 / 3</td>
<td>8 / 8</td>
<td>3 / 3</td>
</tr>
<tr>
<td>6</td>
<td>16 / 16</td>
<td>6 / 6</td>
<td>22 / 22</td>
</tr>
<tr>
<td>7</td>
<td>6 / 6</td>
<td>9 / 9</td>
<td>11 / 11</td>
</tr>
<tr>
<td>8</td>
<td>6 / 6</td>
<td>2 / 2</td>
<td>8b</td>
</tr>
<tr>
<td>9</td>
<td>45 / 45</td>
<td>10 / 10</td>
<td>9b</td>
</tr>
<tr>
<td>10</td>
<td>3 / 3</td>
<td>0 / 8</td>
<td>10b</td>
</tr>
<tr>
<td>11</td>
<td>16 / 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>35 / 35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>5 / 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>177 / 177</td>
<td>66 / 73</td>
<td>165 / 203</td>
</tr>
</tbody>
</table>

Total sampled (T1/T2), n=408 / 453

* T1= initial sampling date (9/7/2001)
T2= sampling date following ~45-day preconditioning period (10/16/2001)

3.3.2. Prevalence Proportions

* E. coli O157:H7 (EC O157:H7) and STEC O157:H7 isolates were obtained from sampling at T1. No isolates were obtained at T2. Table 3.2 reports the herd-level prevalence proportions, the animal-level prevalence proportions, and the prevalence proportions from positive herds at each preconditioning site at T1. Overall point estimates (prevalence proportions) for fecal shedding of EC O157:H7 or STEC O157:H7 at T1 and T2 are reported and are compared statistically with exact methods (Table 3.2).
Table 3.2: Escherichia coli O157:H7 and Shiga-Toxigenic Escherichia coli O157:H7 prevalence proportions from positive herds and overall prevalence estimates for calves participating in the 2001 Louisiana Calf-to-carcass program

<table>
<thead>
<tr>
<th>Prevalence Proportion (PP) and associated 95% Confidence Interval (95% CI)a upon arrival (T1) and after preconditioning (T2)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EC O157:H7</strong></td>
</tr>
<tr>
<td>PP</td>
</tr>
<tr>
<td>Herd-level Prevalence (T1)</td>
</tr>
<tr>
<td>Site A</td>
</tr>
<tr>
<td>Site B</td>
</tr>
<tr>
<td>Site C</td>
</tr>
<tr>
<td>All Sites/T1</td>
</tr>
<tr>
<td>Animal-level Prevalence (T1)</td>
</tr>
<tr>
<td>Site A</td>
</tr>
<tr>
<td>Site B</td>
</tr>
<tr>
<td>Site C</td>
</tr>
<tr>
<td>All Sites/T1</td>
</tr>
<tr>
<td>All Sites/T2</td>
</tr>
<tr>
<td>Animal-level Prevalence, + herds (Site-Herd, T1)</td>
</tr>
<tr>
<td>A-9</td>
</tr>
<tr>
<td>A-12</td>
</tr>
<tr>
<td>A-3</td>
</tr>
<tr>
<td>A-6</td>
</tr>
<tr>
<td>C-1</td>
</tr>
</tbody>
</table>

a Exact 95% confidence interval for proportion
b Difference in proportions, Fishers P: p<0.01

3.3.3. Composite Characterization and PFGE

Isolates were genetically and phenotypically characterized as described above.

Isolates were considered Escherichia coli (E. coli) O157:H7 based on ELISA results and PCR amplification of rfbO157 and fliC157 genes. Isolates were further classified as shiga-toxigenic (STEC O157:H7) if PCR reactions for stx1 or stx2, or both were positive. Genetic
characterization data and PFGE subtype classification for the isolates obtained during the CTC study are reported in Table 3.3.

Table 3.3: Composite polymerase chain reaction characterization and pulsed-field gel electrophoresis classification of Escherichia coli O157:H7 isolates and Shiga-Toxigenic Escherichia coli O157:H7 isolates from the 2001 Louisiana CTC study

<table>
<thead>
<tr>
<th>Site-Herd</th>
<th>n</th>
<th>rfbO157</th>
<th>fliC</th>
<th>stx1</th>
<th>stx2</th>
<th>eaeA</th>
<th>hlyA</th>
<th>PFGE type¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-9</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>A</td>
</tr>
<tr>
<td>A-12</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>B*</td>
</tr>
<tr>
<td>A-3</td>
<td>4</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>C**</td>
</tr>
<tr>
<td>A-6</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>D</td>
</tr>
<tr>
<td>C-1</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>E, F***</td>
</tr>
</tbody>
</table>

¹ PFGE subtype based on the classification scheme by Tenover et al {35} for within herd classification, PFGE subtypes for isolates between herds (epidemiologically unlinked) based on a difference of 2 or more bands {22}{27}{36}{37}

* One of the two isolates from A-12 was not available for PFGE (isolate died)
** Isolates considered indistinguishable
*** Isolates from C-1 considered possibly related

3.4. Discussion

Louisiana’s 2001 estimated calf crop was 405,000 calves, approximately 1% of the United States total (53). As mentioned previously, calves are marketed in a variety of ways. Our goal was to use an existing system to obtain a cross-sectional sample of weaned Louisiana beef calves destined for the feedlot in order to estimate the prevalence of STEC O157:H7 fecal shedding. STEC O157:H7 has not been reported in Louisiana beef cattle. Based on previous studies we expected to find: 1) low animal-level prevalence of STEC O157:H7 fecal shedding in weaned calves coming off pasture at T1, 2) higher prevalence in the cohort following commingling of positive and negative animals placed on the transition ration and, 3) evidence of horizontal transmission of unique STEC O157:H7 subtypes in initially (T1) negative animals sampled at T2.
Sampling for the CTC study was not statistically based. Each producer chose the number of caves to be enrolled from their farms, and results in Table 3.1 demonstrate the marked variability in the number of animals consigned from each producer. The producers who consigned a limited number of calves used the program for production information on growth rates and carcass traits but sold the majority of their calf crop through other outlets.

Generally, the producers who consigned larger numbers of calves used the CTC program to market the majority of their calf crop. Rothman and Greenland describe selection bias as distortions resulting from procedures used to select subjects for study and from factors that influence voluntary participation in studies (44). Selection bias that affects the validity and generalizability of our cross-sectional point prevalence estimates was inherent in the study because sampling was based on convenience rather than statistically based.

The sample size (n=408) for the cohort of calves was adequate to detect statistically significant differences in the proportion of STEC O157:H7 or EC O157:H7 fecal shedding at T1 compared to T2 at low prevalence (Table 3.2). The variability observed in the number of animals consigned from different herds was not unusual for calves voluntarily in preconditioning programs or when animals are aggregated from other marketing outlets (sale barns, contract buyers, etc.). In fact, cohorts of calves destined for feedlots are often obtained from several herds. Furthermore, the absolute number of animals and the proportion of the producer’s calf crop from a given herd vary substantially from herd to herd. Louisiana CTC program administrators indicated that the assembled cohort was consistent with that of previous years (12).

Prevalence proportions and 95% CIs are shown in Table 3.2. Five of the twenty-nine herds enrolled in the CTC program were found to be shedding STEC O157:H7 or EC
O157:H7. Our findings are consistent with those of the previously mentioned studies on cow-calf farms and in weaned calves, from other regions in the U.S., that demonstrated a low animal-level prevalence (0.7%, CI 0.2, 2.1) of STEC O157:H7 (34,46). The animal-level prevalence of the O157:H7 serotype identified in our study was 2.5% (CI 1.2, 4.5). Herd-level prevalence (17.2%) was lower than that of the two previous studies (34,46). The small numbers of calves that were enrolled from some of the herds, make interpretation of the herd-level prevalence estimates problematic. We feel that the O157:H7 serotype, STEC O157:H7 in particular, would have been found in more herds, if statistically based sampling criterion could have been used.

