DISSERTATION

ECOLOGY AND PERSISTENCE OF *ESCHERICHIA COLI* O157:H7 IN FEEDLOT CATTLE AND CHARACTERIZATION OF MOLECULAR MECHANISMS RESPONSIBLE FOR ATTACHMENT

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY BRANDON ADOLPH CARLSON ENTITLED ECOLOGY AND PERSISTENCE OF *ESCHERICHIA COLI* O157:H7 IN FEEDLOT CATTLE AND CHARACTERIZATION OF MOLECULAR MECHANISMS RESPONSIBLE FOR ATTACHMENT BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

ECOLOGY AND PERSISTENCE OF *ESCHERICHIA COLI* O157:H7 IN FEEDLOT CATTLE AND CHARACTERIZATION OF MOLECULAR MECHANISMS RESPONSIBLE FOR ATTACHMENT

Studies were conducted to elucidate the shedding dynamics and ecology of *Escherichia coli* O157:H7 in feedlot cattle. Feedlot cattle (N=788) were evaluated for *E. coli* O157:H7 shedding six times during the final 120 d of finishing. Fecal samples were analyzed for *E. coli* O157:H7 with IMS and confirmed with multiplex PCR. During the first two collections, where all 788 steers were samples, 39.8 and 33.6% of steers were shedding an *E. coli* O157:H7 isolate possessing *eae, stxI,* and *stxII.* Through subsequent sampling, 1% of steers were characterized as persistent *E. coli* O157:H7 shedders (PS) where as 1.4% of steers were never shedding a detectable amount of the organism. Molecular characterization of *E. coli* O157:H7 isolates obtained from PS (n=80) and transient *E. coli* O157:H7 shedders (n=52) revealed a diverse but closely related population of isolates and identified a predominant subtype that accounted for 53% of the isolates characterized that was not dependent ($P > 0.05$) on animal shedding status. Pathogenic potential of *E. coli* O157:H7 isolates representing different subtypes was delineated with a Caco-2 cell (intestinal epithelial cell line) attachment assay. There was an inverse relationship ($P < 0.05$) between genetic diversity and attachment efficacy; as diversity from the dominant subtype increased, ability to attach to Caco-2 cells

diminished. Additional attachment assays were initiated to evaluate the influence of virulence genes upon *E. coli* O157's ability to attach to Caco-2 cells. *E. coli* O157 isolates without either *stx*, no *stxI,* and no *stxII* genes resulted in attachment abilities of 76.7, 65.5 and 57.7%, respectively; all of which were greater ($P < 0.05$) than an *E. coli* O157:H7 that was isolated from a food implicated in human disease and possessed both *stx* genes. Cytotoxicity assays were utilized to verify that differences in attachment efficacy, exhibited by *E. coli* O157 isolates of various virulence genotypes, were independent of cellular destruction.

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CHAPTER I

OBJECTIVES OF DISSERTATION

Beginning in 1996, the harvest segment of the beef industry was mandated by the federal government to implement mitigation strategies designed to control *Escherichia coli* O157:H7 contamination of beef carcasses during the animal-to-carcass conversion process. Initially, the beef industry focused their efforts on the design and implementation of effective antimicrobial interventions applied during beef harvest subsequently providing the foundation of the enhancement of beef carcass microbiological quality. The next logical progression in further enhancing the efficacy of the beef industry's food safety interventions is to elucidate the ecology of *E. coli* O157:H7 to permit the implementation of effective antimicrobial interventions applied during the finishing of fed cattle to reduce the pathogenic load entering the processing facility.

Thus, the objectives of studies presented within this dissertation are to determine if *E. coli* O157:H7 persistence in feedlot cattle truly exists while concurrently elucidating the ecology of *E. coli* O157:H7 persistence in feedlot cattle. Specifically, initiate studies to probe the molecular mechanisms responsible for *E. coli* O157:H7's ability to attach to intestinal epithelium and, to persist in the intestine.

CHAPTER II

LITERATURE REVIEW

2.1. *Escherichia coli* O157 and Public Health

Escherichia coli serotype O157:H7 is a motile, gram-negative, rod-shaped bacterium within the *Enterobacteriaceae* family that was first discovered to cause human disease in the early 1980's (Nataro and Kaper, 1998). It was not until 1982, when *E. coli* O157:H7 was isolated from stool samples obtained from patients who exhibited distinctive gastrointestinal distress characterized by severe crampy abdominal pain, initial watery diarrhea that advanced to bloody diarrhea, all while exhibiting little or no fever, that the organism had ever been detected and associated with human disease (Riley et al., 1983). More importantly, *E. coli* O157:H7 infection can progress into hemolytic uremic syndrome (HUS) which is the most common cause of acute renal failure in children (Siegler, 1995). In an effort to demonstrate the significance of foodborne illness upon public health, Mead et al. (1999) utilized data from public health laboratories to extrapolate society's burden from foodborne illness. Alone, *E. coli* O157:H7 was estimated to cause 73,480 illnesses with a 0.295 hospitalization rate and a 0.0083 case fatality rate; 85% of these illnesses were attributed to foodborne transmission (Mead et al., 1999). Additionally, the researchers believed that underreporting of *E. coli* O157:H7 illnesses could increase the previously reported data by approximately 20 fold (Mead et al., 1999). In 1996, the Centers for Disease Control and Prevention (CDC) began monitoring foodborne diseases in 10 states to ascertain the influence of foodborne disease

on society's welfare. *E. coli* O157:H7 infections peaked in 1999 and progressed in a downwards trend until 2004, even surpassing the goal set forth by the United States Department of Health and Human Services (USDHHS) - Healthy People 2010 initiative (USDHHS, 2000) by 2001 (CDC, 2009a). However, *E. coli* O157:H7 infections have increased since 2004; particularly in persons > 50 years of age where *E. coli* O157:H7 infections accounted for 53.3% of all foodborne illnesses resulting in hospitalization during 2008 (CDC, 2009a). Indicative of *E. coli* O157:H7's continued risk to public health is the increase of case fatality rate from earlier estimations to 2.8% in children < 4 years of age (CDC, 2009a) and the subsequent amplification of risk associated with aging of the US population (CDC, 2003).

2.2. *E. coli* O157 and the Beef Industry

It is generally accepted that ruminants, predominately bovines and bovine-derived products, serve as a major route of *E. coli* O157:H7 transmission to humans. This theory was highlighted in 1982 when 47 individuals became ill following the consumption of undercooked ground beef that was contaminated with *E. coli* O157:H7 (Riley et al., 1983) and again in 1993 when more than 700 individuals were infected with *E. coli* O157:H7 following consumption of contaminated ground beef that did not receive a sufficient lethality treatment, subsequently resulting in four deaths (Bell et al., 1994; Tuttle et al., 1999). Ensuing regulatory action by the Food Safety and Inspection Service of the United Stated Department of Agriculture (FSIS) required all federally inspected beef plants to adhere to the "Zero Tolerance" policy in which all visible contamination must be removed before carcass washing and chilling (Sofos and Smith, 1998). With intentions to even further reduce the risk of producing beef products contaminated with

E. coli O157:H7, FSIS required beef plants to adopt the hazard analysis critical control point (HACCP) system (FSIS, 1996). The HACCP system requires an establishment to conduct a hazard analysis to identify all potential hazards (i.e., chemical, microbiological and physical) that could likely occur during production, identify critical control points in the process where a hazard can be prevented, eliminated or reduced to an acceptable level, and validation procedures which ensure that the critical control points effectively mitigate the identified hazards that are likely to occur during production.

The complexity and highly segmented structure of the beef industry consequently resulted in the identification of the harvest sector as the most logical sector at which to implement pathogen mitigation strategies, as the majority of nation's beef herd is processed through a relatively small number of plants. Correspondingly, the focus of pathogen mitigation development has historically been directed at carcass decontamination applied during the conversion of animals to carcasses. Demand to develop effective and practical carcass decontamination interventions persuaded research institutions to evaluate the antimicrobial efficacy of many antimicrobial interventions. Subsequently, all of the carcass decontamination interventions currently employed in beef harvest facilities (e.g., knife trimming, steam-vacuuming, pre- and post-evisceration carcass washes, organic acid sprays and steam pasteurization) were all developed in response to FSIS's regulatory policy and deemed to be effective in improving the microbiological quality of carcasses (Koohmaraie et al., 2005; Sofos and Smith, 1998; Huffman, 2002). Further investigation of methods to enhance the efficacy of an establishment's pathogen mitigation strategy identified that when multiple antimicrobial interventions are applied sequentially, antimicrobial efficacy is considerably amplified

compared to when antimicrobial interventions are applied independently, a process referred to as "Multiple-Hurdle Technology" (Bacon et al., 2000; Leistner and Gould, 2002). The incorporation of effective pathogen mitigation strategies are responsible for the overall decreasing trend in the number of beef product recalls due to contamination with *E. coli* O157:H7 experienced by the beef industry from 2002 to 2007 (FSIS, 2009a).

In 2007, after five years of a minimal number of recalls of beef products contaminated with *E. coli* O157:H7, the beef industry experienced a record-breaking year of recalls of adulterated product accentuated by a single 21.7 million pound recall of ground beef (FSIS, 2009a). Additionally, the percentage of ground beef samples testing positive for *E. coli* O157:H7 almost doubled from 0.24% in 2007 to 0.47% positive in 2008; the percentage of positives in 2009 are poised to exceed 2008 levels (FSIS, 2009b). Increases in the number of beef product recalls and in the number of positive samples prompted the CDC to state "occurrence of large multistate outbreaks point to gaps in the current food safety system and the need to continue to develop and evaluate food safety practices as food moves from the farm to the table" (CDC, 2009a). Thorough research has evaluated and confirmed the effectiveness of the antimicrobial intervention systems employed within the beef industry's harvest facilities (Bacon et al., 2000; Elder et al., 2000; Arthur et al., 2002, 2004; Woerner et al., 2006). Thus, the next logical progression to complement post-harvest antimicrobial interventions is to reduce the amount of *E. coli* O157 contained in/on cattle entering the plants (Callaway et al., 2004; Loneragan and Brashears, 2005). Research has implicated the hide as the principal source of carcass microbial contamination (Bacon et al., 2000; Elder et al., 2000) and thus, garnered most of the attention regarding pre-harvest pathogen mitigation. Several studies have

demonstrated the improvement in carcass hygiene when hide-on decontamination interventions are utilized during processing (Nou et al., 2003; Bosilevac et al., 2004, 2005). It stands to reason that if *E. coli* O157 levels on hides were reduced there would be a subsequent amelioration effect on carcass hygiene.

The relationship between *E. coli* O157 fecal shedding and carcass prevalence is well documented (Woerner et al., 2006; Loneragan and Brashears, 2005; Arthur et al., 2004; Elder et al., 2000). While the hide and, more specifically, the hide removal process present the most significant challenge in precluding *E. coli* O157 contamination on beef carcasses; the greatest contributor to hide prevalence is live animal fecal shedding (Loneragan and Brashears, 2005). Sampling fecal pats in feedlot pens three days before slaughter emphasized the significance of *E. coli* O157 fecal prevalence. In pens where 20% of fecal pats were positive, 2.5% and 0.6% of post-evisceration carcasses and carcasses in the cooler, respectively, were positive for *E. coli* O157 (Woerner et al., 2006). However, in pens with fecal pat prevalence below 20%, none of the resulting carcasses was positive for *E. coli* O157 when sampled at post-evisceration or in the cooler (Woerner et al., 2006). Utilization of logistic regression to model the influence of *E. coli* O157 fecal prevalence on *E. coli* O157 hide prevalence revealed the significance of fecal prevalence as an indicator of hide prevalence (Loneragan and Brashears, 2005). The *E. coli* O157 fecal shedding burden associated with public health is not exclusive to contaminated beef products; recent outbreaks of human illness have accentuated the indirect burden that bovine fecal shedding of *E. coli* O157 has on public health. A historical illustration of that indirect burden was a case where a town's water supply was contaminated with *E. coli* O157 following an unusually heavy rainfall event that washed

E. coli O157 contaminated bovine fecal material into the water basin where the town derived its water (Matsell and White, 2009). A more recent incident, that infected considerably more people in several states, and crippled the produce industry, was an instance where a vector (feral swine) were contaminated in a bovine environment and consequently broadcast the pathogen over produce fields (Jay et al., 2007). *E. coli* O157 fecal shedding and subsequent hide contamination also present increasingly adverse health risk to human participants who attend and interact with animals at petting zoos and livestock shows (Keen et al., 2007; Chapman et al., 2000). Taken together, the previous information warrants the significant burden of *E. coli* O157 in live animal populations and its potential deleterious effects on public health, and ultimately necessitates its control.

2.3. *E. coli* O157 Shedding and Persistence in the Bovine Reservoir

E. coli O157 shedding is tremendously variable and occurs in all classifications of cattle. Following the 1993 *E. coli* O157:H7 outbreak in several western states, the Animal Plant Health Inspection Service (APHIS) of the USDA initiated an extensive evaluation of *E. coli* O157 prevalence in US feedlots (Hancock et al., 1997). This particular study recruited 1,411 feedlots in 13 states and collected 11,881 fecal samples with 1.8% of the samples positive for *E. coli* O157 (Hancock et al., 1997). Unbeknownst to the authors at the time, they potentially underestimated *E. coli* O157 prevalence as they collected their samples from October to December, a time period now known for reduced *E. coli* O157 prevalence. In years since the APHIS study, numerous *E. coli* O157 prevalence studies have been conducted. In an analysis of pertinent literature published from 1997 to 2003, Hussein and Bollinger (2005) summarized *E. coli* O157 fecal

prevalence in feedlot cattle to be between 0.3 to 13.3%. However, recent studies report *E. coli* O157 fecal prevalence in feedlot cattle to range between 0.0 to 77.8% (Berg et al., 2004; Woerner et al., 2006; Cobbold et al., 2007; Dewell et al., 2005, 2008; Fox et al., 2008). Researchers also have established the "hot season" theory in which *E. coli* O157 fecal frequency and, subsequently, hide prevalence spike in the summer months (Barkocy-Gallagher et al., 2003; Khaitsa et al., 2003; Cobbold et al., 2007) and generally coincides with an increase in outbreaks of *E. coli* O157 associated disease (CDC, 2007). A potential mechanism responsible for the "hot season" *E. coli* O157 spike may be related to day length and melatonin levels (Endrington et al., 2006), a hypothesis that needs to be investigated further.

