THESIS

MOLECULAR ECOLOGY OF *LISTERIA* SPP., *SALMONELLA, ESCHERICHIA COLI* O157:H7, AND NON-O157 SHIGA TOXIN PRODUCING *E. COLI* IN NORTHERN COLORADO WILDERNESS AREAS

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ABSTRACT

MOLECULAR ECOLOGY OF *LISTERIA* SPP., *SALMONELLA, ESCHERICHIA COLI* O157:H7, AND NON-O157 SHIGA TOXIN PRODUCING *E. COLI* IN NORTHERN COLORADO WILDERNESS AREAS

Foodborne disease is a substantial concern in the United States and receives a great deal of attention from the government, industry, and the media. Government initiatives have alleviated some of the burden; however, without improved knowledge of the molecular epidemiology of the pathogens in a variety of environments, a comprehensive understanding of foodborne disease will remain out of reach. *Listeria monocytogenes, Salmonella, Escherichia coli* O157:H7 and non-O157 Shiga toxin producing *E. coli* (STEC) play a prominent role in the incidence of bacterial foodborne illness in the United States. Molecular subtyping methods are used extensively in foodborne disease surveillance, yet there is a knowledge gap regarding the presence, transmission and molecular ecology of these pathogens in non-food associated environments. We collected foodborne pathogen isolates from pristine wilderness environments to obtain subtyping data that may aid in the interpretation of clinical and food isolates particularly during outbreak investigations. Furthermore, the identification of subtypes present in different environments but not commonly linked to human disease

may provide key information regarding the evolution of virulence in these organisms. To achieve these goals, five wilderness locations in Colorado were selected to represent pristine locations and three areas (approximately $100m^2$) within each location were designated; each area was sampled once during the spring, summer, and fall seasons in 2009 and 2010. A total of 450 soil, 450 water, 90 drag swab (surface soil) and 276 fecal samples were collected. Five soil samples and five water samples from each area were composited and all samples were microbiologically analyzed to detect *Listeria* spp. (i.e., *L. monocytogenes* and other *Listeria* spp.), *Salmonella, E. coli* O157:H7, and non-O157 STEC. After non-selective pre-enrichment, samples were divided and microbiologically analyzed to detect each target organism using modified versions of the United States Food and Drug Administration Bacteriological Analytical Manual. Up to four presumptive colonies for each target organism from each sample were confirmed by PCR to detect gene fragments specific to each respective organism. Overall, three samples tested positive for *L. monocytogenes*, including two fecal samples and one water sample. Nineteen samples contained *Listeria* spp. other than *L. monocytogenes*, 14 of which were determined to be *Listeria welshimeri* by *sigB* sequencing. The remaining five *Listeria* spp. were presumptively identified as *Listeria rocourtiae* sp. nov. by 16s rDNA sequencing; however, these isolates demonstrated notably different biochemical properties than *L. rocourtiae. Salmonella* was found in two samples, including one water and one fecal sample, and five non-O157 STEC were found in one fecal, one sediment, and three water samples. *E. coli* O157:H7 was not detected in the natural environments in Northern Colorado surveyed in this study. A molecular serotyping PCR assay revealed two *L. monocytogenes* isolates belonging to the 1/2 b serogroup and two isolates

belonging to the 1/2a serogroup, with a single fecal sample containing two different *L. monocytogenes* serogroups (1/2a and 1/2b). Pulsed field gel electrophoresis typing results indicated three unique DNA fingerprints among the *L. monocytogenes* isolates. Two unique strains were isolated from a single fecal sample; with one strain isolated from two different fecal samples collected from the same area. All five non-O157 STEC- and both *Salmonella*-positive samples had unique PFGE fingerprints. All subtyping data has been deposited in PathogenTracker (www.pathogentracker.com), a publicly available WWW database. In conclusion, results from this study demonstrate a rare presence of foodborne pathogens in pristine environments and the utility of molecular subtyping from distinct environments. Further characterization of the foodborne pathogen isolates obtained from non-food associated environments in this study will expand our knowledge on the molecular ecology of foodborne pathogens in nature.