The animal-level prevalence proportions for EC O157:H7/STEC O157:H7 fecal shedding differed significantly (p< 0.01) at time T1 compared to time T2 (Table 3.2). As mentioned, several feedlot studies have described a higher prevalence among animals in the feedlot on feed for shorter time periods than animals well acclimated to the feedlot (25,57,58). Colonization of cattle by STEC O157:H7 appears to be transient, with fecal shedding being variable, ranging from 2-3 weeks to months (5,6,11). While transient in cattle, STEC O157:H7 can persist in bovine feces for two to three months depending on environmental temperatures and moisture content of the feces (60).

A study showing calves to be susceptible to horizontal or calf-to-calf transmission at very low doses of the bacterium, led Besser et al. to suggest that the low infectious dose provides a high risk of infection for susceptible calves exposed to STEC O157:H7. This rapid transmission between susceptible calves could explain the observed temporal increases in fecal shedding when a susceptible cohort is exposed to STEC O157:H7 (6). Thus, we expected to see an increase in fecal shedding following the forty-five-day feeding period and
not the absence of STEC O157:H7 that was observed. Possible explanations for the absence of fecal shedding at T2 include: 1) climate changes during the forty-five-day feeding period, 2) the presence of antibiotic in the ration, or 3) transmission and subsequent fecal shedding that ended prior to T2.

Seasonal changes in fecal shedding have been shown in cattle. The increased STEC O157:H7 fecal shedding by cattle during warmer months coincides with increases in human disease incidence. Nadirs in STEC O157:H7 fecal shedding, associated with cooler temperatures, similarly corresponds to decreases in human disease incidence (4,8,24,36). Hancock et al. state that the “harmony” that exists between seasonal increases in STEC O157:H7 fecal shedding in cattle and increases in human disease incidence is evidence of causation, thereby implicating cattle in the transmission of STEC O157:H7 to people (23). Concurrent studies in Louisiana dairy cattle detected a limited amount of STEC O157:H7 fecal shedding in October of 2001. It is plausible that by mid-October (T2) the seasonal factors, or other unknown factors, that coincide with colder months were in effect, thereby limiting STEC O157:H7 colonization of susceptible calves. A review of 2001 weather by the Louisiana Agriculture Statistics Service (LASS) indicates that the statewide mean temperature in October 2001 was 64.7°F (54). This is considered to be cooler than normal (67.4°F) for Louisiana and represents a transition in seasonal temperatures from the preceding two months in 2001 of August (81.7°F) and September (76.4°F) (54).

Oxytetracycline was a component in the transition ration at 200 grams/ton in the CTC program and used as labeled for prevention and treatment of shipping fever complex. Most antibiotics in feed target gram-positive bacteria. The ration fed to the CTC calves is labeled for the prevention and early treatment of shipping fever (primarily gram-positive respiratory
pathogens). However, oxytetracycline is a broad-spectrum, bacteriostatic drug (42).

Antibiograms were performed on the isolates. STEC O157:H7 have been shown to be susceptible to many antibiotics including tetracycline. Tetracycline resistance has also been reported in STEC O157:H7 but was not present in any of our isolates (37,49).

The minimum inhibitory concentrations (MICs, ug/ml) for oxytetracycline on the EC O157:H7 isolates and STEC O157:H7 isolates from T1 in our study indicated that all were susceptible (data not shown). If the calves that were shedding EC O157:H7 or STEC O157:H7 consumed enough of the transition ration to reach MIC levels in the intestinal tract, then the susceptible bacteria might have been eliminated secondary to the antibiotic. Studies have shown that growth promoting antibiotic concentrations can vary widely in the gastrointestinal tract (31). Pharmacokinetic properties of oxytetracycline make determination of intralumenal concentrations difficult to estimate. Bioavailability following oral administration is reported to be 60–80%; however, the presence of food in the digestive tract can reduce the amount of tetracycline absorbed by 50% or more (42). This would potentially increase concentrations in the intestinal tract.

Further complicating matters, oxytetracycline may be progressively inactivated as it passes through the intestinal tract because of chelation with ingesta or fecal material (42). Firm conclusions regarding the effect of antibiotic supplementation cannot be made in an uncontrolled trial. With respect to the oxytetracycline supplemented transition ration, we observed an absence of EC O157:H7 or STEC O157:H7 fecal shedding in the previously positive cohort of calves following the forty-five-day feeding period.

Antimicrobials in animal feeds are reported to enhance animal growth by 4–5% (31). While the mechanism of growth promotion is unknown, the practice is common in modern
agriculture. Some authors have suggested that antimicrobials may reduce the gastrointestinal microbial flora limiting competition for nutrients and reducing the bacterial density along the intestinal lumen, permitting the nutrients to be absorbed by the animal and used for growth (31).

There are growing concerns by consumers and health officials regarding antibiotic resistance of foodborne pathogens that may be associated with the practice of adding growth-promoting antibiotics to animal feeds (10). Additional concerns related to STEC include the induction of stx-converting phages when bacteria are exposed to subinhibitory concentrations of antibiotics.

Several in vitro studies have demonstrated induction of stx-converting bacteriophages and the release of stx following exposure to subinhibitory concentrations of antibiotics (21,31,35). In human clinical studies, administration of antibiotics has been shown to increase the risk of HUS in children, possibly due to increased release of stx from enterohemorrhagic E. coli (EHEC) O157:H7 in the intestine (62). Grif et al. found that stx release in response to subinhibitory concentrations of antibiotics differed by bacterial strain (21). Kohler et al. tested several growth promoting antibacterials, but not oxytetracycline, in vitro and found that certain antibacterial growth promoters can induce stx-converting phages. The authors suggested that in vivo induction of phages from lysogenic STEC might increase free stx-converting phage in the intestine and subsequently cause the spread of stx and creation of new STEC pathotypes (31).

As mentioned, STEC O157:H7 fecal shedding in cattle was reported to be transient and variable in duration, ranging from 2–3 weeks to several months (5,6,11). Another possibility for the absence of fecal shedding observed at the T2 sampling time was that
transmission, colonization, and fecal shedding occurred in the cohort of calves prior to the sampling. We feel that other factors discussed above (weather, antibiotics) contributed to the lack of STEC O157:H7 fecal shedding observed at T2, but cannot rule out the possibility that transmission and increased shedding occurred prior to the second sampling date. Early transmission and the cessation of fecal shedding could be due to a combination of factors including acclimation to the transition ration.

An unexpected and unusual finding in our study was the isolation of EC O157:H7 that were \textit{stx} negative. All the \textit{stx} negative EC O157:H7 isolates were from three different herds of origin (A-3, A-9, and A-12) at PC site A (Table 3.2). The EC O157:H7 isolates were classified as three distinct PFGE subtypes (A, B, and C) consistent with their herd of origin. All other characterization data from the \textit{stx} negative EC O157:H7 isolates was consistent with the virulence factor complement of STEC O157:H7 isolates from other studies and the STEC O157:H7 isolates obtained in our study.

The significance of the \textit{stx}-deficient EC O157:H7 isolates (n=7) is unclear. Schmidt \textit{et al.} have identified EC O157:H7 strains that do not produce \textit{stx} but are associated with human disease (47). Diarrhea and cases of HUS were associated with \textit{stx}-deficient EC O157:H7. The authors concluded that \textit{stx} has a significant role in HC, may not be necessary for all manifestations of the diseases typically associated with STEC O157:H7, and that some cases of HUS might result from virulence factors other than \textit{stx}.