The lack of sensitive and specific methods to enumerate *E. coli* O157 has slowed the widespread quantification of *E. coli* O157 shed in bovine feces. However, studies have reported that naturally infected cattle can shed *E. coli* O157 at levels of 4 to 5 log CFU/g of feces (Brichta-Harhay et al., 2007; Cobbold et al., 2007; Stephens et al., 2008), but that most shed *E. coli* O157 at concentrations less than 2 log CFU/g (Omisakin et al., 2003). Animals that shed > 3 log CFU/g of feces have been referred to as "super shedders" (Low et al., 2005). While it is conceivable that any viable *E. coli* O157 shed in bovine feces maintains the potential to proliferate and expatiate prevalence levels, there are certain research groups that believe that super shedders are responsible for upwards of 80% of *E. coli* O157 shed in bovine feces (Matthews et al., 2006b; Cobbold et al., 2007). Few studies have evaluated the natural duration of persistence of *E. coli* O157 fecal shedding in feedlot cattle. Intensive (multiple samples collected per day) fecal sampling, conducted on two different cohorts of 6 to 11 month old dairy calves, revealed two

animals in each cohort (14 and 12.5% of total) persistently shedding *E. coli* O157 for 4 and 15 d, respectively (Robinson et al., 2004). Observation of *E. coli* O157 fecal shedding in feedlot cattle determined that a small number of animals (n = 8) shed the organism for a maximum of 4.5 weeks, with the remaining sample population shedding for an average duration of 2.5 weeks (Khaitsa et al., 2003). A similar *E. coli* O157 shedding duration of approximately one month was estimated after sampling cohorts of dairy cows (Besser et al., 1997). Experimental inoculation of calves with *E. coli* O157:H7 established a maximum shedding duration of at least 14 d (Naylor et al., 2003), 43 d (Sanderson et al., 1999), 70 d (Besser et al., 2001) and 140 d (Cray and Moon 1995). Experimental inoculation of cattle > 0.5 years of age resulted in persistent fecal shedding for at least 29 d (Grauke at al., 2002), 66 d (Sheng et al., 2004), and 98 d (Cray and Moon 1995). Assessment of published literature supports a general trend of increased *E. coli* O157:H7 shedding duration when evaluated in neonates and calves < 1 year of age. As animal age increases, persistent shedding shortens and becomes increasingly intermittent.

2.4. Genetic Diversity of *E. coli* O157 in the Bovine Reservoir

Initial investigation into the genetic diversity of *E. coli* O157 suggested that there could possibly be hundreds or even thousands of *E. coli* O157 subtypes within a specific cattle population (Rice et al., 1999). However, the previous study characterized *E. coli* O157 isolates with multiple *E. coli* O157 virulence genotypes (i.e., compared *E. coli* O157 isolates that were not genetically identical given PCR results), inherently biasing the number of PFGE subtypes observed in their study. Conversely, recent research supports an ever growing body of evidence pertaining to the highly conservative nature of *E. coli* O157:H7 within cattle populations (Robinson et al., 2004; Scott et al., 2006;

Stanford et al., 2005b; Besser et al., 1997; Shere et al., 1998). Correspondingly, all 54 *E. coli* O157 isolates obtained from two different dairies and characterized by PFGE resulted in only one subtype (Robinson et al., 2004). Analysis of genetic diversity of *E. coli* O157:H7 collected from four dairies located in a 30 km area in southern Alberta also demonstrated a highly clonal genetic homology as three dominant subtypes, detected in every dairy, accounted for a majority of the isolates characterized (Stanford et al., 2005b). It can be argued that dairy environments have an increased likelihood of high *E. coli* O157:H7 relatedness because of the lack of animal turnover, which generally are thought to be the main source of new *E. coli* O157:H7 subtypes, which is typical of commercial feedlots. However, even with increased cattle turnover, feedlots can maintain a highly related population of *E. coli* O157:H7. Analysis of 103 *E. coli* O157:H7 isolates obtained from a commercial feedlot with PFGE resulted in identification of three subtypes, with subtypes differing by no more than two bands and all isolates clustered within 80% similarity (Scott et al., 2006). Although delineation of 230 feedlot *E. coli* O157:H7 isolates with PFGE resulted in 56 diverse subtypes, 60% belonged to four closely-related subtypes (LeJeune et al., 2004). Indistinguishable *E. coli* O157:H7 subtypes were recovered from two feedlots that were approximately 100 km apart and did not share any common source of animals that entered the feedlot (Van Donkersgoed et al., 2001). The researchers subsequently hypothesized that wild birds are vectors of *E. coli* O157:H7 and potentially serve as the transmission source of indistinguishable *E. coli* O157:H7 subtypes between two feedlots.

2.5. *E. coli* O157 Attachment and Colonization to Mammalian Epithelial Cells

In both disease and asymptomatic colonization, *E. coli* O157 must adhere to the intestinal epithelial layer of the host to evade the host's normal peristaltic flow. *E. coli* O157 exploits a repertoire of genes to initiate a highly sophisticated and organized cascade of machinery to encourage preliminary contact and subsequent intimate attachment. Motility has never been questioned regarding its role in a pathogen's virulence, but recent research has elucidated additional functions of accessory organelles during the preliminary relationship between host and bacterium. Flagella, hallmark indicators of motility, are believed to confer adherence to bovine intestinal epithelial cells by browsing eukaryotic surfaces and binding to accessible receptor(s), potentially mucin and perhaps other gylcoconjugates (Mahajan et al., 2009). Although the actual mechanism that flagella utilize to initiate adherence is not fully comprehended, research has demonstrated that deficient flagella expression (Bretschneider et al., 2007) and immunization against flagella (McNeilly et al., 2008) diminishes colonization of cattle with *E. coli* O157:H7. In recent years, researchers have begun investigating the function of other genes located outside the *E. coli* O157:H7 pathogenicity island and their capacity as liaisons in bacterial attachment. Recent discoveries have included the long polar fimbrae (Fitzhenry et al., 2006) and the *E. coli* common pilus (ECP)(Rendón et al., 2007). Long polar fimbrae are thought to encourage attachment of *E. coli* O157 to intestinal epithelium through similar mechanisms as flagella. The deletion of genes responsible for fimbrae expression resulted in an attenuated *E. coli* O157 attachment phenotype when compared to wild type *E. coli* O157 (Fitzhenry et al., 2006; Torres et al., 2008). Although the role of the ECP is not completely understood, research has demonstrated that its expression accentuates the attachment efficacy of pathogenic *E. coli* by stabilizing

the bacterium during the initial stages of adherence (Rendón et al., 2007). Although the mechanism through which *E. coli* O157 accessory organelles support initial adherence of the bacteria to the eukaryotic cell is not completely illuminated, this subtle interaction may be sufficient to elicit the release of catecholamines from the stressed epithelial cells. Research that has indicated the catecholamines, such as epinephrine and norepinephrine, can perform as signal molecules to *E. coli* O157 and induce production of virulence factors subsequently ameliorating attachment ability (Bansal et al., 2007).

Although many mechanisms encourage recognition of, and attachment to, eukaryotic host cells, colonization is dependent upon the genetic scaffolding contained on the Locus of Enterocyte Effacement (LEE). The LEE is a 35 to 45 kb pathogenicity island that is comprised of up to 50 genes, of which *tir* and *eae* orchestrate intimate attachment (McDaniel and Kaper, 1997). Also incorporated into the LEE are the *esp* family of genes which encode a Type III secretion system (TSS) that *E. coli* O157 exploits to insert its translocated intimin receptor (Tir) into the eukaryotic host cell and consequently, expressed on the cell's surface (Torres et al., 2005). Initial attachment of *E. coli* O157 to the host cell stimulates a highly coordinated and very specific cascade of events. The TSS is recruited to insert Tir into the host cell permitting intimin, which is expressed on *E. coli* O157's surface, to bind to Tir. At the site of intimate attachment, reorganization of host cell actin is initiated resulting in the formation of a "pedestal" under the bacterium, ultimately supporting the host cell-bacterium association (Wales et al., 2005). The formation of the pedestal is hallmark of the attaching and effacing (A/E) lesion and are unique to only the enteropathogenic and enterohemorrhagic pathotypes of *E. coli* (Torres et al., 2005). Numerous studies have evaluated the significance of Tir and

intimin during *E. coli* O157 colonization of epithelial cells. Through utilization of mutagenesis studies, the roles of Tir (Sheng et al., 2006; Girard et al., 2007) and intimin (Cornick et al., 2002; Cookson and Woodward, 2003; Sheng et al., 2006) in persuading colonization, were validated. The influence of other virulence factors has been evaluated for their ability to influence colonization. In one illustration, Stx II was found to increase eukaryotic expression of nucleolin, which demonstrates affinity for intimin (Robinson et al., 2006) but was refuted when stx mutants maintained the ability to colonize as efficiently as that wild type (Sheng et al., 2006).

2.6. Gastrointestinal Location of *E. coli* O157 and Histopathology

Although cattle are generally regarded as the major reservoir of *E. coli* O157:H7, it is not completely lucid where in the bovine G.I. tract the organism colonizes. Recent research has acknowledged the colon (Grauke et al., 2002; Stoffregen et al., 2004; Dean-Nystrom et al., 2008), specifically an area designated as the rectal-anal junction (RAJ), as the principal site of *E. coli* O157 colonization (Naylor et al., 2003; Sheng et al., 2004; Low et al, 2005; Lim et al., 2007) and is particularly vital in *E. coli* O157 fecal excretion (Cobbold et al., 2007). In cattle naturally infected with *E. coli* O157, tissue samples located 1 cm from the RAJ carried higher quantities of *E. coli* O157 than samples located 15 cm from the RAJ (Low et al., 2005). An obvious trend of increased *E. coli* O157 concentration in areas closest to the RAJ was observed on tissue samples obtained from experimentally infected, naturally colonized, and calves exposed to infected animals (Naylor et al., 2003). It was concluded that animals are more likely to become consistent long-term shedders when they are infected with *E. coli* O157 placed directly at the RAJ rather than through oral inoculation (Sheng et al., 2004).

Cattle infected with *E. coli* O157 and that become asymptomatic carriers generally remain clinically normal (Cray and Moon, 1995) because they lack the Stx receptor globotriaosylceramide (Gb_3) in their G.I. tract, preventing clinical symptoms of infection (Pruimboom-Brees et al., 2000). However, even with the lack of the Gb_3 receptor, *E. coli* O157:H7 strains that produce Stx II elicited an immune response in 6 to 8 week old calves (Hoffman et al., 2006). There appears to be a greater association with animal age and susceptibility to clinical symptoms rather than the deficiency of the Gb_3 receptor. Experimental infection of young calves (< 12 h old or 30 – 36 h old) with 10 logs of *E. coli* O157 resulted in severe diarrhea and systemic A/E lesions throughout the lower G.I. tract (Dean-Nystrom et al., 1997). Contradictorily, one-day-old calves remained clinically normal after inoculation with eight logs of *E. coli* O157 (Sanderson et al., 1999), an inoculation level believed to sufficiently encourage A/E lesion development (Dean-Nystrom et al., 1999). Once animals reach 3 weeks of age, susceptibility to *E. coli* O157 infection (10 logs) appears to diminish; resulting in slight body temperature increases and watery diarrhea that lasts for a couple of days following inoculation, and no formation of A/E lesions (Cray and Moon, 1995; Brown et al., 1997). Still, infection of three to four month old calves with ten logs of *E. coli* O157 caused watery diarrhea (Dean-Nystrom et al., 1999) and A/E lesion formation (Dean-Nystrom et al., 1999, 2008; Stoffregen et al., 2004), and which ultimately demonstrated ability to translocate to the gall bladder where it was able to produce A/E lesions (Stoffregen et al., 2004; Dean-Nystrom et al., 2008). The general consensus that *E. coli* O157 colonization is not associated with clinical symptoms in animals of at least one year of age (Grauke et al.,

2002; Cray and Moon, 1995) prevents the simple identification and removal of asymptomatic *E. coli* O157 carriers from populations of feedlot cattle.

The elucidation of the molecular mechanisms that encourage attachment and result in *E. coli* O157 colonization will permit the development of specific mitigation strategies that can interfere with colonization mechanism, ultimately reducing the *E. coli* O157 infection, amplification and shedding, consequently improving safety of beef carcasses downstream in process flow.

CHAPTER III

Escherichia coli O157:H7 Strains that Persist in Feedlot Cattle are Genetically Related and Demonstrate an Enhanced Ability to Adhere to Intestinal Epithelial Cells

ABSTRACT

A longitudinal study was conducted to investigate the nature of *Escherichia coli* O157:H7 colonization of feedlot cattle over the final 100 to 110 days of finishing. Rectal fecal grab samples were collected from an initial sample population of 788 steers every 20-22 d and microbiologically analyzed to detect *E. coli* O157:H7. The identities of presumptive colonies were confirmed using a multiplex PCR assay that screened for gene fragments unique to *E. coli* O157:H7 (*rfbE* and $fliC_{h7}$) and other key virulence genes (*eae*, *stxI*, and *stxII*). Animals were classified into a persistent shedding (PS), transient shedding (TS) or nonshedding (NS) status if they consecutively shed the same *E. coli* O157:H7 genotype (based on multiplex PCR profile), exhibited variable *E. coli* O157 shedding, or never shed morphologically typical *E. coli* O157, respectively. Overall, 1.0% and 1.4% of steers were classified as PS and NS animals, respectively. Characterization of 132 *E. coli* O157:H7 isolates from PS and TS animals by pulsed field gel electrophoresis (PFGE) typing yielded 32 unique PFGE types. One predominant PFGE type represented 53% of all isolates characterized and persisted in cattle throughout the study. Isolates belonging to this predominant and persistent PFGE type

demonstrated an enhanced ($P < 0.0001$) ability to adhere to Caco-2 human intestinal epithelial cells as compared to isolates representing less common PFGE types, but equal virulence expression. Interestingly, attachment efficacy decreased as genetic diversity from the predominant and persistent subtype increased. Our data support that certain *E. coli* O157:H7 strains persist in feedlot cattle, which may be partially explained by an enhanced ability to colonize the intestinal epithelium.

3.1. Introduction

Escherichia coli serotype O157:H7 was first linked to human illness in the early 1980s, when it was determined to cause severe abdominal pain with initially watery diarrhea that progressed to grossly bloody diarrhea accompanied by little or no fever (Riley *et al.,* 1983). Initially, *E. coli* O157:H7 can cause non-bloody diarrhea through attachment to, and subsequent destruction of, intestinal microvilli (Gyles, 2007). In addition to microvilli damage, serious health complications can arise due to the ability of *E. coli* O157:H7 to produce Shiga toxins (StxI and StxII). Shiga toxins are very potent cytotoxins that are absorbed into the intestinal microvasculature and initiate apoptosis of vascular epithelium resulting in hemorrhagic colitis (Ray and Lui, 2001). Persistent uptake of these toxins may lead to more severe manifestations of disease such as hemolytic uremic syndrome (HUS), which may ultimately result in kidney failure (Gyles, 2007). Most recent estimates identify *E. coli* O157:H7 as the cause of at least 70,000 cases of foodborne illness annually in the U.S., with 4% of cases developing life-threatening HUS (Mead *et al.,* 1999). Epidemiological studies have implicated the consumption of meat, dairy products, produce, and water contaminated by animal feces, as well as person-to-person contact and direct contact with farm animals or their

environment, as routes of *E. coli* O157:H7 transmission leading to human illness (Mead and Griffin, 1998).