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CHAPTER I

LITERATURE REVIEW

Molecular Ecology of Foodborne Pathogens in Nature

1.1 Foodborne Illness

Foodborne illness is a considerable public health burden and has severe economic impacts in the United States. Recent literature estimated that 9.4 million episodes of foodborne illness are caused by 31 major pathogens each year in the United States (U.S.; Scallan et al., 2011). Together, *Listeria monocytogenes, Salmonella, Escherichia coli* O157:H7 and non-O157 Shiga toxin-producing *E. coli* (STEC) are responsible for over 33% of bacterial foodborne illnesses attributed to 31 major pathogens in the U.S. The annual cost of foodborne illness due to medical care and quality of life losses in the U.S. is estimated at $157 billion (Scharff, 2010). In 2011, Scallan and colleagues estimated that *L. monocytogenes* causes 1,591 domestically acquired foodborne disease cases, including 255 deaths, annually in the U.S. Non-typhoidal *Salmonella* was estimated to cause 1.03 million foodborne disease cases, including 378 deaths, while *E. coli* O157:H7 was estimated to cause 63,153 foodborne illness cases, including 20 deaths. Non-O157 STEC was estimated to cause 112,752 foodborne illnesses annually; however, data are skewed and the number of non-O157 STEC related deaths has not been determined.

Combined, *L. monocytogenes, Salmonella,* and STEC O157 cause approximately 653 deaths out of the 861 deaths annually due to domestically acquired bacterial foodborne pathogens in the U.S. These figures represent estimates based on adjustments due to the fact that foodborne disease is vastly underreported (Scallan et al, 2011).

The Foodborne Diseases Active Surveillance Network (FoodNet) identifies the number of laboratory-confirmed infections of foodborne pathogens through population-based surveillance in ten states. In 2009, the incidence per 100,000 of *L. monocytogenes, Salmonella*, STEC O157, and non-O157 STEC was 0.34, 15.19, 0.99, and 0.57 respectively. In comparison to the 1996-1998 data, the incidence of disease attributed to *L. monocytogenes, Salmonella* and *E. coli* O157 decreased by 26%, 10% and 41%, respectively; baseline data was not available for non-O157 STEC [Centers for Disease Control and Prevention (CDC), 2010]. While significant progress has been made over the past 13 to 15 years to reduce the incidence of *E. coli* O157 and *L. monocytogenes* infections, the incidence of salmonellosis has remained fairly constant.

Progress in reducing the incidence of foodborne illnesses attributed to *E. coli* O157 and *L. monocytogenes* may in part be attributed to aggressive efforts put forth by the meat industry to control these pathogens through interventions during processing and product formulations. Some interventions have been implemented to reduce the number of foodborne infections caused by key pathogens. Koohmaraie and colleagues (2005) summarized numerous interventions, including hide, carcass and trim interventions, which have lowered the contamination of *E. coli* O157:H7 in beef. The combination of multiple interventions, called hurdles, has been used extensively in the food industry to improve the safety and quality of foods. For example, the use of multiple-sequential

decontamination interventions, including steam vacuuming, pre-evisceration carcass washing, pre-evisceration organic acid solution rinsing, hot water carcass washing, post-evisceration final carcass washing, and post-evisceration organic acid solution rinsing was found to improve the microbial quality of beef carcasses (Bacon et al., 2000). Formulation of ready-to-eat foods with antimicrobials has also been implemented to control growth of *L. monocytogenes* in finished ready-to-eat meat products during refrigerated storage, which may have resulted from cross-contamination of such products by the plant environment following the lethality step (Stopforth et al., 2005). In 1997, the U.S. government set forth an objective in the Healthy People 2010 Initiative to reduce the incidence of foodborne diseases by fifty percent for key pathogens to be achieved by the year 2010 (U.S. Department of Health and Human Services, 2010). This goal was achieved in 2009 for *E. coli* O157:H7; however, the Healthy People 2010 goal has yet to be met for *L. monocytogenes* and *Salmonella*, supporting the critical need for additional efforts to control these pathogens in foods.