There are at least three possibilities for the presence of \textit{stx}-deficient EC O157:H7: 1) \textit{stx} is not present in these isolates because they have not been exposed to shigatoxin-converting bacteriophage, 2) \textit{stx} was present in the isolates previously, but they have subsequently lost the phage-mediated virulence factor, or 3) PCR for \textit{stx1} and \textit{stx2} did not
detect target sequences because of some inhibitory factor intrinsic to these isolates or possibly because the isolates possess a \textit{stx}2 variant not detected by the primer pair used.

Non-shigatoxigenic EC O157:H7 have been recognized in cattle and have been associated with disease in humans. Itoh \textit{et al.}, in study of Japanese slaughterhouses, isolated \textit{stx}-deficient EC O157:H7 that were positive for other putative virulence factors (28). The authors proposed that \textit{stx}-deficient EC O157:H7 might widely exist in cattle. Numerous U.S. studies that have used protocols that would detect these isolates have rarely reported the detection of \textit{stx}-deficient EC O157:H7 in cattle.

Karch \textit{et al.} have reported the frequent loss of \textit{stx} in \textit{E. coli} isolates upon routine subculture (29). Feng \textit{et al.} have also reported spontaneous loss of \textit{stx}1 and \textit{stx}2 in their laboratory and suggested that the \textit{stx}-deficient strain was the progeny of the STEC O157:H7 strain (16). The mechanism by which \textit{stx} genes are lost is not clear. In our CTC study and concurrent studies in dairy cattle all the isolates were systematically subcultured for additional testing. Only the isolates from the three herds shown in Table 3.2 did not possess \textit{stx}1 or \textit{stx}2 genes.

The positive controls and other \textit{stx}2 positive isolates that were run in the same batch as the \textit{stx}-deficient isolates indicated that the PCR assay worked correctly. Unknown factors, unique to these isolates, could have interfered with or inhibited the PCR reaction. Another possibility is that the isolates possess a \textit{stx}2 variant that is not annealed to and amplified by the primer pair we used for \textit{stx}2.

Variants of \textit{stx}2 include \textit{stx}2c, 2d, 2e, and 2f (17,40,48). STEC O157:H7 isolates of human origin have been shown to possess variable \textit{stx} genotypes including the \textit{stx}2c variant (17,18). Recognition of new \textit{stx}2 variants and their ability to spread \textit{stx} genes horizontally
between STEC strains or accumulate in a single strain complicates characterization of STEC isolates (18,48). Furst et al. state that the emergence of new stx variants complicates selection of primer pairs for diagnostic purposes (18). Resolution of the issues regarding the stx-deficient EC O157:H7 isolates will be a component of ongoing studies.

A single unique PFGE profile was recognized in each herd, except herd C-1. Based on the criterion of Tenover et al., these two STEC O157:H7 isolates from herd C-1 were considered “possibly related.” Previous studies have demonstrated that PFGE changes occur with the loss of stx genes (39). In our study, EC O157:H7 and STEC O157:H7 isolates were not isolated from the same herd. Laegreid et al., in their study of weaned calves, stated that most herds had one or two closely related patterns, but five herds in their study had STEC O157:H7 isolates with two distinct patterns.

In our study, sampling was not statistically based, and the number of calves sampled from participating herds was highly variable with the potential for large variation due to sampling techniques. Additionally, we archived only one isolate from each positive sample and subsequently subjected it to restriction enzyme digestion and PFGE. Conclusions regarding the number and diversity of subtypes in our study are limited. We were not able to demonstrate horizontal transmission of PFGE subtypes. Our data suggest that unique strains of EC O157:H7 and STEC O157:H7 may be endemic in epidemiologically unlinked herds and that subtypes within a single herd can vary in their PFGE pattern.

In implementing future studies we propose the following. To obtain a valid cross-sectional estimate of the prevalence of STEC O157:H7 in weaned Louisiana beef calves we would like to prospectively form a cohort of calves using statistically based random sampling criteria. To determine if the medicated transition ration had any effect on fecal shedding, we
would also like to enroll a control cohort that would not be fed the ration. Furthermore, the use of XbaI in PFGE might identify unique subtypes not differentiated by the enzyme we used in the study.

Summarizing, we observed that the prevalence proportion of STEC O157:H7 in the Louisiana cohort of weaned beef cattle was consistent with that of previous studies in weaned range calves. The presence of EC O157:H7 that were stx-deficient was an unexpected finding in our study. We expected, but did not observe, increased fecal shedding or the spread of STEC O157:H7 subtypes in the cohort of calves at T2. The absence of fecal shedding following the forty-five-day feeding period might be attributable to seasonal influences, inhibitory concentrations of oxytetracycline in the transition ration, or transient colonization and fecal shedding prior to T2. Based on our study and previous studies of weaned beef cattle on farms, intervention at the farm level may not be optimal for potential strategies aimed at reducing STEC O157:H7 risk in humans. However, cooperating preconditioning programs or other points of pre-feedlot aggregation of calves might be useful points at which to incorporate intervention strategies or perform controlled trials in the future.

3.5. References


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4.1. Introduction

Shiga-toxigenic *Escherichia coli* O157:H7 (STEC O157:H7) is recognized as an “emerging” cause of human diarrheal disease (25). Severe disease manifestations include hemorrhagic colitis (HC), thrombotic thrombocytopenic purpura (TTP), and hemolytic uremic syndrome (HUS) (17). The primary reservoir of STEC O157:H7 is thought to be ruminant species that are asymptptomatically colonized by the bacteria. Ruminants, particularly cattle, have been shown to be the source of STEC O157:H7 in human clinical cases related to foodborne, waterborne, and direct contact transmission (17). Additionally, deer have been implicated in foodborne outbreaks of STEC O157:H7 and apparently are asymptptomatically colonized as well (13,23,28). The role of deer in the epidemiology of STEC O157:H7 in cattle populations and human disease is unclear.

Bengis *et al.* described the transmission of infectious diseases at the wildlife/livestock interface and proposed that wherever the interface occurs, it should be regarded as bidirectional (4). They further described “certain enteropathogenic bacterial diseases” as indigenous or endemic multi-species diseases that are generally maintained in livestock and/or free-ranging wildlife and have an almost worldwide distribution. Woolhouse has suggested that “emerging diseases” are primarily zoonotic opportunists responding to changing host and pathogen ecologies (41). The emergence of new pathogens has been described as a multifactor process that includes interaction and transmission of zoonotic pathogens in wildlife, domestic animals, and the human population (11).
The livestock industry is becoming more aware of the economic impact of diseases that might be maintained and transmitted by wildlife (4,36). The Center for Emerging Issues (USDA-APHIS-CEI) lists deer as a wildlife species in which STEC O157:H7 has been found and lists the potential significance as “human infection” and “transmission to cattle” (37). Populations of deer in many parts of the United States have increased greatly during the last twenty years. Interaction between wildlife, including deer, and traditional domestic animals occurs and has a role in disease transmission (4,33).

Some authors have suggested, based on studies in cattle, that STEC O157:H7 does not exhibit host specificity (18). The organism has been isolated from multiple animals and a variety of sources in the farm environment, suggesting that the STEC O157:H7 reservoir is not limited to cattle (24). White-tailed deer (*Odocoileus virginianus*; WTD) are recognized as a potential source for dissemination of zoonotic organisms in the environment and in watersheds (10,31). Domestic cattle and WTD often share rangeland and indistinguishable genetic subtypes of STEC O157:H7 have been identified in WTD and cattle from the same farm (14,30). STEC O157:H7 might be transmitted to WTD by “spill-over” from cattle and then be transmitted back (“spill-back”) to cattle from WTD (11). This type of “spill-over” and “spill-back” transmission coupled with agricultural intensification and changes in technology/industry have been suggested as the general mechanism for the emergence of new infectious diseases in humans (11).