It is generally accepted that cattle and other animals are a major reservoir of *E. coli* O157:H7, but it remains unclear if animals become colonized for prolonged periods with *E. coli* O157:H7, or if they transiently shed the organism following repeated exposure to the organism through ingestion of contaminated feedstuffs, water or through exposure to other contaminated environmental sources. Based on results from numerous epidemiological studies (Barkocy-Gallagher *et al.,* 2003; Besser *et al.,* 1997; Galland *et al.,* 2001; Khaitsa *et al.,* 2003; LeJeune *et al.,* 2004), *E. coli* O157:H7 prevalence in feedlot cattle is highly variable and can range from below 1% up to 80%. Several other studies (Besser *et al.,* 2001; Brown *et al.,* 1997; Grauke *et al.,* 2002) have shown evidence of persistent *E. coli* O157:H7 colonization in individual cattle, providing support that at least some animals are susceptible to persistent *E. coli* O157:H7 colonization. Multiple experimental inoculation studies (Cray and Moon, 1995; Grauke *et al.,* 2002; Naylor *et al.,* 2003; Sheng *et al.,* 2004) showed that *E. coli* O157:H7 persists in the bovine gastrointestinal (G.I.) tract from at least 14 d to up to 140 d post-infection. Studies have implicated the lower G.I. tract and, specifically, the recto-anal junction (RAJ) as the major location of *E. coli* O157:H7 colonization and proliferation (Buchko *et al.,* 2000; Cobbold *et al.,* 2007; Grauke *et al.,* 2002; Naylor *et al.,* 2003); however, the organism also can be found throughout the bovine G.I. tract (Besser *et al.,* 2001; Brown *et al.,* 1997; Laven *et al.,* 2003; Rasmussen *et al.,* 1993; Tkalcic *et al.,* 2000).

It stands to reason that if *E. coli* O157:H7 prevalence among cattle presented to harvest is reduced, there would be a subsequent decrease in the probability of beef

product contamination, while still adhering to good manufacturing procedures. Although there is consensus on the importance of pre-harvest pathogen mitigation and its role in minimizing entry of *E. coli* O157:H7 into harvest facilities, there is contention about the significance of "super-shedders" (animals that excrete large quantities of a pathogen for varied amounts of time) on *E. coli* O157:H7 transmission dynamics at the pre-harvest level (Cobbold *et al.,* 2007; Matthews *et al.,* 2006a and b; Naylor *et al.,* 2003). Utilizing statistical modeling, researchers have estimated that the prevalence of "super-shedders" in a population is, on average, 4% and that these animals excrete 50 times more *E. coli* O157:H7 than other animals colonized by this organism (Matthews *et al.,* 2006a). Additionally, the same researchers suggested that approximately 80% of *E. coli* O157:H7 transmission is generated by a few "super-shedders" (Matthews *et al.,* 2006b).

Research from our group discovered a unique association between pen-floor fecal pat *E. coli* O157:H7 prevalence and carcass contamination by this pathogen (Woerner *et al.,* 2006). When pen-floor fecal pat prevalence exceeded 20%, carcasses corresponding to the same pen of animals had *E. coli* O157:H7 prevalence of 14.3, 2.9, and 0.7% at pre-evisceration, post-evisceration, and after final intervention, respectively. However, when pen-floor fecal pat prevalence was below 20%, pre-eviscerated carcass prevalence levels declined to 6.3% with no detectable *E. coli* O157:H7 contamination of post-eviscerated and post-final intervention carcass samples (Woerner *et al.,* 2006). Thus, it is our hypothesis that animals which persistently excrete normal levels of *E. coli* O157:H7 over prolonged periods (persistent shedders), rather than animals that periodically shed abnormally high levels (super-shedders), present the most significant source of *E. coli* O157:H7 contamination within the food continuum. Although previous studies suggest

that cattle may become persistently colonized by *E. coli* O157:H7 and shed the organism in their feces for prolonged periods, molecular subtyping data are required to further investigate whether cattle are persistently colonized by the same strain (i.e., molecular subtype) or if they are repeatedly exposed to different strains through contaminated feedstuffs, water or other environmental sources. Correspondingly, the objectives of this study were to determine if naturally colonized feedlot cattle persistently shed *E. coli* O157:H7, using combined cultural microbiological analyses and molecular subtyping approaches, and to probe the factors (i.e., agent, host, environmental, or a combination of these factors) that contribute to the complex ecology of *E. coli* O157:H7 persistence at the pre-harvest level.

3.2. Materials and Methods

3.2.1. Study design. Holstein (N = 788) steers consuming a high-concentrate finishing ration at a commercial feedlot in eastern Kansas (all research protocols were reviewed and approved by the Colorado State University Animal Care and Use Committee, Approval: 05-233A-01) that had never been exposed to direct-fed antimicrobials (e.g., Bovamine®) were enrolled in the current study. Steers were housed in five pens -- three of the pens shared fence lines and the two remaining pens were independently located in other areas of the feedlot -- and all animals were fed the same finishing diet during the sampling period. Animals were permanently removed from the study population following treatment for any clinical illness.

Rectal fecal grabs were collected every 20 to 22 d during the final 120 d (June – October) of the finishing period. Every animal was sampled during the first two collection periods to establish each animal's *Escherichia coli* O157:H7 shedding status

along with the prevalence of shedding among this population of feedlot cattle (Table 3.1). Animals that varied in *E. coli* O157:H7 shedding status during the first two sample collections were exempted from further sample collection as the focus of this study was to further investigate the "persistent shedder" status. The four remaining sample collections focused on identification of animals that were either consecutively *E. coli* O157:H7 positive or, consecutively *E. coli* O157:H7 negative, as determined by results from each previous sample collection. Additionally, a random subset of animals that varied in their *E. coli* O157:H7 shedding status was included during each of the final four sample collections to determine if these animals reverted back to their original shedding patterns.

At every pre-determined collection period, steers were processed through conventional processing facilities where rectal fecal grab samples were collected from each targeted animal. Feces were transferred to a sterile Whirl-Pak bag (Nasco, Modesto, CA) and subsequently placed in a cooler with ice packs. Following sampling, the fecal samples were transported to the Pathogen Reduction Laboratory, Center for Meat Safety & Quality at Colorado State University (Fort Collins, CO) where they were stored at 4°C until microbiological analysis (within 48 h after collection).

3.2.2. Fecal *E. coli* O157:H7 analysis. Fecal samples were enriched according to procedures outlined by Barkocy-Gallagher et al. (2005). Following incubation, fecal slurries were stored at 4°C until they were subjected to immunomagnetic bead separation (IMS). IMS was conducted as described by Barkocy-Gallagher et al. (2002), and ultimately 50 μl of each sample was plated onto Rainbow agar (Biolog Inc., Hayward, CA) supplemented with 10 mg/L of novobiocin (Sigma-Aldrich, St. Louis, MO) and 0.8

mg/L of potassium tellurite (Sigma), as well as Sorbitol MacConkey agar (Becton, Dickinson and Company, Sparks, MD) supplemented with 20 mg/L of novobiocin and 2.5 mg/L of potassium tellurite (mSMAC). Rainbow plates were incubated for 24 ± 2 h at 37°C and mSMAC plates were incubated for 36 ± 2 h at 37°C. After incubation, up to 3 colonies displaying *E. coli* O157:H7 morphology were selected from each medium and initially screened for the O157 antigen using the RIM *E. coli* O157:H7 latex agglutination test (Remel, Lenexa, KS). All agglutination-positive colonies were cultured into 5 ml of TSB (24 ± 2 h at 37°C) and streaked onto mSMAC (36 ± 2 h; 37°C) for purity.

Presumptive colonies were confirmed to be *E. coli* O157:H7 by a multiplex PCR assay performed in 96-well plate format in 25 μl reactions including a primer master mix containing forward and reverse primers at concentrations specified previously (Hu et al., 1999) to amplify each of the following genes: *rfbE* (encodes the O157 antigen), $fliC_{h7}$ (encodes the H7 antigen), *eae* (encodes intimin), *stxI* (encodes Shiga toxin I), and *stxII* (encodes Shiga toxin II), GoTaq® Green Master Mix (Promega, Madison, WI), nuclease-free water and DNA template. PCR cycling conditions were followed as outlined in Hu et al. (1999). PCR products were separated by electrophoresis in 2% agarose gels, stained with ethidium bromide and visualized with UV illumination. Multiplex PCR profiles were assigned based on presence/absence of each targeted gene. Isolates where amplicons corresponding to *rfbE*, $fliC_{h7}$, and at least one *stx* gene were detected were designated as *E. coli* O157:H7. For the purpose of this study, we chose to follow-up on animals that consecutively shed the most common *E. coli* O157:H7 genotype (the presence of all five genes by multiplex PCR). Up to three *E. coli* O157:H7 isolates from

each positive fecal sample were stored in 15% glycerol at –80°C for further characterization.

3.2.3. Enumeration of *E. coli* O157:H7 persistent-shedder animals. A five-tube MPN assay (FDA, 2001) was used to enumerate *E. coli* O157:H7 in fecal samples collected from persistent shedders just before slaughter to determine whether or not they excreted elevated *E. coli* O157:H7 quantities in their feces. A 10 g aliquot of each fecal sample was combined with 90 ml of Butterfield's phosphate buffer (Becton; BPB) and pummeled in a stomacher for 2 min. Three 1:10 serial dilutions were prepared from each BPB sample. From each of the sample's three serial BPB dilutions, 1 ml was removed and added to each of five different tubes of Lauryl tryptose broth (Becton; LTB) resulting in a total of 15 tubes for each sample. Inoculated LTB tubes were incubated for 24 h at 37°C. After incubation, all turbid LTB tubes were streaked on mSMAC and appropriately incubated with morphological typical colonies confirmed by multiplex PCR as previously described.

3.2.4. Gastrointestinal tissue and content samples. All animals identified as persistent shedders (PS; n = 8) and non-shedders (NS; n= 11), as well as a sub-sample of animals identified as transient shedders (TS; n = 18), were harvested at a commercial harvesting facility in the upper Midwest. Entire gastrointestinal (G.I.) tracts (esophagus, reticulum, rumen, omasum, abomasum, gall bladder, small intestine, large intestine, colon, and bung) were collected from each animal and transported to a vacant area of the facility to allow for sample collection. Additionally, livers from each animal were examined for the presence of abscesses. Tissue and content samples were collected aseptically from the reticulum, rumen, omasum, abomasum, duodenum (proximal to the anterior side of the

first loop), ileocecal valve, distal colon (~ 60 cm proximal to the anus), rectal-anal junction and two mesenteric lymph nodes (from a position ~ 30 cm proximal to the anterior root of the mesentery and the ileal cecal colic node; only tissue samples from the lymph nodes) for microbiological analysis. Tissue samples were washed with sterile phosphate buffered saline + 0.05% Tween 20 (Sigma; PBS) to remove visible organic matter before placement into a Whirl-Pak (Nasco) bag. All microbial samples were placed in ice pack-filled coolers and shipped to Pathogen Reduction Laboratory, Center for Meat Safety & Quality at Colorado State University (Fort Collins, CO).

Gastrointestinal tissue and content samples were microbiologically analyzed as described above. Ten-gram aliquots of epithelial layer of each tissue were aseptically removed and placed into a Whirl-Pak bag containing 90 ml of phosphate buffered tryptic soy broth (TSB - PO_4; Barkocy-Gallagher et al., 2005). Lymph nodes were first, aseptically, trimmed of excess adipose tissue and a 10 g aliquot of each tissue sample was placed into Whirl-Pak bags containing 90 ml of TSB –PO_4. All tissue samples were pummeled (IUL Instruments, Barcelona, Spain) for 2 min and processed as detailed above. Additionally, pH measurements were obtained by making an additional 1:10 dilution of G.I. content samples and distilled water, pummeling (IUL Instruments) for 2 min and measuring by submerging a glass pH electrode (Denver Instruments, Arvada, CO) into the sample.

Gastrointestinal tissue samples from select PS (n = 3) and NS (n = 3) animals were fixed in 4% paraformaldehyde and shipped to the Colorado State University Pathology Diagnostic Laboratory where they were embedded in paraffin using an automated tissue processor, sectioned at 5 μm on a microtome and stained with

hematoxylin and eosin. Histopathological evaluation/characterization was performed by a trained pathologist who was blinded with respect to sample identification information.

3.2.5. Pulsed-Field Gel Electrophoresis. PFGE typing of *E. coli* O157:H7 isolates was performed using the standardized CDC, PulseNet protocol (CDC, 2001). At least one isolate from all six sample collections from each PS animal and representative isolates available from TS animals were selected for characterization by PFGE. All isolates were previously confirmed to be *E. coli* O157:H7 with *eae, stxI* and *stxII* before PFGE analysis. Briefly, isolates were grown on tryptic soy agar (Becton; TSA) plates and incubated at 37°C for 18 h. Bacterial cultures were imbedded in 1% agarose (SeaKem Gold Agarose, Cambrex Bio Science Rockland, Inc., Rockland, ME), lysed, washed, and digested with *Xba*I overnight at 37°C. Restricted agarose plugs were then placed into 1% agarose gels and electrophoresed on a CHEF Mapper XA (BioRad Laboratories, Hercules, CA) for 21 h with switch times of 2.16 s to 54.17s. *Xba*I digested *Salmonella* ser. Braenderup (H9812) DNA was used as a reference size standard (Hunter et al., 2005). Agarose gels were stained in ethidium bromide and resultant images were captured with a FOTO/Analyst Investigator System (FOTODYNE, Inc., Hartland, WI). PFGE patterns then were analyzed and compared using the Applied Maths Bionumerics (Applied Maths, Saint-Matins-Latem, Belgium) (v3.5) software package. Similarity clustering analyses were performed with Bionumerics software using the unweighted pairs group matching algorithm and the Dice correlation coefficient (Hunter et al., 2005).

3.2.6. Cell Attachment Assay. The attachment efficiency of *E. coli* O157:H7 isolates representing the predominant PFGE subtype was compared to that of the *E. coli* O157:H7 isolates representing the more genetically diverse PFGE subtypes by performing

attachment assays using the Caco-2 human intestinal epithelial cell line. Up to ten ante-mortem fecal isolates representing each of the following categories (i) dominant PFGE subtype, (ii) subtypes closely related to the dominant subtype, (iii) subtypes possibly related to the dominant subtype, and (iv) distantly related to the dominant subtype (based on the criteria previously defined; Tenover et al., 1995) were selected for characterization by this Caco-2 attachment assay. Caco-2 cells were seeded into 24-well flat bottom plates (Corning Inc., Corning, NY) at a density of 1 x 10^5 cells/well in Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY; DMEM) containing 20% heat inactivated fetal bovine serum (Gibco) without antibiotics and grown to confluency (approximately 72 h). *E. coli* O157:H7 overnight cultures were prepared by inoculating a single well-isolated colony into a 10 ml tube of brain heart infusion (Becton; BHI) broth and incubating at 37°C for 12 to 18 h without shaking. Overnight *E. coli* O157:H7 cultures (1 ml) were pelleted by centrifugation (11,337 x *g*; 5 min) and re-constituted in 1 ml of PBS. Confluent Caco-2 monolayers were infected with approximately 2 x 10^7 *E. coli* O157:H7/well. After infection for 3 h at 37°C, non-adherent bacteria were removed by washing three times with PBS. Caco-2 cells were lysed by addition of 0.5 ml of ice-cold sterile ultrapure water and vigorous pipetting followed by vortexing the cell suspension. Adherent *E. coli* O157:H7, along with overnight *E. coli* O157:H7 cultures, were enumerated by spread plating appropriate serial dilutions onto BHI plates, in duplicate. BHI plates were incubated at 37°C for 24 h and resultant colony forming units were enumerated. The attachment efficiency of each *E. coli* O157:H7 isolate was determined as a percentage of the initial inoculum that was recovered as adherent *E. coli*

O157:H7 cells. The attachment efficiency of each isolate was measured in duplicate wells in at least three independent experiments.

3.2.7. Statistical Analysis. The G.I. content pH data were analyzed with PROC MIXED of SAS (SAS version 9.3; SAS Institute, Cary, NC). Analysis of variance techniques were employed to determine if there were differences ($P < 0.05$) among main effects, including G.I. tissue location and shedding status, as well as all appropriate interactions.

The proportion of the study population expected to be classified as PS or NS animals by chance alone was calculated by multiplying the prevalence estimates of animals shedding and not shedding *E. coli* O157:H7 with the five-gene multiplex PCR profile at each of the six time points. These joint probabilities represented the expected frequency in each category, which was compared to the observed number in each category by using chi-square goodness-of-fit tests. Within-table variance (i.e., independence of the expected and observed outcomes) was determined using P value of <0.05.

Chi-square analysis was utilized to detect differences of subtype frequency between shedding status groups using PROC FREQ procedure of SAS. Initially, data were collapsed to form three subtype categories – dominant subtype, subtypes that differed from the dominant subtype by 1 to 3 bands, and subtypes that differed from the dominant subtype by 4 to > 7 bands before analysis. The estimated probability of shedding a given PFGE subtype during the ante-mortem collections were analyzed using a repeated measures Generalized Estimating Equations (GEE) marginal logistic model with PROC GLIMMIX of SAS using the empirical difference in standard errors.

Differences between predicted probabilities were considered significant at P values of <0.05.

The difference between log transformed values for cells inoculated into each well and log transformed counts for adherent cells recovered in each well served as the dependent variable to compare Caco-2 attachment data. ANOVA techniques were employed to determine if there was a difference between attachment abilities of different PFGE subtype categories as previously described. Differences in attachment efficiency were analyzed using PROC MIXED of SAS with least squares means generated for each PFGE subtype category. Ultimately, least squares means were separated using pairwise t-tests incorporating a Tukey's adjustment with significant inferences noted when differences between means were detected at the $P < 0.05$ level.

3.3. Results

3.3.1. *Escherichia coli* O157:H7 carriage in feedlot steers. All animals enrolled in this study were sampled during the first two sample collections, which allowed us to determine the overall prevalence of *E. coli* O157:H7 in the study population. Presumptive *E. coli* O157:H7 isolates were characterized by a five-gene multiplex PCR that detects gene fragments unique to serotype O157:H7, along with genes encoding three key virulence determinants (i.e., *eae, stxI,* and *stxII*). Overall, 45.5 and 58.7% of the study population shed *E. coli* belonging to serotype O157 and carrying at least one *stx* gene in collections 1 and 2, respectively (Table 3.1). Over the first two collection periods, animals shed *E. coli* O157 isolates with five virulence genotypes as determined by their multiplex PCR profiles (Table 3.1). During the first two collection periods, *E. coli* O157:H7 isolates possessing the *eae*, *stxI* and *stxII* genes (five-gene multiplex PCR

profile) were shed most frequently, as 39.8% and 33.6% of animals shed isolates with this genotype during the first and second collections, respectively (Table 3.1).

Since the purpose of this study was to determine if cattle become persistently colonized by *E. coli* O157:H7, which would imply the same *E. coli* O157:H7 strain is able to persist in the G.I. tract of a given animal over time, our strategy for the remaining four sample collections was to target animals that consistently shed the predominant genotype (e.g., five-gene multiplex PCR profile). As a result, the prevalence of less common genotypes could not be determined after the first two sample collections and we focused our efforts on monitoring persistence of the dominant genotype throughout the remainder of the study (Table 3.2). Specifically, persistent shedding (PS) animals were defined as those animals that shed an *E. coli* O157:H7 isolate carrying *eae*, *stxI* and *stxII* (five-gene positive multiplex genotype) over the six collection periods. Animals that intermittently shed an *E. coli* O157:H7 isolate with a genotype carrying all three virulence factors were classified as transient shedding (TS) animals, while animals that never shed morphologically typical *E. coli* O157 (i.e., inability to rapidly ferment sorbitol) during the 120 d sample collection period were classified as non-shedder (NS) animals. Overall, based on these criteria, eight of 788 animals (1.0%) were classified as PS animals, while 11 of 788 animals (1.4%) never shed a detectable amount of morphologically typical *E. coli* O157 and were thus classified as NS animals. Chi-square goodness-of-fit tests revealed the independence of the observed and expected frequency of PS status ($P < 0.001$), while the number of animals classified as having NS status was similar to that expected by chance alone ($P = 0.23$). The remaining 769 animals were classified as TS animals since these animals shed an *E. coli* O157 isolate carrying at least

one *stx* gene at least once over the duration of this study. The distribution of both PS and NS animals was balanced over the five pens, where each pen contained at least one animal of each *E. coli* O157:H7 shedding status. These results demonstrated that small subpopulations of cattle within a feedlot population appear to become persistently colonized by *E. coli* O157:H7 for extended periods of time during the final 120 d of finishing.

Feces from the eight animals classified as PS, collected during the final ante-mortem sampling, were enumerated using five-tube MPN methodology. Only one PS animal (PS-7) shed *E. coli* O157:H7 at levels (46 MPN/g) detectable by our method. The remaining seven PS animals shed *E. coli* O157:H7 below the detectable limit of 1.8 MPN/g (data not shown). Our results indicated that an animal that becomes persistently colonized by *E. coli* O157:H7 does not necessarily shed the organism at high levels in its feces.

3.3.2. Gastrointestinal tissue and gastrointestinal content analysis. *Escherichia coli* O157:H7 was detected on tissue and in G.I. content samples collected from both upper and lower sites along the G.I. tract in PS and TS animals (Table 3.3). Although insignificant, PS animals generated more lower G.I. tissue and content samples that tested positive for *E. coli* O157:H7 when compared to TS animals. Only one PS animal had an upper G.I. tissue sample (omasum) and one anterior root lymph node tissue sample that tested positive for *E. coli* O157:H7. There were two upper G.I. tissue samples (reticulum and omasum) testing positive for *E. coli* O157:H7 in two different TS animals. No gall bladder samples were positive for *E. coli* O157:H7 regardless of previous shedding status (Table 3.3).

3.3.3. Histology and pathology. Histological analyses of tissue samples collected from PS and TS animals in this study did not reveal any notable differences between tissues of PS and NS animals. All tissue samples from PS and NS animals were characterized as normal or having minor lesions commonly found in G.I. tissues characteristic of fed cattle. In addition, livers examined from animals representing PS, TS and NS groups were free of visible surface lesions (data not shown). Thus, there do not appear to be significant physiological differences associated with animals that become persistently colonized by *E. coli* O157:H7 as compared to animals that were not colonized with this organism.

3.3.4. Molecular characterization. At least one fecal isolate was selected to represent each ante-mortem collection period for each of the eight PS animals along with all post-mortem isolates (for a total of 82 isolates), and a random representative set of isolates was selected from 16 TS animals along with any post-mortem isolates (for a total of 50 isolates) resulting in a set of 132 isolates that were characterized by PFGE typing. The 132 *E. coli* O157:H7 isolates analyzed by PFGE typing were classified into 32 different subtypes (Fig. 3.1; Table 3.4). A single, predominant PFGE subtype (subtype F; Fig. 3.1; Table 3.4) accounted for 53% of all 132 isolates characterized. A Chi-square test of independence showed that Subtype F was similarly distributed among PS and TS animal populations ($P > 0.05$) and this subtype persisted throughout the study (Fig. 3.2). Twenty-two of the 32 unique PFGE subtypes were observed among PS animals, and 17 of these 32 unique PFGE subtypes were exclusive to PS animals. Interestingly, only five PFGE subtypes (E, F, H, I, and N) overlapped between PS and TS animal populations (Table 3.4). All eight PS animals shed subtype F during the first sample collection, at

least twice over the entire collection period, and for at least two consecutive sample collection periods (Table 3.5). One PS animal (PS-4) shed subtype F over the entire collection period, while two other PS animals (PS-1 and PS-7) shed subtype F consecutively over the first four sample collections (Table 3.5). During fecal sample collections, PS animals shed 15 PFGE subtypes in addition to subtype F, with 10 of those subtypes only differing from F by three or fewer bands (Table 3.4). Tenover et al. (1995) characterized differences of three or fewer bands as compared to a reference strain (subtype F was used as the reference strain) to be closely related. The remaining five subtypes differed from subtype F by four or more bands and were thus not considered to be closely related to PFGE subtype F, according to the Tenover et al. (1995) criteria. The post-mortem tissue and G.I. content sample collection yielded an additional ten unique PFGE types among PS and TS animals that were not present during ante-mortem fecal sample collections, where four of these post-mortem PFGE subtypes were closely related to the dominant ante-mortem subtype F.

The ability of the predominant *E. coli* O157:H7 subtype to persist in the G.I. tracts of feedlot cattle was evidenced by the percentage of animals that shed this subtype in their feces throughout the study. More specifically, the estimated probabilities of encountering subtype F were 89.1, 77.0, 78.5, 59.3, 36.0, and 30.2% during sample collections 1, 2, 3, 4, 5, and 6, respectively (Fig. 3.2). Although there was a decreasing trend ($P < 0.05$) in the probability of shedding the dominant subtype, the dominant subtype was still detected and actually accounted for 31.6% (6/19) of the isolates collected during the final ante-mortem collection. Interestingly, our results also suggest that the dominant *E. coli* O157:H7 molecular subtype (subtype F) underwent micro-

evolutionary changes throughout the study as evidenced by emergence of molecular subtypes that were closely related to the dominant subtype and the decline in the presence of the dominant subtype as the study progressed (Fig. 3.2). These results indicated that a dominant *E. coli* O157:H7 strain (subtype F) and other closely related strains persisted in the population of feedlot cattle over a 120 d period.

In an effort to further investigate the ability of *E. coli* O157:H7 to persist in the feedlot environment, we molecularly characterized an additional *E. coli* O157:H7 isolate from a previous study by Childs et al. (2006) that was collected from the environment of the same feedlot enrolled in the current study more than two years before our cattle arrived. This particular *E. coli* O157:H7 isolate (referred to as "Kansas") was analyzed first with multiplex PCR to determine its genotype. After it was determined that the Kansas isolate had the same genotype as the persistent *E. coli* O157:H7 strain from the current study (i.e., five-gene multiplex PCR profile), the Kansas isolate was characterized by PFGE typing and compared to isolates from our current study (Fig. 3.1). The Kansas isolate only differed by a single band from the dominant subtype and maintained 86% similarity (Fig. 3.1), supporting long-term persistence of closely related *E. coli* O157:H7 strains in the feedlot environment.

3.3.5. Cell attachment. The abilities of *E. coli* O157:H7 isolates belonging to the dominant PFGE subtype (subtype F), closely related PFGE subtypes (< 3 band difference from subtype F), possibly related subtypes (between 4 and 6 band difference from subtype F), and divergent subtypes (> 7 band difference from subtype F) to adhere to the Caco-2 human intestinal epithelial cell line were compared. Attachment efficiency was expressed as the percent of the initial inoculum that adhered to host cells and mean

attachment efficiency for the strain categories described above ranged from 10.7 to 53.9% (Fig. 3.3). *E. coli* O157:H7 isolates belonging to the persistent PFGE subtype (subtype F) demonstrated an enhanced ($P < 0.05$) ability to adhere to human intestinal epithelial cells when compared to closely related, possibly related and genetically divergent subtypes. A trend was observed between genetic diversity and attachment efficacy; as genetic diversity from subtype F increased (based on the number of band differences), ability to attach to Caco-2 cells diminished (Fig 3.3). As a reference, an *E. coli* O157:H7 isolate obtained from a food sample associated with an outbreak of human illness (ATCC 43895) with the same genotype (five-gene multiplex PCR profile) was included in all cell attachment assays. *E. coli* O157:H7 isolates representing the dominant subtype demonstrated a more than 3-fold increase in attachment efficacy than the outbreak associated isolate (Fig 3.3). Results from Caco-2 attachment assays suggest that *E. coli* O157:H7 subtypes that persist in cattle have an enhanced ability to adhere to human intestinal epithelial cells.

3.4. Discussion

To date, there has not been as an extensive investigation of the molecular ecology of *E. coli* O157:H7 persistence and shedding in naturally colonized feedlot cattle. Our results demonstrated that closely related *E. coli* O157:H7 strains may persist in the feedlot ecosystem (i.e., cattle and the feedlot environment) for extended periods, which may be in part explained by an enhanced ability of these persistent strains to adhere to intestinal epithelial cells. Additionally, we demonstrated that most (97.6%) feedlot steers shed Shiga toxin-encoding *E. coli* O157 during the final 120 d of the feeding period. Molecular characterization of *E. coli* O157:H7 isolates revealed that a predominant *E.*

coli O157:H7 strain persisted throughout the study and that this persistent strain diversified over the duration of the study. Further phenotypic characterization of isolates belonging to the persistent strain, along with closely related and more genetically divergent PFGE subtypes, using a cell culture attachment assay, revealed increased attachment efficiency of the persistent *E. coli* O157:H7 strain found in this feedlot population. Our results illustrate that certain *E. coli* O157:H7 strains may persist in cattle populations and that, ultimately, these strains may represent an increased human health risk due to the increased likelihood of these strains entering the human food supply and their subsequent enhanced ability to attach to human intestinal epithelial cells.

Most feedlot cattle appear to shed Shiga toxin-encoding *E. coli* O157 at some point during the final phase of finishing. During collections 1 and 2, we detected an *E. coli* O157 isolate carrying at least one Shiga toxin encoding gene in 45.5 and 58.7% of all animals, respectively. Before now, Shiga toxin-encoding *E. coli* O157 prevalence, a more indicative estimate of true risk (e.g., a Shiga toxin-encoding *E. coli* O157 would elicit regulatory response if transferred to the carcass; FSIS, 2008), was reported to have a point prevalence ranging between 0.3 to 19.7% in feedlot cattle (Hussein and Bollinger, 2005). More importantly, we observed that 97.6% of steers shed a Shiga toxin-encoding *E. coli* O157 isolate at least once during the final 120 d of the feeding period, levels that, before now, have never been reported. During our first two sample collection periods, 39.8 and 33.6% of the animals shed an *E. coli* O157:H7 isolate belonging to the same genotype (i.e., carrying the *eae, stxI,* and *stxII* genes), a higher prevalence than previously reported (Cobbold et al., 2007; Elder et al., 2000; Hussein, 2007; Scott et al., 2006; Stanford et al., 2005a; Van Donkersgoed et al., 1999, 2001).

Small subpopulations of feedlot cattle appear to become persistently colonized by *E. coli* O157:H7. Few studies have evaluated the persistence of *E. coli* O157:H7 fecal shedding in large populations of feedlot cattle naturally colonized by this human pathogen. We found that 1% of feedlot steers persistently shed the same *E. coli* O157:H7 genotype (i.e., a strain carrying *eae, stxI,* and *stxII*) during the final 120 d of the feeding period. Intensive (multiple samples collected per day) fecal sampling, conducted on two different cohorts of 6 to 11 month old dairy calves, revealed two animals in each cohort (14 and 12.5% of total) persistently shedding *E. coli* O157:H7 for 4 and 15 d (Robinson et al., 2004). Observation of *E. coli* O157:H7 fecal shedding in feedlot cattle determined that a small number of animals (n = 8) shed the organism for a maximum of 4.5 weeks, about three-quarters of the amount of time we found *E. coli* O157:H7 persistence to last, with the remaining sample population shedding for an average duration of 2.5 weeks (Khaitsa et al., 2003). Within dairy cattle, duration of *E. coli* O157:H7 shedding was estimated to be approximately one month (Besser et al., 1997). These previous studies on naturally colonized populations were limited by the exclusion of molecular subtyping to characterize isolates in order to determine if animals were persistently colonized by the same *E. coli* O157:H7 strain or if they were continuously exposed to, and subsequently shed, genetically diverse strains. Experimental inoculation of calves with *E. coli* O157:H7 established shedding durations between 14 and 140 d (Besser et al., 2001; Cray and Moon, 1995; Naylor et al., 2003; Sanderson et al., 1999). Alternatively, experimental inoculation of cattle (> 0.5 years of age) increased the minimum days of persistent fecal shedding to 29 d but reduced the maximum length to only 98 d (Cray and Moon, 1995; Grauke et al., 2002; Sheng et al., 2004). Assessment of

published literature supports a general trend of increased *E. coli* O157:H7 shedding duration in neonates and calves < 1 year of age. As animal age increases, persistent shedding is curtailed and becomes increasingly intermittent.