Armstrong *et al.* suggested specific mechanisms, including intensive agricultural production practices coupled with changes in technology, industry, and consumer habits, to explain STEC O157:H7 emergence in humans (3). Thus, WTD potentially have an indirect role in the epidemiology of STEC O157:H7 in cattle and people. Deer not only have a
potential indirect role in the epidemiology of STEC O157:H7 carriage through the food chain, they also pose direct human health risks for people who handle and/or consume WTD colonized by STEC O157:H7, including meat from the farmed deer industry (8,10,28,30).

Several reports have confirmed that deer are directly involved in the transmission of STEC O157:H7. A sporadic case and an outbreak of STEC O157:H7 have been associated with the consumption of contaminated venison (23,28). Keene et al. reported an Oregon disease outbreak investigation that found STEC O157:H7 infection in people associated with the consumption of Black-tailed deer jerky (23). In this outbreak, six confirmed cases and five presumptive cases were identified. Isolates from the human clinical cases, unconsumed jerky, uncooked meat from the same deer, the meat saw used to butcher the deer, and fragments of the deer hide were indistinguishable genetic subtypes. STEC O157:H7 was also isolated from 9% of fecal samples (n=32) collected in a nearby forest.

A sporadic case reported by the Connecticut Department of Health directly linked venison consumed by a seven-year-old boy following a hunting trip by his father to the STEC O157:H7 isolated from his stool (28). In this case, the isolates recovered from the boy and frozen venison were identical genetic subtypes. Another larger STEC O157:H7 outbreak that involved consumption of unpasteurized apple juice implicated and deer as a potential source of the STEC O157:H7 contamination in the orchards where the apples originated (10). Investigators hypothesized that the apple juice was contaminated when laborers picked up apples from the ground that were contaminated with deer feces and included them with the apples harvested from trees, although the source of the outbreak was not confirmed.

Epidemiologic studies of STEC O157:H7 in WTD have indicated that free-ranging WTD are colonized at low prevalence proportions. Rice et al. found 1.8% of WTD (n=108)
and 2.6% of beef cattle (n=191) shed the organism using the methods described by Sanderson et al. (30,32). Seven cattle and WTD isolates from this study were genetically indistinguishable. Based on these findings, the authors suggested that strain sharing between wild and domestic ruminants could play a role in maintaining the organism in bovine populations.

A WTD *E. coli* (EC) O157:H7 prevalence study in Kansas that used an enrichment and immunomagnetic separation (IMS) protocol, found that 2.4% of fresh WTD fecal samples (n=212) picked up from the ground were culture positive (33). IMS has been demonstrated to be more sensitive than direct plating techniques in detecting STEC O157:H7 (9,16,24,35). Isolates were not characterized for shigatoxin (stx) genes. Characterization of *stx*, which is thought to be the defining virulence factor in STEC, allows further classification of EC O157:H7 isolates as STEC O157:H7. The authors acknowledge that they were uncertain, despite specific sampling criteria, that each fecal sample collected represented an individual deer and not multiple samples from the same deer. Additionally, the sampling period, which included multiple visits to two cow-calf farms, was during the winter months (late September through April). While this is at-risk period for hunters, studies in cattle have demonstrated winter nadirs in fecal shedding of STEC O157:H7. Therefore, sampling during the winter may bias prevalence estimates in WTD. Seasonal shedding patterns have not been demonstrated in WTD as they have in dairy and beef cattle (18).

Renter *et al.*, using selective enrichment and IMS isolation techniques, individually identified and tested WTD fecal samples at harvest during the 1998 hunting season in southeastern Nebraska (29). The study was designed to determine distribution patterns and estimate prevalence for EC O157:H7 (stx profile was not characterized). Their results did not
demonstrate a spatial or a geographic pattern that was statistically significant. The animal-level prevalence estimate was 0.25% (n=1608). Again, the study was conducted during the winter, which is the period that hunters would be at greatest risk for infection. Renter et al. and Sargeant et al. did not confirm the presence of stx in their field isolates (29,33).

Fischer et al. conducted experimental and field studies that indicated that WTD can be colonized and shed STEC O157:H7 (13). Six WTD (three-month-old) were orally administered a $10^8$ CFU cocktail of STEC O157:H7 strains. One strain was a deer isolate from the outbreak in Oregon (described above), two were human isolates, and two isolates were from cattle. Results from the experimental studies suggested that fecal shedding in WTD might be transient. The results were similar to the results from studies in other ruminant species.

At necropsy, Fischer et al. recovered STEC O157:H7 throughout the entire digestive tract of the deer following oral inoculation. Gross pathological lesions and adherent bacteria on mucosal surfaces were absent in the deer. Furthermore, attaching and effacing (AE) lesions were not detected on histopathologic examination of tissue sections.

Fischer et al. also demonstrated horizontal transmission when they isolated STEC O157:H7 from an uninoculated deer that was co-housed with a deer shedding STEC O157:H7 (13). By day two post-contact, the uninoculated deer shed STEC O157:H7 in its feces. Interestingly, eight STEC O157:H7 strains were isolated from two deer at the end of the contact transmission trial that were genotypically identical to the Oregon deer strain used in the original mixture of isolates. The authors suggested that some type of host specificity might account for this observation.
Data from two field studies conducted by Fischer et al. in 1997 estimated that the prevalence was 0% (n=310) in fresh deer fecal samples collected from the ground. In the second study, fecal samples collected from the ground and from hunter-harvested WTD were positive for STEC O157:H7 at a prevalence proportion of 0.6% (n=469). In 1998, fecal samples from hunter-harvested WTD were taken at the site that was positive in 1997, and STEC O157:H7 was not detected (n=140) (13).

Experimental work suggests that STEC O157:H7 transiently colonizes WTD. Prevalence studies have shown that STEC O157:H7/EC O157:H7 colonize WTD at low prevalence during the winter. In order to determine STEC O157:H7 colonization of WTD in Louisiana we conducted studies to: 1) estimate the point prevalence of STEC O157:H7 fecal shedding in hunter-harvested WTD (HH-WTD) during the at-risk period for venison contamination (HH-WTD study), and 2) estimate the point prevalence and describe seasonal trends in STEC O157:H7 fecal shedding in a captive herd of WTD by serial fecal culture for one year (Longitudinal study).

4.2. Materials and Methods

4.2.1. White-Tailed Deer (Odocoileus virginianus)

4.2.1.1. Hunter-Harvested White-Tailed Deer Study

In collaboration with the Louisiana Department of Wildlife and Fisheries (LDWF) Deer Study Group, we collected fecal samples at hunter check-in stations on Wildlife Management Areas (WMA) during the 2001 rifle season (38). WMAs that were sampled were selected based on the previous year’s harvest in an attempt to maximize the number of samples that we could obtain. Fecal samples (~20 grams) were collected per rectum with a clean glove, maintained at ambient temperature, transported to the laboratory, and placed in
culture media within twenty-four hours. Sampling was convenience based and dependent on hunter success.

4.2.1.2. Longitudinal Study

The Louisiana State University Agricultural Center (LSU AgCenter) maintains a herd of approximately 125 WTD to support research efforts on deer management and veterinary science (20). The research herd is maintained in high-fenced pastures and fed a grain-based concentrate. Individual deer are identified with ear tags. Beginning in December 2000, we collected fecal samples by entering the pen enclosures, observing individually identified deer, and picking up the fresh ground-deposited fecal pellets (~20 grams) from those individually identified deer. Cross-sectional samples were taken every other month (six cross-sectional samples), sampling was convenience based and animals were not handled or confined. Fecal samples were maintained at ambient temperature, transported to the laboratory, and placed in media within twenty-four hours.

4.2.2. Culture Methodology

Because the sensitivity of methods significantly influences the magnitude of the prevalence estimates, culture techniques were selected that have higher sensitivity than conventional enrichment and direct plating techniques (9,16,24,35). The culture techniques have been described previously and have primarily been used for culturing cattle feces (12,22).