It is plausible that the eight animals we found to be colonized by the same *E. coli* O157:H7 strain throughout the duration of the study continued to be re-exposed to the organism via animal-to-animal transmission or contaminated pen environment, subsequently resulting in the persistent *E. coli* O157:H7 shedding status. Animal-to-animal transmission was previously observed when un-inoculated young calves (10 weeks old) commingled with calves inoculated with *E. coli* O157:H7 began shedding the organism (Besser et al., 2001). However, animal-to-animal transmission of *E. coli* O157:H7 was found to be very inefficient and unlikely in a group of five- to eight-month old calves (Sheng et al., 2004), further substantiating our classification of these animals to be persistently colonized with *E. coli* O157:H7 – particularly since the PS animals were fed and maintained in different pens. Additionally, it appears that observations from one time point to the next are not independent with regard to positive animals. Furthermore, previous literature suggests that while the microbiological assays are highly sensitive, the sampling methodology is quite insensitive (Echeverry et al., 2005). Thus, the true PS population was likely underestimated and the true NS population overestimated; if so, there is even more true dependency from time point to time point than that observed herein.

***E. coli* O157:H7 may show specificity for colonization of the lower gastrointestinal tract with no histopathological differences between PS and NS animals.** We did not find conclusive evidence regarding the preferential site of *E. coli*

O157:H7 colonization due to the limited prevalence of the organism in the G.I. tract tissue and content samples collected post-mortem, most presumably a result of transportation stress (Barham et al., 2002) and extended lairage (Minihan et al., 2003) at the plant. A comparable situation was reported for a population of sheep inoculated with *E. coli* O157:H7 that shed detectable amounts of the organism in the feces, but the organism could not be detected from any G.I. tract tissue or content samples following necropsy (Grauke et al., 2002). Despite limited *E. coli* O157:H7 prevalence, we found at least one ileal-cecal and rectal-anal junction tissue sample to be positive for *E. coli* O157:H7 in animals both identified as PS and TS animals. We also found three positive tissue samples from the fore-stomach; the earliest G.I. site believed to be the site of *E. coli* O157:H7 propagation (Brown et al., 1997). Recent research has acknowledged the colon (Grauke et al., 2002; Stoffregen et al., 2004), specifically an area designated as the rectal-anal junction (RAJ) as the principal site of *E. coli* O157:H7 colonization (Low et al., 2005; Naylor et al., 2003; Sheng et al., 2004) and this colonization site is particularly vital to *E. coli* O157:H7 excretion in feces (Cobbold et al., 2007). In cattle naturally colonized by *E. coli* O157, tissue samples located 1 cm from the RAJ carried higher quantities of *E. coli* O157 than samples located 15 cm from the RAJ (Low et al., 2005). An obvious trend of increased *E. coli* O157:H7 concentration in areas closest to the RAJ was observed on tissue samples obtained from experimentally infected, naturally colonized and calves exposed to infected animals (Naylor et al., 2003). It was concluded that animals are more likely to become consistent long-term shedders when they are infected with *E. coli* O157:H7 placed directly at the RAJ rather than through oral inoculation (Sheng et al., 2004). While our findings do not confirm the preferential site

of *E. coli* O157:H7 colonization in naturally infected cattle, they do corroborate the lower G.I. tract as the more ideal site of colonization.

Histopathological evaluation of the all of the G.I. tracts obtained from PS, TS and NS animals did not reveal any gross abnormalities or discernable lesions. More specifically, no attaching and effacing (A/E) lesions, which are characteristic of *E. coli* O157:H7 colonization, were identified in PS animal G.I. tissue samples. All animals enrolled in the study remained healthy throughout the entire course of sample collection and cattle colonized with *E. coli* O157:H7 do not generally present clinical symptoms (Cray and Moon, 1995). There does, however, appear to be an association with animal age and susceptibility to clinical symptoms for animals exposed to very high levels of *E. coli* O157:H7. Experimental infection of young calves (< 12 h old or 30 – 36 h old) with ten logs of *E. coli* O157:H7 resulted in severe diarrhea and A/E lesions throughout the lower G.I. tract (Dean-Nystrom et al., 1997). Contradictorily, one-day-old calves remained clinically normal after inoculation with eight logs of *E. coli* O157:H7 (Sanderson et al., 1999), an inoculation level believed to sufficiently encourage A/E lesion development (Dean-Nystrom et al., 1999). Once animals reach three weeks of age, susceptibility to *E. coli* O157:H7 infection (10 logs) appears to diminish; resulting in slight body temperature increases and watery diarrhea, but only lasting for a couple of days following inoculation and no formation of A/E lesions (Brown et al., 1997; Cray and Moon, 1995). Still, infection of three- to four-month-old calves with ten logs of *E. coli* O157:H7 caused watery diarrhea (Dean-Nystrom et al., 1999) and A/E lesion formation (Dean-Nystrom et al., 1999; Stoffregen et al., 2004) and in one case resulted in translocation of the bacteria to the gall bladder where it was able to produce A/E lesions

(Stoffregen et al., 2004). The general consensus that *E. coli* O157:H7 colonization is not associated with clinical symptoms in animals of at least one year of age (Cray and Moon, 1995; Grauke et al., 2002) is further validated by the lack of pathological symptoms observed for the animals enrolled in our study. Furthermore, histopathological comparisons between PS and NS animals support that host-associated factors do not appear to be as significant as *E. coli* O157:H7's own ability to orchestrate the persistent colonization in cattle greater than one year of age.

A single *E. coli* O157:H7 strain may persist in a population of feedlot cattle. We observed a single predominant PFGE subtype that accounted for 53% of all isolates characterized; furthermore, 87% of the isolates belonged to the predominant PFGE subtype, or to PFGE subtypes that were closely related to the predominant subtype (only 1 - 3 bands different; Tenover et al., 1995). Our data contributes to a growing body of evidence indicating a highly conservative nature of *E. coli* O157:H7 within cattle populations. More specifically, along with several previous studies (Besser et al., 1997; Robinson et al., 2004; Scott et al., 2006; Shere et al., 1998; Stanford et al., 2005b), our study supports that a given population of feedlot cattle appears to become colonized by a single predominant and a few closely related strains. The predominant PFGE subtype within our study was disseminated into each animal pen, each sample collection date, and was shed by each PS animal on at least two consecutive sample collections. Similarly, in another study, all 54 *E. coli* O157 isolates obtained from two different dairies belonged to the same PFGE subtype (Robinson et al., 2004). Analysis of genetic diversity of *E. coli* O157:H7 collected from four dairies located in a 30 km area in southern Alberta also demonstrated a highly clonal *E. coli* O157:H7 population as three dominant subtypes,

detected at every dairy, accounted for a majority of the isolates characterized (Stanford et al., 2005a). It can be argued that dairy farm environments have an increased likelihood of sustaining highly clonal *E. coli* O157:H7 populations because of the lack of animal turnover as compared to commercial feedlots where new animals presumably serve as the main source of new *E. coli* O157:H7 subtypes. However, feedlots also appear to maintain highly related populations of *E. coli* O157:H7, even with increased animal turnover rates. For example, analyses of 103 and 230 *E. coli* O157:H7 isolates obtained from two different commercial feedlots with PFGE revealed that isolates clustered with 80% similarity (Scott et al., 2006) and 60% of isolates belonged to four closely-related subtypes (LeJeune et al., 2004), respectively. Additionally, indistinguishable *E. coli* O157:H7 subtypes were recovered from two feedlots that were approximately 100 km apart and did not share any common source of animals that entered the feedlot (Van Donkersgoed et al., 2001). Previously, we demonstrated that transit to, or holding at, the processing plant can introduce *E. coli* O157:H7 isolates with diverse PFGE subtypes (Childs et al., 2006). We observed similar results, as ten unique *E. coli* O157:H7 subtypes were obtained from either G.I. tract tissue or content samples that were never observed during ante-mortem sampling.

Persistence of predominant *E. coli* O157:H7 subtypes in beef cattle feedlots was characterized and determined to last for several years (LeJeune et al., 2004). These strains of *E. coli* O157:H7 persist in the environment (Bach et al., 2005) and can potentially be rapidly disseminated throughout a cattle population (Scott et al., 2006). We compared the PFGE banding pattern of the dominant PFGE subtype observed during our current study to an *E. coli* O157:H7 (with the same genotype) obtained from an

environmental sample (Childs et al., 2006) of the same feedlot two years before the arrival of our cattle and determined the two subtypes to be highly related with only one band difference and only 14% divergent from the dominant subtype (Fig. 3.1). Our findings present further evidence that certain *E. coli* O157:H7 strains likely persist in the feedlot environment, subsequently colonizing exposed animal inhabitants.

***E. coli* O157:H7 strains that persist in cattle populations demonstrate an enhanced ability to adhere to intestinal epithelial cells.** Based on our observations that a predominant PFGE subtype and other closely related PFGE subtypes represent the majority of *E. coli* O157:H7 isolates obtained from the population of feedlot cattle studied here, and that these subtypes persisted in these cattle throughout the study, we hypothesized that these strains may have evolved to represent an "ecotype" that adapted to colonize and persist in the G.I. tract. We explored this hypothesis through characterization of *E. coli* O157:H7 isolates representing the predominant and persistent PFGE subtype (subtype F), along with isolates representing PFGE subtypes that were closely related, possibly related and distantly related to subtype F. Our investigation illustrated that *E. coli* O157:H7 isolates that represent the predominant PFGE subtype demonstrate an enhanced ($P < 0.05$) ability to adhere to the Caco-2 human intestinal epithelial cell line. Interestingly, as genetic diversity from the predominant PFGE subtype is increased, attachment efficacy deteriorates. *E. coli* O157:H7 isolates representing the predominant subtype demonstrated a superior ability to attach to Caco-2 cells than did a reference *E. coli* O157:H7 isolate from an outbreak of human illness, thereby substantiating the human pathogenic potential of *E. coli* O157:H7 strains that persist in feedlot cattle.

We characterized the attachment efficacy of *E. coli* O157:H7 isolates using a human intestinal epithelial cell line because of the lack of an immortal bovine intestinal epithelial cell line. Although our initial objectives were to elucidate the nature of *E. coli* O157:H7 colonization and persistence in the bovine G.I. tract, we discovered that *E. coli* O157:H7 that persist in feedlot cattle appear to have an accentuated ability to adhere to human intestinal epithelial cells which is imperative to disease manifestation. *E. coli* O157:H7 depends on intimin and the translocated intimin receptor (Tir) for intimate adherence to the host cell (Kaper et al., 2004). The role of the intimin and its importance in bacterial adherence has been investigated and validated in bovine models (Cornick et al., 2002; Girard et al., 2007; Sheng et al., 2006) and appears to be no different in human cell lines (Cookson and Woodward, 2003). We do not discount the significance of intimin in bacterial attachment; but taking into consideration that all of the *E. coli* O157:H7 isolates screened during the attachment assay contained the gene responsible for intimin production, our results provide evidence of other influential mechanisms responsible for attachment efficacy. Further work is required to elucidate the molecular mechanisms responsible for the disparity of attachment efficacies between diverse *E. coli* O157:H7 strains that encode the same virulence determinants.

3.5. Conclusions

We have provided compelling evidence that, within a population of healthy feedlot cattle, a small subpopulation of animals appear to become persistently colonized by closely related *E. coli* O157:H7 strains. We found no physiological differences between animals we classified as PS, TS and NS based on our observations of animal health status and post-mortem histopathology. In addition, PS and TS animals appeared

to become colonized by a single predominant *E. coli* O157:H7 molecular subtype along with other closely-related molecular types supporting emergence of new genotypes. Finally, our findings provide provoking evidence that cattle may be more likely to be colonized by *E. coli* O157:H7 molecular subtypes that demonstrate an accentuated human pathogenic potential as evidenced by the enhanced ability of persistent strains to adhere to human intestinal epithelial cells. Additionally, it stands to reason that there is an increased likelihood of these *E. coli* O157:H7 subtypes to be transferred through the production continuum, and subsequently, into the human population because of their increased prevalence in feedlot cattle. Our results highlight the importance of pre-harvest food safety interventions to reduce the load of *E. coli* O157:H7 that enters the human food supply and support that those such efforts should be targeted at strains that persist in cattle populations which seem to represent the greatest risk to human health. Further research is needed to elucidate the underlying pathogen factors associated with persistent colonization of healthy cattle by *E. coli* O157:H7, including work to further probe molecular mechanisms associated with enhanced adhesion to intestinal cells, and to develop mitigation strategies to control *E. coli* O157:H7 in feedlot populations with the ultimate goal of reducing the risk of human infection.

Table 3.1. Distribution of *E. coli* O157 virulence genes among animals collected during the first two ante-mortem collections periods.

	Collection 1[b]		Collection 2	
Genotype[a]	Animals Shedding	% of Animals	Animals Shedding	% of Animals
rfb, fliC$_{h7}$, eae, stxI, stxII	314	39.8	265	33.6
rfb, stxI	32	4.1	156	19.8
rfb, stxI, stxII,	3	0.4	0	0.0
rfb, eae, stxI, stxII	9	1.1	42	5.3
rfb, fliC$_{h7}$, stxI, stxII	1	0.1	0	0.0
Total	359	45.5	463	58.7

[a] *rfb* (encodes the O157 antigen), *fliC$_{h7}$,* (encodes the H7 antigen) *eae* (encodes intimin), *stxI* (encodes Shiga toxin I) and *stxII* (encodes Shiga toxin II).

[b] All 788 animals were sampled during collection 1 and 2.

Table 3.2. Prevalence of *E. coli* O157:H7 among feedlot cattle over the final phase of finishing.

Collection	Number of Animals Sampled	Number of Positives[a]
1	788	314
2	788	265
3	476	206
4	197	39
5	100	28
6	35	15

[a] Positive samples were those that contained an *E. coli* O157:H7 with the *eae, stxI,* and *stxII* virulence genes. This *E. coli* O157:H7 genotype was targeted as it was the most common genotype identified during the first two sample collections and the purpose was to gain insight into persistent shedding of *E. coli* O157:H7.

Table 3.3. Distribution of post-mortem gastrointestinal tissues and contents positive for *E. coli* O157:H7 among persistent and transient shedders.