Briefly, ten grams of fresh deer fecal pellets were placed in 90 ml of gram negative broth (Difco, Sparks, MD) supplemented with cefsulodin (10 mg/l, Sigma, St. Louis, MO), vancomycin (8mg/ml, Sigma, St. Louis, MO) and cefixime (0.05 mg/ml, Lederle, Pearl River, NY) and allowed to soften for fifteen minutes. The softened fecal pellets were then
manually emulsified and incubated for six hours at 37°C. Immunomagnetic separation (IMS) was performed on a 1 ml aliquot using Dynabeads anti-\textit{E. coli} O157 uniform, paramagnetic, polystyrene microscopic beads. The anti-\textit{E. coli} O157 beads have adsorbed and affinity purified antibodies against \textit{E. coli} O157 covalently bound to the surface (Dynal Inc., Lake Success, NY).

The immunomagnetic bead/broth suspension was then washed three times with 1 ml of phosphate buffered saline containing 0.05% Tween 20 (Sigma, St. Louis, MO) (PBS-Tween20) following capture of the paramagnetic beads using a magnetic particle concentrating separation rack (Dynal Inc., Lake Success, NY). Following the final wash the bead/bacteria complex was resuspended in 100 µl of PBS-tween20.

An aliquot of 50 µl of the bead/bacteria complex was spread plated on sorbitol MacConkey agar (ctSMAC) containing cefixime (0.05mg/l) and potassium tellurite (2.5mg/l) and incubated at 37°C for 18–24 hours. A maximum of three colonies with typical STEC O157:H7 phenotypic characteristics were selected as suspects and placed into 5 ml of MacConkey broth and 2 ml of trypticase soy broth (TSB) for 18–24 hours at 37°C.

4.2.3. Confirmation by Enzyme-Linked Immunosorbent Assay (ELISA)

Indirect enzyme-linked immunosorbent assay (ELISA) was performed for identification of isolate serotype (22). The ELISA was performed using murine monoclonal antibodies (MAb) to H7 antigen, anti-H7 MAb 2B7, and O157 antigen, anti-O157 MAb 13B3 (19,40). Briefly, 1 ml aliquots of the TSB and MacConkey cultures were heat killed by placing them in boiling water for 10 minutes. A positive control (\textit{E. coli} O157:H7; ATCC 43888), negative control (\textit{E. coli} O78:H11; ATCC 35401) and samples were added to ninety-six well plates and incubated at 37°C for one to two hours. Following incubation, the plates
were washed with ELISA wash buffer. MAb 13B3 or MAb 2B7 was then added, followed by incubation at 37° C for 10 minutes, and wash steps. A horseradish peroxidase labeled goat anti-mouse antibody was added followed by incubation and wash steps. One component ABTS (2,2’-azino-di (3-ethyl-benzthiazoline-6-sulfonate); KPL, Guilford, UK) substrate was then added and allowed to react for ten to twenty minutes at room temperature before stopping the reaction with 1% SDS (sodium dodecyl sulfate). Absorbance was read at dual wavelengths of 405/490. Reactions were considered positive at 0.2 + the optical density (OD) of the negative control.

Isolates reactive with MAb 13B3 (O157 positive ELISA) were inoculated in TSB and evaluated for motility using phase-contrast microscopy. Isolates that had the correct phenotype (1-2 mm, sorbitol-negative colonies) on ctSMAC, fermented lactose in MacConkey broth (blue to yellow color change), positively reacted with anti-O157 MAb 13B3, and positively reacted with anti-H7 MAb 2B7 or were non-motile (NM) were considered to be \textit{E. coli} O157:H7 or \textit{E. coli} O157:NM.

Additionally, isolates were tested by Sensititre gram-negative (AP80) autoidentification plates (Accumed International, Westlake, OH) and confirmed as \textit{E. coli} by reactions to thirty-two substrates. A single isolate from each positive sample was archived in a brain heart infusion (BHI) broth-glycerol suspension at –80° C for future characterization.

4.2.4. Characterization by Polymerase Chain Reaction (PCR)

Isolates were characterized by polymerase chain reaction (PCR) for \textit{rfb}_{O157} and \textit{fliC}_{H7}, and the putative virulence factors \textit{stx}1, \textit{stx}2, \textit{eae}A and \textit{hly}A. Somatic (O157), flagellar (H7) and virulence factor genes were amplified using previously published primer pair sequences. Duplex reactions were run for \textit{stx}1 and \textit{stx}2, as well as \textit{eae}A and \textit{rfb}_{O157}. PCR for \textit{hly}A and
were run as uniplex reactions. PCR cycling conditions were as previously described (15,27).

Amplified gene products were subjected to electrophoresis on a 2% agarose gel and then stained with ethidium bromide. Gel images were captured digitally, photographed and scored. Clear, well-defined bands of the correct size for tefb_{O157} (259 base pairs (bp)), fliC_{H7} (625 bp), stx1 (180 bp), stx2 (255 bp), eaeA (384 bp) and hlyA (534 bp) that were consistent with the positive control were considered positive PCR reactions. A well-characterized STEC O157:H7 strain was used as the positive control (ATCC 43895). A 100 bp DNA ladder was used as the standard. E. coli O157:H7 or O157:NM isolates were considered to be STEC O157:H7 or STEC O157:NM if PCR reactions for stx1, stx2 or both were positive.

4.2.5. Statistical Analysis

Prevalence estimates with 95% confidence intervals (CI) were calculated as the number of positive samples divided by the total number of samples (PEPI 4.0). Fisher’s exact statistics were utilized to estimate CIs for null values. Sampling in the longitudinal study was convenience-based. To evaluate the probability of the population (n=125) being disease free (zero culture positive samples) at each cross-sectional sample, given our sampling size, we calculated probabilities for various minimum prevalence levels (FreeCalc v.2, Angus Cameron 1998). Data were entered and stored in Excel 2000.

4.3. Results

4.3.1. Hunter-Harvested White-Tailed Deer Study

One stx-deficient, sorbitol-fermenting EC O157:H7 isolate was obtained during the HH-WTD study. Colonies were selected that had the correct phenotypic characteristics as described in the materials and methods section. Review of our laboratory records indicated
that the isolate had the correct phenotype but appeared to be slightly pink (partial fermentation). After streaking for isolation, the isolate readily fermented sorbitol confirming that it was sorbitol-positive. Sensititre autoidentification further confirmed that the isolate was sorbitol-positive as well as beta-glucuronidase positive, and identified it as E. coli (100% probability). PCR demonstrated clear, well-defined bands of the correct size for rfb\textsubscript{O157}, fliC\textsubscript{H7}, eaeA, and hlyA.

**Table 4.1. Prevalence proportion and 95% confidence intervals for *Escherichia coli* O157:H7 fecal shedding in hunter-harvested white-tailed deer at selected Louisiana Wildlife Management Areas (WMAs) during 2001**

<table>
<thead>
<tr>
<th>Date</th>
<th>WMA</th>
<th>n(pos) / n(tot)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>10/27/01</td>
<td>Boise-Vernon</td>
<td>0/94</td>
<td>0, 3.8</td>
</tr>
<tr>
<td>11/3/01</td>
<td>West Bay</td>
<td>0/26</td>
<td>0, 13.2</td>
</tr>
<tr>
<td>11/3/01</td>
<td>Jackson-Bienville</td>
<td>0/12</td>
<td>0, 26.5</td>
</tr>
<tr>
<td>11/17/01</td>
<td>Buckhorn/Tensas River NWR</td>
<td>0/58</td>
<td>0, 6.2</td>
</tr>
<tr>
<td>11/23/01</td>
<td>Sherburne</td>
<td>0/18</td>
<td>0, 18.5</td>
</tr>
<tr>
<td>11/23/01</td>
<td>Three Rivers</td>
<td>1\textsuperscript{a}/35= 2.9%</td>
<td>0.1, 14.9</td>
</tr>
<tr>
<td>12/1/01</td>
<td>Sherburne</td>
<td>0/95</td>
<td>0, 3.8</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>1/338= 0.3%</td>
<td>0.0, 1.6</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Isolate characterized as a sorbitol-positive, stx-deficient *Escherichia coli* O157:H7

The sampling date, sampling location (WMA), number of fecal samples (n(tot)), prevalence proportion, and 95% CI for EC O157:H7 are shown in Table 4.1. The overall prevalence proportion of EC O157:H7 fecal shedding was 0.3%. EC O157:H7 that possessed stxs (STEC O157:H7) were not detected.

Six WMAs were sampled at their respective hunter check-in stations. WMAs were distributed throughout the state. Figure 4.1 shows the location of the WMAs.
4.3.2. Longitudinal Study

The LSU AgCenter WTD research herd (n=125) was sampled every other month for one year. Table 4.2 lists the number of samples collected at each sampling date during the study. We isolated STEC O157:H7 from one deer in the herd during August (2001). The prevalence proportion of STEC O157:H7 fecal shedding in August was 1.8% (n=55).
We followed routine culture and isolation techniques as outlined in the materials and methods section. The serotype of the EC O157:H7 isolate was initially confirmed by broth ELISA. However, a viable isolate could not be recovered from MacConkey broth for archiving. We performed PCR on the isolate DNA directly from the MacConkey broth and characterized the isolate as the O157:H7 serotype possessing stx1 and stx2 genes (STEC O157:H7). The isolate also possessed eaeA and hlyA. Figure 4.2 shows composite results of the PCR characterization. The primers used are reported in the methods section. Positive and negative controls were used on each gel.
Figure 4.2. Composite results from MacConkey broth demonstrating polymerase chain reaction for the Shiga-Toxigenic *Escherichia coli* O157:H7 isolated from a White-Tailed Deer at the LSU AgCenter Idlewild Research Station (2% agarose gel).

4.4. Discussion

The prevalence of STEC/EC O157:H7 in WTD has been reported to be low (13,29,30,33). We sampled 338 HH-WTD at six WMAs during the 2001 Louisiana hunting season. One deer (1/338=0.3%) was found to be culture positive for EC O157:H7. Our findings are consistent with other studies conducted during the winter months that have reported that EC O157:H7 infrequently colonized WTD.
Characterization of the isolate indicated that it did not possess stxs but possessed other putative virulence factors common in the STEC O157:H7 clone recognized as a cause of human disease. Phenotypically the isolate was unusual in that it was sorbitol positive and beta-D-glucoronidase (GUD) positive.

There are at least three possibilities for the absence of the stx genes in the isolate: 1) stx is not present in the isolate because it has not been exposed to shigatoxin-converting bacteriophage, 2) stx was present in the isolate previously, but it has subsequently lost the phage-mediated virulence factor, or 3) PCR for stx1 and stx2 did not detect target sequences because of some inhibitory factor intrinsic to this isolate or possibly because the isolate has stx variations that were not amplified by our primer pair.

The EC O157:H7 isolates’ sorbitol fermentation and positive beta-glucuronidase reaction were unusual and unexpected. The laboratory records indicated that the colony was clonal on ctSMAC and had the correct phenotype, although it was slightly pink (partial fermentation) when it was selected. The isolate reacted with the H7 mononclonal antibody and was motile on phase-contrast microscopy. Sorbitol positive STEC O157:NM that are also GUD positive are a common STEC serotype isolated from HUS patients in Germany and have been associated with outbreaks of disease (2,7,21). Sorbitol fermenting strains of EC O157:H7 have recently been reported in Australia, but are thought to be rare in the U.S. and Canada (5,35). Cattle are reported to be a reservoir of these strains and a source of human disease in Europe but not in the U.S. and Canada (6). Sorbitol fermenting strains of EC O157:H7 have not been reported in deer. Further characterization of the isolate will be a component of ongoing studies.
Several authors in Germany have reported stx-deficient, sorbitol fermenting strains of EC O157:H7/NM that have been associated with human clinical cases of diarrhea and HUS (1,34). They suggest that these atypical EC O157:NM strains are responsible for human disease. Furthermore, the authors hypothesize that stx production in not obligatory for the pathogenicity of STEC O157 in humans (1,34).

Results of the longitudinal study conducted in the LSU AgCenter’s WTD research herd did not show a seasonal trend in STEC O157:H7 fecal shedding. We expected that if STEC O157:H7 was present and isolated in the deer herd, we would be able to describe seasonal changes in fecal shedding. One STEC O157:H7 isolate was obtained during the study during the month of August. Unfortunately the isolate died in MacConkey broth, but was characterized with PCR. Because our sampling strategy was convenience-based we did not set target sample sizes. Semi-tame WTD are difficult to work with and typically require tranquilization with a dart gun to perform routine physical exams or medical treatments. In order to avoid morbidity and mortality associated with handling and confinement, we elected to take a convenience sample from the herd. Ignoring that the herd was STEC O157:H7 positive in August, we used calculated freedom from disease probabilities (Null hypothesis: population is diseased) to evaluate the actual number sampled at each time point. The freedom from disease probabilities evaluating the number sampled from the herd (Table 4.2) demonstrate that our convenience samples did not produce a sample size that was adequate to label the population (n=125) as negative (95% confidence level) at the minimum prevalence levels reported in the literature (1% and 2.5%).

Summarizing the HH-WTD study, our findings are consistent with other studies that have found a low prevalence of EC O157:H7 in WTD during the winter. The lone isolate
from the study was a stx-deficient EC O157:H7 that fermented sorbitol, possessed GUD activity, and was motile. GUD positive STEC O157:H7 have been reported in Japan (26). This isolate is atypical and its potential public health significance is unclear. The previously mentioned studies in Germany indicate that an isolate of this type may be pathogenic in humans. Our culture method and criteria for selecting colonies does not normally detect isolates with a sorbitol positive phenotype. Atypical isolates that ferment sorbitol or are GUD positive, or demonstrate other phenotypic variability confound diagnostic techniques targeting the common sorbitol negative, GUD negative STEC O157:H7 clone (39).

In the longitudinal study, we isolated STEC O157:H7 from one deer in the herd during the summer, but we were unable to demonstrate seasonal trends in fecal shedding given one positive isolate. Our finding of low prevalence in the WTD herd is consistent with field studies in wild WTD that have observed low prevalence. The sample sizes were inadequate to rule out STEC O157:H7 fecal shedding in the herd. The STEC O157:H7 isolate was genetically characterized (PCR) despite dying in the MacConkey broth. Future studies targeting a statistically based sample size might demonstrate seasonal patterns of STEC O157:H7 fecal shedding if they exist in the LSU AgCenter WTD research herd or other captive deer herds.

In conclusion, STEC O157:H7 and atypical EC O157:H7 strains were isolated from WTD in our studies. The public health significance of the stx-deficient, GUD positive, sorbitol fermenting EC O157:H7 strain is not clear. Seasonal shedding patterns were not demonstrated but might be shown in future studies of captive WTD herds. Based on our studies and the current literature, WTD are colonized at low prevalence during the winter. WTD might have a role in the epidemiology of STEC O157:H7 in cattle populations because
WTD and cattle share rangeland. Additionally, people that consume venison that might be contaminated by STEC O157:H7 or atypical EC O157:H7 strains should be aware of the potential health risks.