		Persistent Shedder[a]		Transient Shedder[b]	
Location	Site	Tissue	Content	Tissue	Content
Upper G.I. Tract	Reticulum	-	-	1	-
	Omasum	1	-	1	-
	Rumen	-	-	-	-
	Abomasum	-	-	-	-
	Duodenum	-	-	-	-
Lower G.I. Tract	Ileal-Cecal Junction	1	1	1	-
	Colon	-	1	-	-
	Rectal-Anal Junction	1	1	1	2
	Ileal Cecal Colic Node	-	-	-	-
	Anterior Root Node	1	-	-	-
	Gall Bladder	-	-	-	-

[a] n = 8
[b] n = 18

Table 3.4. Description of pulsed-field gel electrophoresis characterization of *E. coli* O157:H7 isolates from persistent shedder and transient shedder animals collected ante- and post-mortem.

		Persistent Shedder		Transient Shedder	
PFGE Type	Band Difference from Type F	Isolates[a]	Animals	Isolates	Animals
A	3	1(0)	1	0(0)	0
B	4	0(0)	0	0(1)	1
C	4	1(0)	1	0(0)	0
D	2	0(3)	1	0(0)	0
E	1	0(2)	1	1(0)	1
F	Dominant Subtype	40(0)	8	24(6)	11
G	3	0(5)	2	0(0)	0
H	3	2(0)	2	3(0)	3
I	2	8(0)	3	2(0)	1
J	4	0(4)	2	0(0)	0
K	1	1(0)	1	0(0)	0
L	1	1(0)	1	0(0)	0
M	1	0(0)	0	2(0)	1
N	6	1(0)	1	1(0)	1
O	4	0(0)	0	0(1)	1
P	1	3(0)	1	0(0)	0
Q	2	0(0)	0	1(0)	1
R	3	1(0)	1	0(0)	0
S	2	0(0)	0	3(0)	1
T	1	1(0)	1	0(0)	0
U	1	1(0)	1	0(0)	0
V	2	0(0)	0	1(0)	1
W	1	1(0)	1	0(0)	0
X	3	0(0)	0	0(1)	1
Y	1	0(0)	0	1(0)	1
Z	4	1(0)	1	0(0)	0
AA	> 7	2(0)	1	0(0)	0
AB	> 7	1(0)	1	0(0)	0
AC	> 7	0(1)	1	0(0)	0
AD	> 7	0(0)	0	0(1)	1
AE	3	0(1)	1	0(0)	0
AF	> 7	0(0)	0	0(1)	1
Total		66(16)		39(11)	

[a] Number before parenthesis indicates number of isolates collected ante-mortem whereas number in parenthesis indicates number of isolates collected post-mortem for each PFGE subtype.

Table 3.5. Distribution of pulsed-field gel electrophoresis subtypes for each persistent shedder during each ante-mortem collection period.

	Collection					
Animal	1	2	3	4	5	6
PS-1	F	F	F,	F, I	AB	I
PS-2	F	F, I	F	I	I	I
PS-3	F	F	C	T, K	Z	N
PS-4	F	F	F, U	F	F	F
PS-5	F	F	F	H	I	A
PS-6	F	F	F	L	R	H
PS-7	F	F, W	F	F, A	P	F
PS-8	F	F	F	A	F	AA

Figure 3.1. Partial dendogram containing a representative banding pattern from each of the 32 unique pulsed-field gel electrophoresis banding patterns including the banding pattern from the *E. coli* O157:H7 isolate (Kansas) obtained from a previous study (Childs et al., 2006) conducted two years before the current study. The Kansas isolate was determined to be of the same virulence genotype as the other *E. coli* O157:H7 isolates utilizing multiplex PCR before inclusion in PFGE analysis. The letters located to the right of each unique banding pattern correspond to the PFGE subtype outlined in Table 4.

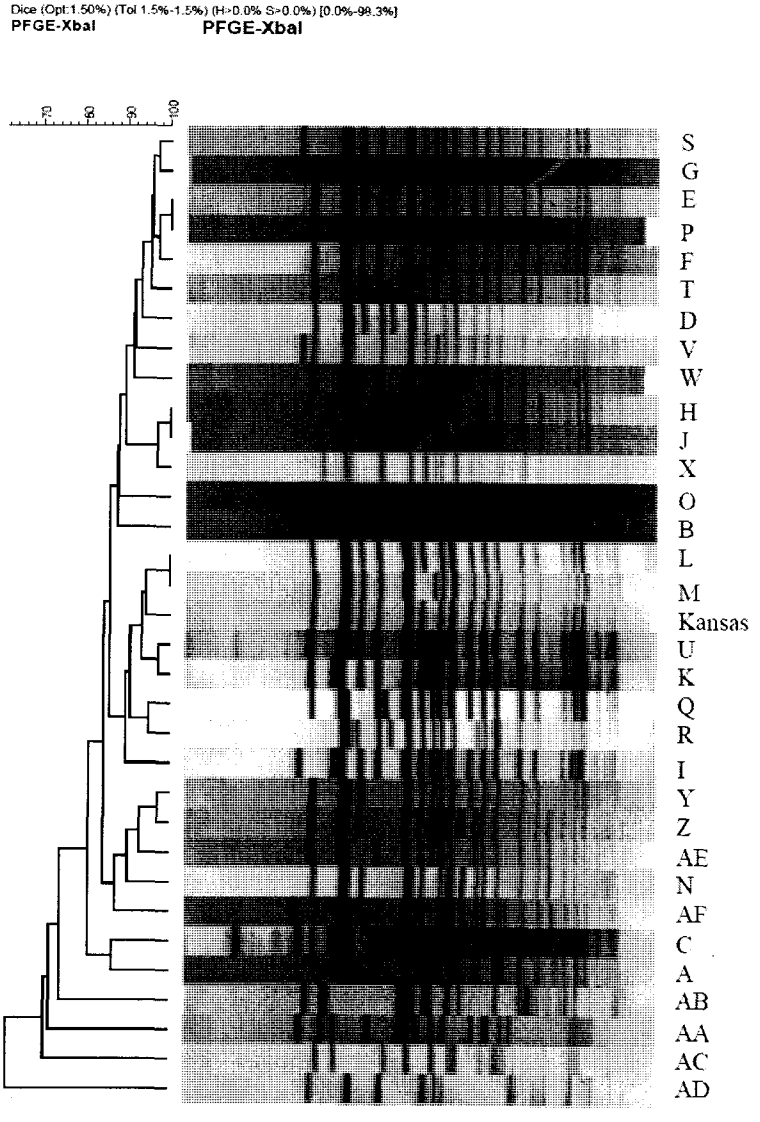


Figure 3.2. Estimated probabilities of encountering the three pulsed-field gel electrophoresis subtype categories during ante-mortem sampling. The probability of encountering an *E. coli* O157:H7 belonging to the dominant subtype, a subtype differing from the dominant subtype by 1-3 bands or a subtype differing from the dominant subtype by 4->7 bands is on the *y* axis while each collection is located on the *x* axis. Isolates possibly related (i.e., 4 to 6 band difference) to the dominant subtype and isolates divergent (>7 band difference) from the dominant subtype were collapsed to form the 4->7 band difference subtype category due to low numbers. The insufficient frequency of the 4->7 band difference subtype category prevented the inclusion of this category in the analysis preventing the calculation of error bars. Error bars represent standard error calculated for each estimated probability.

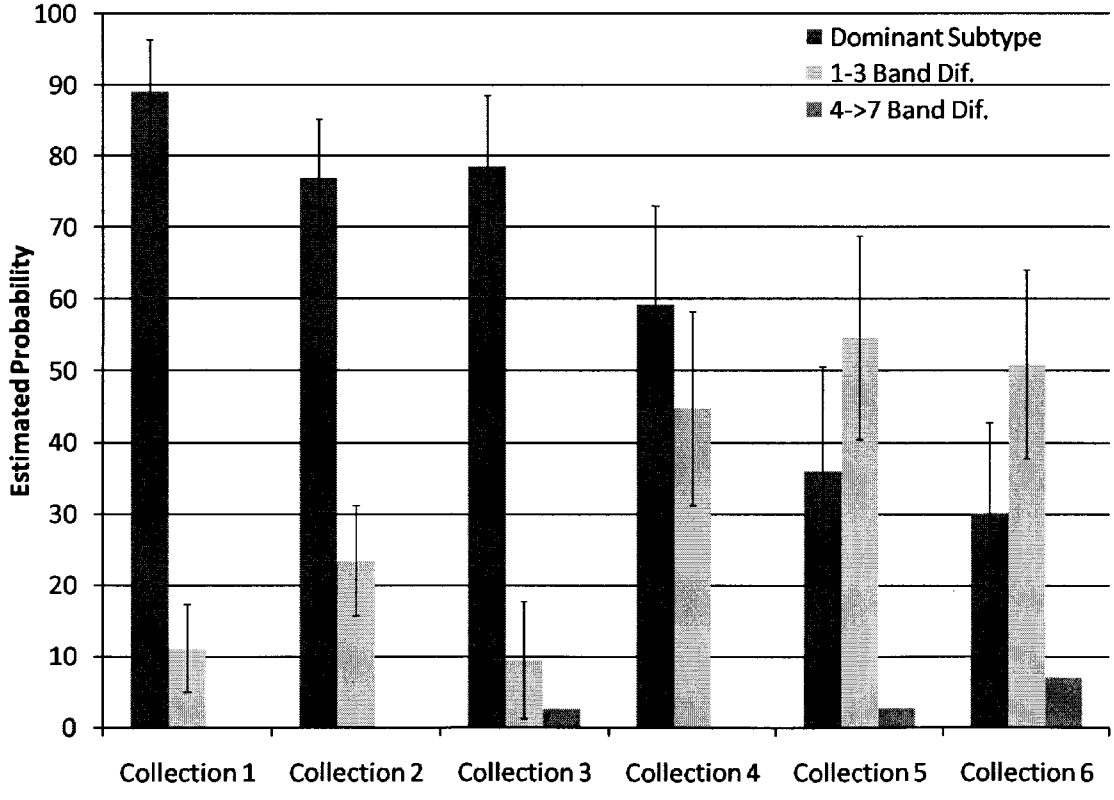
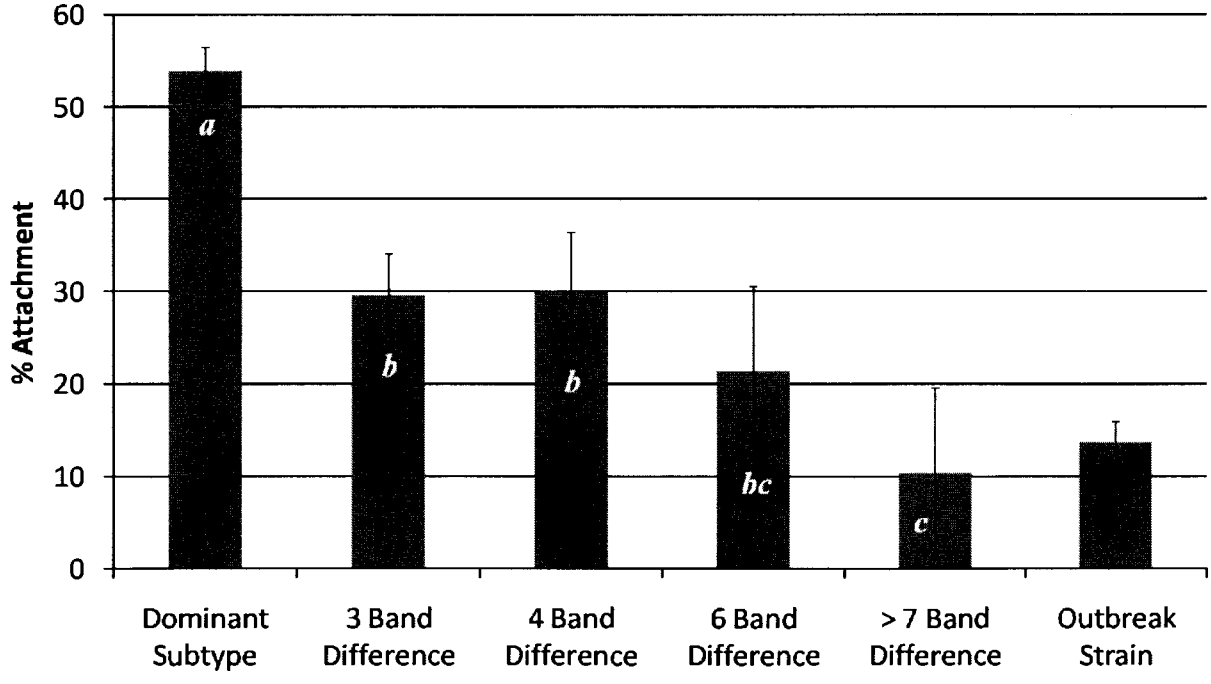


Figure 3.3. Caco-2 attachment efficacies of *E. coli* O157:H7 isolates representing genetically diverse pulsed-field gel electrophoresis subtypes and an *E. coli* O157:H7 isolate known to cause an outbreak of foodborne illness included as a reference. For ease of interpretation, data are presented as the percentage of adherent *E. coli* O157:H7 cells recovered from each well. The PFGE subtype is located on the *x* axis while % attachment is located on the *y* axis. Columns represent least squares means for attachment efficacies obtained from at least two strains (except for the 6 and >7 band difference where only one *E. coli* O157:H7 strain was assayed for each category) for each subtype category over three independent experiments. The error bars represent the standard error calculated for each least squares mean. Columns lacking common letters are different ($P < 0.05$). The outbreak strain was not included in the analysis but included in this graph as a reference.



CHAPTER IV

Influence of Virulence Factors and Genetic Diversity on the Ability of *Escherichia coli* O157 to Attach to Intestinal Epithelial Cells

ABSTRACT

Recent outbreaks of human illness associated with the consumption of foods contaminated with Shiga toxin-producing *Escherichia coli* belonging to the O157 serotype have heightened the public health significance of this organism. During early stages of *E. coli* O157 infection, the organism must adhere to the host's intestinal epithelium subsequently permitting colonization and the production of very potent cytotoxins (Shiga toxins; Stx) which, in burgeoning concentrations, can manifest as hemolytic uremic syndrome. The influence of genes encoding *E. coli* O157 virulence factors (i.e., *eae, stxI,* and *stxII*) and $fliC_{H7}$ was evaluated to determine the genes responsible for the most potent virulence phenotype. *E. coli* O157 isolates that contained only *eae* and $fliC_{H7}$ with neither Stx gene demonstrated an enhanced ($P < 0.05$) ability to attach to Caco-2 intestinal epithelial cells when compared to an *E. coli* O157:H7 isolate that contained *eae* and both Stx genes obtained from a food sample implicated in a disease outbreak. In an effort to explain differences in attachment, cytotoxicity assays were performed to determine Caco-2 cytotoxicity attributed to various *E. coli* O157 virulence genotypes at 3 h and 6 h post-infection. There was an interaction ($P < 0.05$) between genotype and time, but no difference ($P > 0.05$) between *E. coli* O157 genotypes

at 3 h post-infection. At 6 h post-infection, *E. coli* O157:H7 with *eae* and only *stxI* demonstrated the greatest ($P > 0.05$) cytotoxic insult. Probing genetic diversity of *E. coli* O157 isolates with a single nucleotide polymorphism (SNP) assay that evaluated the presence of 39 phylogenetically informative SNPs verified the cytotoxicity results as the *E. coli* O157 isolate with the greatest ($P < 0.05$) cytotoxicity was grouped into a clade containing the *E. coli* O157:H7 isolate responsible for the largest U.S. outbreak. These results contribute to the growing body of evidence that molecular mechanisms responsible for attachment and colonization are likely multi-factorial and independent of cytotoxicity.