4.5. References


CHAPTER FIVE

GENERAL DISCUSSION

5.1. Overview

We designed and implemented cross-sectional studies in dairy cattle, weaned beef cattle, and white-tailed deer (WTD) populations to investigate the epidemiology of Shiga-toxigenic *Escherichia coli* (STEC) O157:H7 in Louisiana. Each of these ruminant populations has been implicated in the transmission and carriage of STEC O157:H7 to humans. The various descriptive field studies were conducted beginning in December 2000 and ending in December 2001.

The epidemiology of STEC O157:H7 in human clinical disease and in ruminants is unclear. Domestic ruminant species, primarily dairy and beef cattle, are thought to be the primary reservoirs of STEC O157:H7. WTD, colonized by STEC O157:H7, are potentially involved in the maintenance of STEC O157:H7 in domestic ruminants and additionally pose a direct risk to people who consume venison. The epidemiology of STEC O157:H7 in domestic ruminants and WTD has not been described in Louisiana.

In addition to describing the results of our observational studies in dairy cattle, weaned beef cattle, and WTD, we addressed some of the enigmatic questions regarding the epidemiology of STEC O157:H7. The seasonal (summer) peaks in cattle fecal shedding of STEC O157:H7 and the latitudinal associated (south to north increase) human STEC O157:H7 incidence are perplexing issues in STEC O157:H7 epidemiology. These seasonal peaks in cattle fecal shedding are thought to be associated with seasonal changes in ambient temperatures. Louisiana is subtropical in climate, with relatively small fluctuations in seasonal temperatures and humidity. In our longitudinal study in dairy cattle, we described
increased seasonal (spring/summer) fecal shedding of STEC O157:H7 that was consistent with other studies.

Some authors have suggested that increases in human disease incidence may be associated with increases in the prevalence of STEC O157:H7 in cattle. Louisiana, like many southern states, has not reported a large number of human STEC O157:H7 cases. Our STEC O157:H7 prevalence results, in each ruminant population, were consistent with (weaned beef cattle and WTD) or greater (dairy cattle) than those reported in comparable U.S. studies.

5.2. Results

Cross-sectional studies in Louisiana dairy herds were designed to: 1) estimate cow-level and herd-level point prevalence of STEC O157:H7 fecal shedding (PP study), 2) describe seasonal shedding patterns in five herds sampled over one year (Longitudinal study), and 3) estimate the site-specific point prevalence in the oral cavity and on the dorsal hide surface (MHF study). The corresponding results for the dairy studies conducted during 2001 are as follows.

In the PP study, the point prevalence estimate in herds (n=13), sampled during the summer, was 38.5%, with a cow-level prevalence of 6.5%. Among positive herds, cow-level prevalence ranged from 3%-34.6%.

The Longitudinal study described the seasonal shedding patterns in five herds sampled in 2001. Three of the five herds sampled in the longitudinal study during the year had at least one positive sample (cow fecal sample) during the study. Among the positive herds a seasonal increase in cow-level prevalence was detected during spring (13.3%) and summer (10.5%), compared to fall (1.0%) and winter (1.0%). Logistic regression analysis of the longitudinal study data indicated that adult dairy cattle during the warm season had
significantly increased odds of being a STEC O157:H7 fecal shedder than those sampled during colder months. Additionally, lactating dairy cows showed increased odds of being STEC O157:H7 fecal shedders compared to non-lactating (dry) cows, although not at statistically significant levels.

In the MHF study, only one isolate was obtained from the hide of a cow and no isolates were obtained from the mouth samples. The cow-level prevalence estimates of STEC O157:H7 in the oral cavity, on the dorsal hide surface, and from feces were 0%, 0.7% and 25.2%, respectively.

One study in beef cattle was conducted. Weaned beef cattle entering a preconditioning program (CTC program) were sampled upon arrival (T1) and at the end of a forty-five feeding period (T2). Calves from various herds were commingled, given preconditioning treatments (vaccinations, anthelmintic treatments, etc.), and fed a grain-based ration containing an antibiotic. Five of the twenty-nine herds (17.2%) enrolled in the CTC program were found to be shedding STEC O157:H7 or *Escherichia coli* (EC) O157:H7 at T1. No cattle were found to be shedding STEC O157:H7 or EC O157:H7 at T2 following the preconditioning. Our findings were consistent with those of the previous studies of cow-calf farms and in weaned calves, from other regions in the U.S., which have demonstrated that STEC O157:H7 colonizes calves prior to aggregation in feedlots. We found 0.7% (CI 0.2, 2.1) of the weaned beef cattle in the CTC cohort shedding STEC O157:H7. The animal-level prevalence of the O157:H7 serotype, which included *stx*-deficient (shigatoxin deficient) isolates, was 2.5% (CI 1.2, 4.5). Herd-level prevalence (17.2%) was lower than reported in previous studies. We expected, but did not observe, increased fecal shedding or the spread of STEC O157:H7 subtypes at T2.
Two field studies in WTD populations were conducted in Louisiana during 2000/2001. The first study was a hunter-harvested WTD (HH-WTD study) prevalence study conducted during the 2001-hunting season. We collected 338 fecal samples from HH-WTD and found one positive WTD fecal sample (1/338=0.3%, CI 0, 1.6). The lone isolate from the study was not characteristic of the common STEC O157:H7 clone because it was stx-deficient, sorbitol positive, and beta-D-glucuronidase (GUD) positive.

In the second field study (Longitudinal study), we isolated STEC O157:H7 from one deer in a captive herd during the summer, but were unable to demonstrate seasonal trends in fecal shedding given one positive isolate. We sampled the herd six times from December 2000 to September 2001. In August, one of fifty-five (1.8%, CI 0, 9.7) fecal samples was positive for STEC O157:H7. The isolate died, but was genetically characterized and found to possess virulence factors that were consistent with the common STEC O157:H7 clone most frequently implicated in human clinical disease.

5.3. Conclusions

We detected STEC O157:H7 in each population of ruminants (dairy cattle, weaned beef cattle, and WTD) during our field studies. Summarizing the studies among dairy cattle, we found high STEC O157:H7 fecal prevalence in adult dairy cows relative to other U.S. studies. We attribute some of the difference in magnitude to the increased sensitivity of the culture methods that we used. We used selective enrichment and immunomagnetic separation (IMS) techniques that have been shown to be more sensitive than conventional culture methods.

Furthermore, we demonstrated seasonal increases in STEC O157:H7 fecal prevalence in adult dairy cattle that were consistent with published results and showed quantitatively that
cows sampled during warmer months are at increased odds of being fecal shedders. The factors that influence seasonal increases in STEC O157:H7 fecal shedding might be related to environmental temperatures or other unknown factors that are not currently defined in cattle. Differential dietary factors or the stress of lactation might account for the increased odds of STEC O157:H7 fecal shedding in lactating cows relative to dry cows that we observed in the longitudinal study. Increasing the sample size of the study and stratifying the effect of lactation by days-in-milk rather than the wide lactation categories that we used might show statistically significant differences in fecal shedding among cows at different stages of lactation/feeding.

In the MHF study, we found high STEC O157:H7 fecal prevalence among the adult cows in the lactation string relative to the level reported in other studies of adult cattle. We also isolated viable STEC O157:H7 from the dorsal hide surface of one cow. Viable STEC O157:H7 on the hide surface pose a risk to susceptible humans that have direct contact with animals at dairy farms, fairs, or petting zoos. Our data suggest that visitors to farms, fairs, and petting zoos should be made aware of potential risks associated with direct contact with dairy cows that might be colonized on hide surfaces with STEC O157:H7.