4.1. Introduction

In the last decade, *Escherichia coli* serotype O157 has been linked to numerous multi-state outbreaks of human illness. Clinical manifestation of an *E. coli* O157 infection range from mild gastrointestinal distress to hemorrhagic colitis (HC) and life-threatening complications such as hemolytic uremic syndrome (HUS), which consequently results in 5% mortality (Nataro and Kaper, 1998). While consumption of undercooked ground beef products contaminated with the organism tends to be the hallmark source of infection, water, the environment, person-to-person contact, animal contact, other copious food vehicles (summarized in Erickson and Doyle, 2007) and, most recently, raw cookie dough (CDC, 2009b) have been implicated in outbreaks of *E. coli* O157 associated illness.

Manifestation of human disease is initiated with the evasion of viable *E. coli* O157 bacteria from the harsh conditions of the stomach to reach the more growth tolerant environment of the lower gastrointestinal (G.I.) tract. Once in the lower G.I. tract, *E. coli*

O157 utilizes a substantial repertoire of molecular machinery to facilitate the complicated process of attaching to the intestinal epithelial lining to prevent elimination through peristaltic flow. Recent literature highlights the significance of flagellin (Mahajan et al., 2009) and long polar fimbrae (Fitzhenry et al., 2006) to promote initial interaction with the host's epithelium. Once introduced to the eukaryotic cell, a cascade of highly regulated and specific processes encoded by the locus of enterocyte effacement (LEE) operon occurs (Torres et al., 2005). Initially, the *E. coli* O157 bacterium exploits a type III secretion system to insert its translocated intimin receptor (Tir; encoded by *tir*) into the eukaryotic host cell. Subsequently, another LEE encoded protein, intimin (encoded by *eae*) which is expressed on the bacteria's surface, forms an intimate adhesion with Tir and is further stabilized through the recruitment of eukaryotic actin and consequential rearrangement of actin to form a "pedestal" on the host cell's surface where the bacteria rests (Mainil and Daube, 2005). This pedestal formation is known as the attaching and effacing (A/E) lesion and is a classical histological characteristic of an *E. coli* O157 infection (Law, 2000).

Once an intimate attachment is achieved, *E. coli* O157 can produce very potent cytotoxins that, when diffused into the circulatory system, may cause chronic nephrotoxicity that can progress into HUS. Shiga toxins 1 and 2 (StxI and StxII; encoded by *stxI and stxII*, respectively) are internalized through receptor-mediated endocytic process following binding with the glycolipid Gb3 (globotriaosylceramide) receptor and once internalized, disrupt the integrity of 60S ribosomes inhibiting protein synthesis, consequently causing cell death (Mainil and Daube, 2005). Although cytotoxic, Stx2 has demonstrated the ability to amplify eukaryotic expression of nucleolin, which exhibits

affinity for intimin, thus enhancing the attachment ability of *E. coli* O157 (Robinson et al., 2006). However, a conflicting report refutes Stx2's role in enhancing *E. coli* O157's attachment capacity (Sheng et al., 2006).

The ability to swiftly characterize *E. coli* O157 on the nucleotide level provides supporting evidence to the theory of the recent emergence of *E. coli* O157 with intensified virulence. Riordan et al. (2008) highlighted the increase in hospitalization and development of HUS in patients from recent spinach and lettuce outbreaks in 2006 to the considerably lower frequencies of hospitalization and HUS experienced during the 1993 North American and 1996 Japanese outbreaks, most presumably due to an increase in *E. coli* O157 virulence. Subsequent single nucleotide polymorphism (SNP) characterization of *E. coli* O157 from outbreaks showed that *E. coli* O157 isolates from 2006 outbreaks linked to spinach and lettuce formed a highly clonal and well-supported phylogenetic clade (clade 8). These studies support the recent emergence of *E. coli* O157 subpopulations with enhanced ability to cause HUS (Manning et al., 2008).

In a previous study conducted by our group (Carlson et al., 2009), we demonstrated the presence of a predominant strain of *E. coli* O157:H7 in a population of feedlot cattle and the amplified pathogenicity potential of the predominant strain characterized by its enhanced ability to adhere to Caco-2 cells. We also identified *E. coli* O157 isolates belonging to different virulence genotypes from the same population of cattle, which were not further characterized with virulence phenotype assays in our previous study (Carlson et al., 2009). The objectives of the current study were to: (i) investigate the affects of the presence/absence of various *E. coli* O157 virulence genotypes, using a set of natural isolates, on ability to attach to Caco-2 cells, (ii)

determine if potential cytotoxicity effects associated with possessing *stxI* and *stxII* genes contributes to observed differences in attachment efficiency of *E. coli* O157 isolates belonging to different virulence genotypes and (iii) combine epidemiological data, virulence phenotype data and phylogenetic analysis of informative SNPs for a set of *E. coli* O157 isolates from a population of feedlot cattle and human clinical isolates from outbreaks to probe associations between attachment efficacy and virulence.

4.2. Materials & Methods

4.2.1. *E. coli* O157 Isolate Selection. *E. coli* O157 isolates, collected from a feedlot steer population, were selected from a subpopulation of isolates based on the presence/absence of the virulence genes *eae, stxI,* and *stxII.* In addition, two *E. coli* O157:H7 isolates, obtained from the same group of cattle and containing *eae, stxI,* and *stxII* that demonstrated significantly different attachment efficacies from our previous study (Carlson et al., 2009) also were selected. Five different *E. coli* O157 virulence genotypes among isolates from our previous study were selected for characterization by *in vitro* virulence phenotype assays (Table 4.1). In addition, a standard laboratory control *E. coli* O157:H7 strain with *eae, stxI,* and *stxII* (ATCC 43895) isolated from a food sample that was implicated in an outbreak of human illness was included in all *in vitro* virulence phenotype assays as a reference. All strains were available as pure, frozen cultures (-80°C).

4.2.2. Caco-2 Cell Attachment Assays. The attachment efficiency of *E. coli* O157 isolates was evaluated using the Caco-2 human intestinal epithelial cell line. Caco-2 cells were seeded into 24-well flat bottom plates (Corning Inc., Corning, NY) at a density of 1 x 10^5 cells/well in Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY;

DMEM) containing 20% heat-inactivated fetal bovine serum (Gibco) without antibiotics and grown to confluency (approximately 72 h). *E. coli* O157 overnight cultures were prepared by inoculating a single well-isolated colony into a 10 ml tube of BHI (Becton, Dickinson and Company, Franklin Lakes, NJ) broth and incubating at 37°C for 18 h without shaking. Overnight *E. coli* O157:H7 cultures (1 ml) were pelleted by centrifugation (11,337 x *g*; 5 min) and re-constituted in 1 ml of PBS. Confluent Caco-2 monolayers were infected with approximately 2 x 10^7 *E. coli* O157/well. After infection for 3 h at 37°C, non-adherent bacteria were removed by washing three times with PBS. Caco-2 cells were lysed by addition of 0.5 ml of ice-cold sterile ultrapure water, followed by vigorous pipetting and vortexing. Adherent *E. coli* O157, along with overnight *E. coli* O157 cultures, were enumerated by spread plating appropriate serial dilutions in duplicate onto BHI plates to determine adherent populations and initial inoculums for each isolate, respectively. BHI plates were incubated at 37°C for 24 h and resultant colony forming units were enumerated. The attachment efficiency of each *E. coli* O157:H7 isolate was determined as a percentage of the initial inoculum that was recovered as adherent *E. coli* O157:H7 cells.

4.2.3. Caco-2 Cytotoxicity Assays. To probe the hypothesis that the observed differences among attachment efficacies for *E. coli* O157 isolates representing different virulence genotypes may be attributed to cytotoxic affects from Stx, we determined Caco-2 cytotoxicity for each isolate representing different virulence genotypes that were characterized by the Caco-2 attachment assays previously described. Caco-2 monolayers and *E. coli* O157 cultures were prepared as previously described. Confluent Caco-2 monolayers (72 h) were infected with approximately 2 x 10^7 *E. coli* O157/well and

allowed to incubate at 37°C. Cytoxicity was measured at 3 and 6 h post-infection using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI) per manufacturer's instructions. Briefly, at predetermined time points, 50 μl of supernatant was removed from each well containing a Caco-2 monolayer infected with an *E. coli* O157 isolate representing virulence genotypes and transferred to a 96-well, clear, flat bottom plate (Corning) containing 50 μl of substrate and allowed to incubate for 30 min at room temperature. Following this incubation, 50 μl of stop solution was added to each well of the 96-well plate and absorbance was recorded at 490 nm. To permit calculation of percent cytotoxicity, positive cell lysis controls were prepared by adding 150 μl of lysis solution to non-infected Caco-2 cells and incubated at 37°C for 1 h before each of the predetermined infection durations for experimental wells (i.e., 2 h and 5 h). Following appropriate incubation duration, lysis controls were handled in the same manner as were the infected *E. coli* O157 experimental sample wells.

4.2.4. SNP Genotyping. We completed single nucleotide polymorphism (SNP) genotyping analyses on a panel of 95 *E. coli* O157 isolates selected to represent the genetic diversity (based on five-gene multiplex PCR and PFGE analyses) of *E. coli* O157 isolates from our previous study (Carlson et al., 2009). *E. coli* O157 DNA was purified according to the manufacturer's instructions using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA). Thirty-four real-time PCR-based SNP genotyping assays were performed to analyze each individual isolate in this study (in total, 3,230 real-time SNP genotyping PCR assays were conducted for all 95 isolates). The 34 SNP loci that we targeted in this study were carefully selected from an original panel of 96 SNP loci in a recent SNP genotyping study (Manning et al., 2008). We found that the combination of these 34 SNP

loci provided the best discriminatory power among the nine genetic lineages (or clades) described in *E. coli* O157 populations. Real-time PCR SNP genotyping was performed using a GenomeLab™ SNPStream® system (Beckman) as detailed previously (Manning et al., 2008).

4.2.5. Statistical Analysis. Populations of *E. coli* O157 recovered during attachment assays were enumerated and converted to log CFU/well and analyzed using ANOVA techniques with the PROC MIXED procedure of SAS (version 9.2, SAS Institute, Cary, NC). Least squares means were computed for the independent variable GENOTYPE and separated using the PDIFF option and Tukey's adjustment with significant differences noted at $P < 0.05$. Attachment data were reported as percent attached [(Recovered *E. coli* O157 ÷ *E. coli* O157 Inoculum) * 100] for ease of interpretation. Absorbance values recorded during cytotoxicity assays also were analyzed using ANOVA techniques evaluating treatment effects of GENOTYPE as the independent variable with the PROC MIXED procedure (SAS) including a repeated measures statement for the independent TIME variable. Least squares means were computed and separated as previously described. Cytotoxicity data are presented as percent cytotoxicity [(Treatment Absorbance ÷ Lysis Control Absorbance) * 100].

4.3. Results and Discussion

We have taken a unique approach to utilize *in vitro* attachment assays and cytotoxicity assays to characterize the pathogenic potential of naturally occurring *E. coli* O157 isolates belonging to different virulence genotypes and thus representing a genetic diverse set of *E. coli* O157 isolates from feedlot cattle. We employed the Caco-2 colonic epithelial cell line to evaluate the attachment capacity of different *E. coli* O157 virulence

genotypes as it is an appropriate *in vitro* surrogate (Mellor et al., 2009) to model the interaction of an enteric pathogen and the epithelium most likely to be colonized during human *E. coli* O157 infection. Additionally, the Caco-2 cell line expresses the Gb3 receptor and thus, is susceptible to Shiga toxin induced apoptosis (Schüller et al., 2004). We evaluated *E. coli* O157 attachment efficiency to Caco-2 cells at 3 h post-infection, as a short attachment period would highlight hyper-virulent phenotypes. We observed a difference ($P < 0.05$) between attachment capabilities among *E. coli* O157 isolates representing different virulence genotypes. *E. coli* O157 isolates without either *stx* gene demonstrated a greater ($P < 0.05$) capacity to attach to Caco-2 cells than did the *E. coli* O157:H7 reference strain. To initially probe whether these observed differences in attachment efficacy were independent of Stx induced lysis of Caco-2 cells, cytotoxicity assays were also performed on the same isolate set. In addition, we probed the genetic diversity of *E. coli* O157 isolates through SNP genotyping and further substantiated the influence of genetic lineages upon pathogenic potential of *E. coli* O157 with the combination of cytotoxic potential. An *E. coli* O157 isolate that demonstrated the greatest ($P < 0.05$) cytotoxic potential was categorized into the clade that contained the *E. coli* O157:H7 clinical isolate linked to the largest *E. coli* O157 outbreak in the U.S.

4.3.1. *E. coli* O157 virulence genotypes demonstrate significant variation in attachment efficacy for Caco-2 cells. There was no difference ($P > 0.05$) in attachment efficacies of *E. coli* O157 isolates with no *stx* genes, no *stxI*, and no *stxII* that resulted in 76.7, 65.5, and 57.7% attachment, respectively (Figure 4.1). While Shiga toxins are known to be potent cytotoxins, research has exhibited their ability to expatiate attachment efficiency of *E. coli* O157. The presence of StxII was found to increase nucleolin

expression by HEp-2 which demonstrates an affinity for intimin, subsequently enhancing the attachment capacity of *E. coli* O157:H7 (Robinson et al., 2006). *E. coli* O157 isolates that contained the *stxII* gene did not express ($P > 0.05$) a greater capacity to attach to Caco-2 cells when compared to *E. coli* O157 isolates that contained the *stxI* gene or no *stx* gene at all. Interestingly, *stx* negative *E. coli* O157 isolates demonstrated a more profound ($P < 0.05$) attachment capacity for Caco-2 cells than did *E. coli* O157:H7 isolates with *eae* and both *stx* genes isolated from bovine feces (Low 5) and a food sample (43895) associated with an outbreak of human disease (Figure 4.1). Even more, *E. coli* O157 isolates without any of the virulence genes screened for in our previous study (Carlson et al., 2009) exhibited similar attachment capacities as compared to other *eae* and *stx* positive *E. coli* O157 isolates, providing conflicting evidence pertaining to previous articles that report an adhesion synergism exhibited by *E. coli* O157 isolates that contain *eae* and *stxII* (Boerlin et al., 1999; Paton et al., 1997). While it is difficult to explain the unexpected attachment capacity exhibited by the *E. coli* O157 isolates that did not contain any of the screened-for virulence genes, we know that these isolates do contain the very potent endotoxin Lipopolysaccharide (LPS). Lipopolysaccharide has been investigated for its role in mediating *E. coli* O157's capacity to adhere to intestinal epithelial cells with conflicting results (Paton et al., 1998; Cockerill et al., 1996). Our findings emphasize the potential of LPS to be sufficient in facilitating *E. coli* O157 attachment to Caco-2 cells.