In the cohort of weaned calves in the CTC study, we observed that the prevalence proportion of STEC O157:H7/EC O157:H7 was consistent with that of previous studies of weaned range calves. As demonstrated in other studies, STEC O157:H7 appears to be widely dispersed throughout the U.S. breeding (cow-calf) populations. Calves are probably exposed and possibly colonized prior to arrival in feedlots. Intervention in cow-calf herds might be difficult to implement because STEC O157:H7 appears to be widely dispersed at the farm level. Certain intervention strategies (e.g., vaccination) might be more amenable to farm-
level intervention. We did not observe increased fecal shedding or the spread of unique STEC O157:H7 subtypes. The absence of fecal shedding following the forty-five-day feeding period might be attributable to seasonal influences, inhibitory concentrations of oxytetracycline in the transition ration, or transient colonization and cessation of fecal shedding prior to sampling at T2. Preconditioning programs or other points of pre-feedlot aggregation of calves might be useful points at which to incorporate intervention strategies or perform controlled trials in the future (see proposed future studies below).

Our studies in WTD demonstrated low prevalence proportions of STEC O157:H7/EC O157:H7 in both studies. We could not demonstrate seasonal trends in fecal shedding during the longitudinal study because we only isolated STEC O157:H7 once. Due to difficulties inherent in working with WTD, we could not achieve optimal sample sizes. An increased effort in sampling more herds might achieve sample sizes that would detect seasonal changes in STEC O157:H7 fecal shedding in WTD similar to those demonstrated in cattle. Our culture methods are not designed to detect sorbitol positive isolates. The isolate from the HH-WTD study was selected because it had the correct phenotype and only partially fermented sorbitol. STEC O157:H7 or EC O157:H7 isolates, like the stx-deficient EC O157:H7 isolate in the HH-WTD study, having atypical phenotypic characteristics would probably be missed by our culture method. Atypical STEC O157:H7 isolates are not as significant in human disease in the U.S. as the common STEC O157:H7 clone. The potential for emergence of novel STEC with unique phenotypes and genetic characteristics complicates diagnostic protocols. Our findings indicate that STEC O157:H7 and EC O157:H7 colonize WTD in Louisiana and that potential risks exist for those who handle WTD and consume venison.
5.4. Proposed Future Studies

It is necessary to reconsider the risk factor epidemiology of STEC O157:H7 in ruminants and repeat earlier studies using newer methods (IMS), considering the differences in the sensitivity of methods. Earlier studies, particularly in dairy cattle, used methods that were not sensitive making association of fecal shedding with risk factors difficult. We suggest longitudinal epidemiology studies in dairy cattle that use sensitive methods and adequate sample sizes to determine consistent associations between dietary factors, production indices, environmental factors or management factors and STEC O157:H7 colonization and fecal shedding.

Determination of the factors accounting for herd-to-herd variation will be a key component in future studies. The generalized estimating equation approach to logistic regression (GEE-LR) is one way to control for herd-to-herd variation in the analysis. The study design must incorporate a larger number (>20) of herds (clusters) and have adequate mechanisms for obtaining herd and individual level data.

Modifying the culture techniques used in the MHF study might detect a higher prevalence of hide contamination and contamination of the oral cavity in dairy cattle. For example, a previous study in beef cattle found high prevalence proportions in the mouth and on the hide but used methods designed for studies in beef cattle feedlots. We suggest repeating the study in dairy cows from multiple herds with high STEC O157:H7 fecal prevalence and varying our culture methodology.

The collaboration with the CTC program operated by the LSU AgCenter was beneficial. Future work with similar programs that provide large numbers of weaned beef calves should be conducted longitudinally using serial sampling to follow the calves through
the feedlot. Louisiana is primarily a cow-calf production state, so further studies in beef cattle should focus on the epidemiology of STEC O157:H7 in the calf crop that will be marketed to feedlots. We propose future studies that will determine the effect of feeding the medicated transition ration to preconditioning calves. Randomly assigning calves to treatment and control cohorts will discern if differences in colonization and fecal shedding of STEC O157:H7 occur secondary to the medicated transition ration. If the cohort consuming the medicated feed were to demonstrate an absence of fecal shedding, as occurred in our pilot study, perhaps this type of preconditioning feeding program could be used to reduce the levels of STEC O157:H7 entering feedlots. The study design should incorporate a decreased sampling interval. Sampling the treatment and control cohorts at two-week intervals will increase the probability of detecting transmission and strain sharing in initially negative calves.

As mentioned, an increased sample size in the farmed WTD herd might have resulted in the demonstration of seasonal changes in STEC O157:H7 fecal shedding. Because the WTD basic ruminant physiology is similar to cattle and experimental evidence suggests that fecal shedding is similar, we would like to repeat the study in several captive herds of WTD. Study design will have to account for the problems inherent in sampling semi-tame deer and include an increased effort targeting statistically based sample sizes in an effort to test the seasonal shedding hypothesis.

Our findings in Louisiana indicate that STEC O157:H7 colonizes each ruminant population at prevalence levels consistent with (weaned beef cattle and WTD) or greater (dairy cattle) than those reported in comparable U.S. studies. We propose that factors other than colonization rates of ruminant populations might explain the geographic differences
observed in human incidence. Studies investigating the population-adjusted incidence of human disease should be performed. Factors that might contribute to the perceived differential levels of human incidence include differential levels of immunity in humans, differential levels of surveillance and reporting by state public health agencies, or failure to use standardized diagnostic procedures by laboratories.

Collaborative studies are ongoing as a direct result of the work presented here. We are currently collaborating with the Louisiana Office of Public Health to perform a descriptive analysis of Enterohemorrhagic *E. coli* O157:H7 human clinical isolates and a comparative characterization analysis of those human isolates and the isolates obtained in our field studies in 2001 and recent isolates obtained from livestock during the 2002 Louisiana State Fair.

Further analysis of the stx-deficient EC O157:H7 isolates found in our HH-WTD study and the CTC study is being conducted collaboratively with scientists at the USDA-Meat Animal Research Center. We plan to probe the genomic DNA with other primer pairs designed to amplify the full-length stx genes, as well as probing with primers designed to amplify stx2 variants. If alternative primer pairs detect stx genes, comparative sequencing will follow to describe sequence variability and to determine sequence homology between reference strains and Louisiana field isolates.

Lastly, we plan to perform composite cluster analysis (Bionumerics software) on all phenotypic and genetic characterization data from the STEC O157:H7 and EC O157:H7 field isolates that we have archived from our studies. We plan to describe how the known epidemiologic information corresponds to clusters of isolates that are formed based on the phenotypic and genetic characterization data.
VITA

John Robert Dunn was born March 7, 1968, in Kansas City, Kansas, to Dr. and Mrs. George Dewey Dunn. He grew up in Nashville, Tennessee, where he graduated from Hillsboro High School in 1986. John then enrolled at Louisiana State University. In December 1991, he was awarded the Bachelor of Science degree in Zoology.

In August 1993, he began veterinary school at Louisiana State University School of Veterinary Medicine. In 1995, John was selected for the Pfizer Education Alliance Award. He also received the 1997 Louisiana Cattlemen’s Award for proficiency in large animal medicine and surgery. John received his Doctor of Veterinary Medicine degree in May 1997. Dr. Dunn practiced veterinary medicine until the spring of 2000 in Zachary, Louisiana.

In January 2000, Dr. Dunn began to pursue the Doctor of Philosophy degree in the Department of Epidemiology and Community Health. During his doctoral training he has taught courses and conducted collaborative research with scientists at the United States Department of Agriculture. During this time he received the 2001 American Veterinary Medical Association leadership conference award. He also received the Phi Zeta Veterinary Research Honor Society’s second place award for his doctoral research. Dr. Dunn’s research has focused on foodborne pathogens and the epidemiology of those pathogens in livestock populations. His interests are in infectious disease epidemiology and public health veterinary practice.

In May 2003, John will receive the degree of Doctor of Philosophy from the School of Veterinary Medicine (epidemiology). He has accepted an offer to join the United States Public Health Service working as an Epidemic Intelligence Service Officer with the Centers for Disease Control and Prevention.