4.3.2. Observed differences in attachment efficiency for *E. coli* O157 isolates belonging to different virulence genotypes appear to be independent of cytotoxic response. Caco-2 cytotoxicity was measured at 3 h and 6 h post-infection (Figure 4.2)

and resulted in a GENOTYPE*TIME interaction ($P < 0.05$). At the 3 h time point, there was no difference in Caco-2 cytotoxicity (50 to 56.6% cytotoxic) across *E. coli* O157 isolates representing different virulence genotypes. However, at 6 h post-infection, the *E. coli* O157 isolate with *eae* and *stxI* (i.e., No StxII) demonstrated the greatest ($P < 0.05$) Caco-2 cytotoxicity (85.8% cytotoxic) while only exhibiting moderate attachment capacity (Fig 4.1). To our knowledge, this is the first report where *E. coli* O157 with *stxI* resulted in a more extensive cytotoxic insult than did *E. coli* O157 isolates that contained either just *stxII* or both *stx* genes. Contrary to the belief that StxII production delineates the most virulent *E. coli* O157 phenotype, an idea developed with the combination of historical epidemiological data (Ostroff et al., 1989) and validation of StxII's cytotoxic potency during *in vitro* cytotoxicity experiments (Baker et al., 2007; Lefebvre et al., 2009); our data provides evidence that *stxI* genotypes maintain the potential to produce a greater cytotoxic insult than *stxII* genotypes, at least when evaluated in intestinal epithelial cells. Additionally, we highlight that *E. coli* O157 isolates obtained from healthy beef cattle have the ability to express hyper-virulent phenotypes in a human intestinal epithelial cell line, a result that conflicts with earlier literature (Baker et al., 2007). The validity of our cytotoxicity model is further substantiated by the findings of Acheson et al. (1998), whom reported on the complexity of Stx-mediated lysis; they described the ability of both StxI and StxII alone to cross a Caco-2 monolayer without obvious cytotoxic repercussion, but Caco-2 destruction resulted when monolayers were exposed to the bacteria that produced Stx. An explanation for our results could be that StxI has a greater affinity for stressed epithelial cells; Schüller et al. (2007) demonstrated StxI's increased binding ability over StxII to intestinal epithelial cells that were inflamed,

potentially elucidating the mechanism responsible for our results. Furthermore, *E. coli* O157 isolates without additional virulence genes and *E. coli* O157 isolates with *eae* and $fliC_{H7}$ induced a greater cytotoxic reaction than did *E. coli* O157 strain 43895 which was implicated in an outbreak of human disease, most presumably due to *E. coli* O157 LPS insult.

4.3.3. Genetic lineage offers greater insight into *E. coli* O157 attachment ability than to *E. coli* O157 cytotoxicity. Phylogenetic analysis of *E. coli* O157:H7 clinical isolates obtained from various outbreaks of human illness identified 39 SNP genotypes and subsequently divided the isolates into nine distinct clades (Manning et al., 2008). These researchers suggested that there has been a recent emergence of hyper-virulent *E. coli* O157 strains (clade 8) that demonstrate a more profound ability to cause severe human disease (Manning et al., 2008). Interestingly, all of the *E. coli* O157 isolates that varied in their virulence genotype (i.e., contained at least *eae, stxI* or *stxII* but not all three) except the *E. coli* O157 isolate that was missing *stxII* were classified into clade 7 (Table 4.1); a clade associated with the ability to cause, albeit less severe, diarrhea. However, attachment to intestinal cells by pathogenic *E. coli* alone can cause watery diarrhea (Robins-Browne and Hartland, 2002). Clade 7 isolates (i.e., O157, No Stx, and No Stx 1 genotypes) demonstrated a numerically greater capacity to attach (Fig 4.1) to Caco-2 cells than did Clade 2 isolates (i.e., No Stx 2, High 5 and 43895), but had a diminished cytotoxic effect at 6 h post-infection (Fig 4.2). The *E. coli* O157 isolate that contained *eae, stxI,* and *stxII* (High 5) and the *E. coli* O157 lacking *stxII* were classified into clade 2, the most numerically frequent clade that is associated with a diminished ability to cause HUS compared to clade 8, which may somewhat explain their virulence phenotype.

However, we provide disputing evidence to an earlier report indicating that *E. coli* O157:H7 strains isolated from diseased humans were constituents of diverse lineages than were strains obtained from healthy cattle (Kim et al., 1999) as we had bovine isolates classified into clades containing clinical *E. coli* O157 isolates (Figure 4.3).

4.4. Conclusion

We have provided evidence that a variety of *E. coli* O157 virulence genotypes isolated from healthy feedlot cattle have expatiated attachment abilities and maintain similar cytotoxic capacities compared to fully virulent *E. coli* O157:H7 isolates. Our research also provides evidence validating the significance of the *stxI* genotype and the production of Stx1 in initiating human disease as *stxI E. coli* O157 virulence genotypes demonstrated significantly more Caco-2 cytotoxic insult than did *stxII E. coli* O157 virulence genotypes. Characterization of bovine *E. coli* O157 isolates with SNP genotyping contributed insight into the pathogenic potential of different *E. coli* O157 virulence genotypes. While our work highlighted a portion of the influence of virulence genotypes, intimate attachment to eukaryotic cells and subsequent destruction of epithelium is highly specific, multi-factorial and presumably influenced by mechanisms not evaluated within the scope of our study. Further work is needed to elucidate the influence of genetic lineage on pathogenic potential of *E. coli* O157 containing virulence factors.

Table 4.1. *E. coli* O157 virulence genotypes (number of isolates for each genotype), virulence genes present, origin and genetic lineages of isolates evaluated in cell attachment and cytotoxicity assays.

E. coli O157 Virulence Genotype (number of isolates screened)	Virulence Genes Present	Origin	Clade
O157 (2)	*rfb*	bovine feces	7
No Stx (2)	*rfb, eae, fliC*$_{H7}$	bovine feces	7
No Stx1 (2)	*rfb, eae, fliC*$_{H7}$, *stxII*	bovine feces	7
No Stx2 (1)	*rfb, eae, fliC*$_{H7}$, *stxI*	bovine feces	2
Low 5 (1)	*rfb, eae, fliC*$_{H7}$, *stxI, stxII*	bovine feces	3
High 5 (1)	*rfb, eae, fliC*$_{H7}$, *stxI, stxII*	bovine feces	2
43895 (1)	*rfb, eae, fliC*$_{H7}$, *stxI, stxII*	ATCC	2

Figure 4.1. Attachment data presented as percent of *E. coli* O157 inoculum (% Attachment) recovered from Caco-2 monolayers 3 h post-infection. All *E. coli* O157 isolates belonging to different *E. coli* O157 virulence genotypes were screened in duplicate wells during three independent assays with LS means calculated from resultant values (n = 6). *E. coli* O157 virulence genotypes (i.e., columns) with differing letters are different, $P < 0.05$.

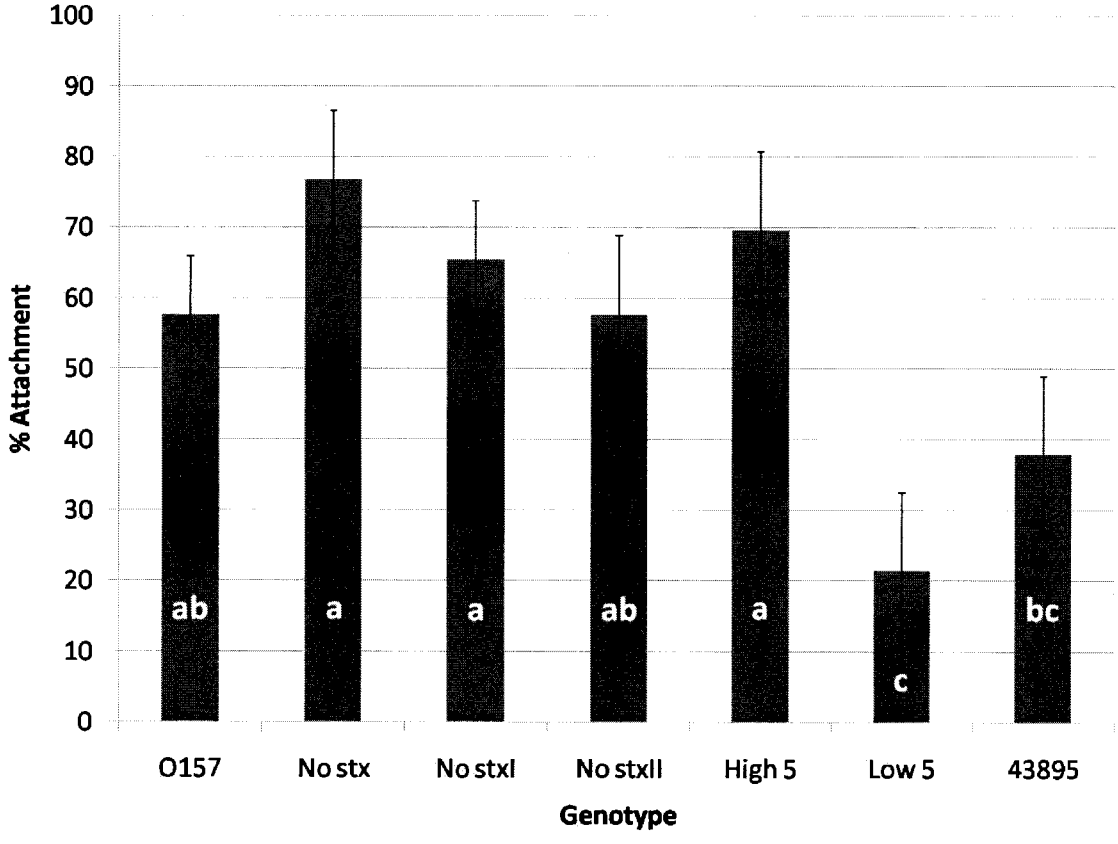


Figure 4.2. Cytotoxicity data presented as percent of Caco-2 lysis exhibited by *E. coli* O157 isolates belonging to different virulence genotypes. All *E. coli* O157 isolates belonging to different *E. coli* O157 virulence genotypes were screened in duplicate wells during eight independent assays with LS means calculated from resultant values (n = 16). *E. coli* O157 virulence genotypes (i.e., columns) with differing letters are different, $P < 0.05$.

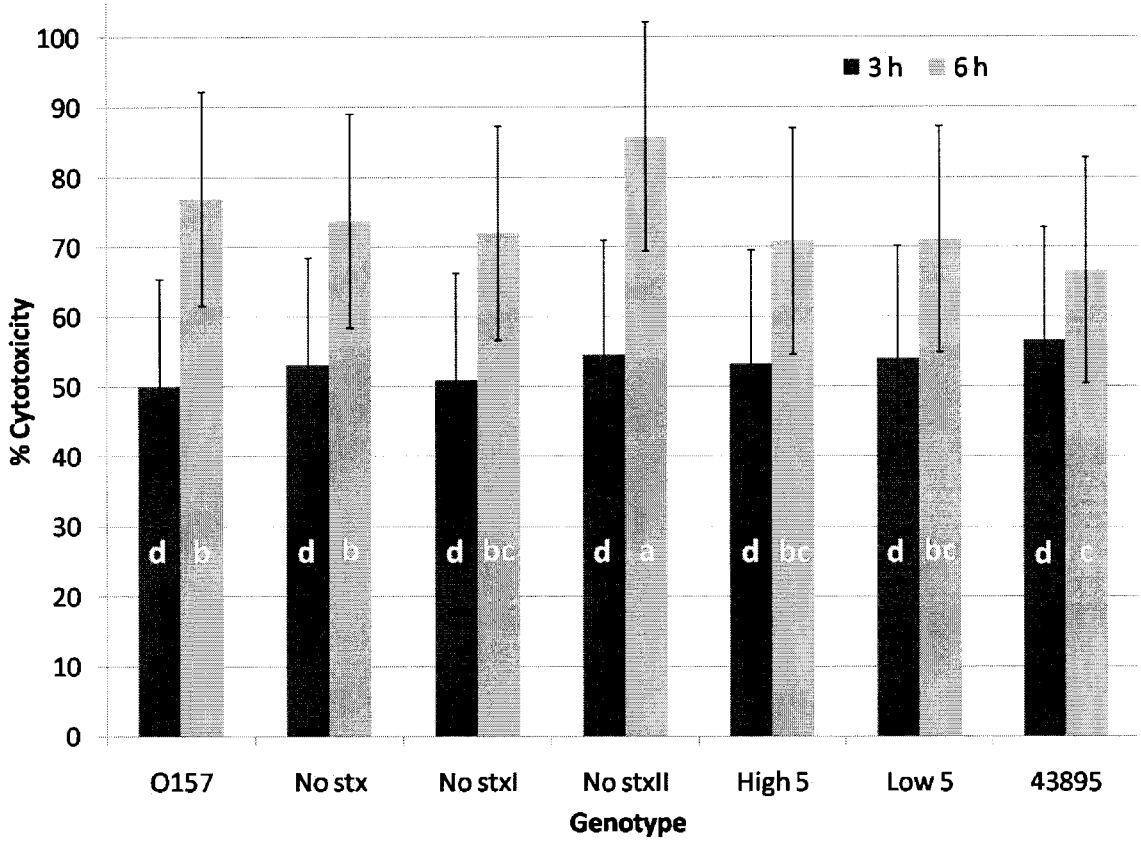
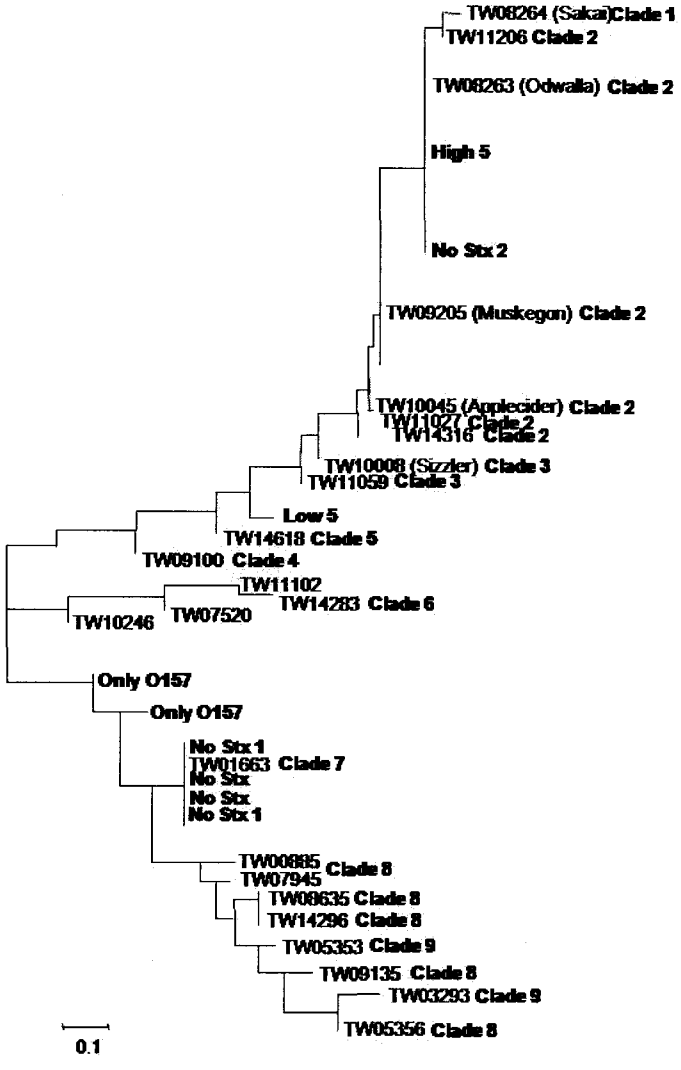


Figure 4.3. Phylogenetic tree containing all of the *E. coli* O157 isolates included in the Caco-2 attachment and cytotoxicity assays. TW isolates were included as clade reference (Manning et al., 2008).



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