1.2. *Listeria* Taxonomy

The genus *Listeria* is composed of Gram-positive, non-sporeforming, catalase-positive, oxidase-negative, and facultative anaerobic cells (Vázquez-Boland et al., 2001). At least six different species belong to the *Listeria* genus, including *L. monocytogenes, L. seeligeri, L. welshimeri, L. innocua, L. ivanovii*, and *L. grayi.* Two new species, *L. marthii* and *L. rocourtiae*, were recently identified as well (Graves et al., 2009; Leclercq et al., 2010). Only two species in the *Listeria* genus are considered pathogenic, with *L. monocytogenes* representing the only pathogen of public and veterinary health significance, while *L. ivanovii* is usually restricted to causing disease in sheep.

1.3. *L. monocytogenes* in Food

L. monocytogenes is thought to live as a saprophyte where it can survive in soil and decaying vegetation (Weis et al., 1975). This organism has been isolated from a variety of food products and in both food and non-food associated environments (Farber and Peterkin, 1991). *L. monocytogenes* is capable of tolerating a variety of environmental stresses and can grow in bile salts, at pH ranges between 4.3 and 9.0 (Hain et al., 2007) and in temperatures ranging from 1-45 degrees Celsius. Bacterial cells are motile at 10 to 30 degrees Celsius (Kamp and Higgins, 2009) and will survive in as much as 20% NaCl (Farber and Peterkin, 1991; Gray and Boor, 2006). The ability for cells to grow at refrigeration temperatures during shelf-life storage is a major concern for food safety (Walker et al., 1990; Vasquez-Boland et al., 2001).

L. monocytogenes was first demonstrated to be transmitted through contaminated food in 1981 when several deaths and stillbirths were attributed to contamination of coleslaw (Schlech et al., 1983). Outbreaks have since linked this organism to a variety of foods, particularly ready-to-eat (RTE) foods, which include foods that do not receive additional heat treatment by the consumer prior to consumption. Since *L. monocytogenes* is readily inactivated though cooking and pasteurization, cross-contamination with food-associated environments (e.g., food processing plant or retail) after the lethality step (e.g., cooking or pasteurization) is the major route through which *L. monocytogenes* contaminates foods (Farber and Peterkin, 1991). Foods contaminated with this organism that have been linked to listeriosis include but are likely not limited to dairy, vegetable, and meat products (Schuchat et al., 1991). The risk of infection with *L. monocytogenes* is increased in the elderly, pregnant women, neonates, human immunodeficiency virus-

infected individuals, and those undergoing immunosuppressive therapies and may lead to meningitis, sepsis and abortion (Fshi et al., 2001). Due to the severity of listeriosis illness, numerous studies have reported the prevalence in a variety of foods and food-processing environments to try and understand the molecular ecology and transmission patterns of this organism. For example, Sauders and colleagues (2009) found *L. monocytogenes* in the environment of 60% of retail establishments and in 2.7% of ready-to-eat foods tested at retail. In another comprehensive survey, *L. monocytogenes* was found in 1.82% of more than 30,000 ready-to-eat food samples collected at retail establishments in Maryland and California (Gombas et al., 2003). Most recently, 1.7 to 10.8% in environmental samples collected from small or very small ready-to-eat meat processing plants contained *L. monocytogenes* (Williams et al., 2011).

1.4. *L. monocytogenes* Virulence

The virulence determinants of *L. monocytogenes* have been studied extensively and the mechanism of host cell invasion is fairly understood. Many virulence factors contribute to its ability to survive and spread throughout the host. Several of these key virulence factors are located on a single gene cluster referred to as LIPI-1 (*Listeria* pathogenicity island 1). LIPI-1 consists of the *plcA-prfA, mpl-actA-plcB,* and *hly* genes along with other open reading frames with unknown functions (Vázquez-Boland et al., 2001). LIPI-1 is a 10 kb gene cluster found in the two pathogenic species of *Listeria* which encodes LLO (*hly*), ActA (*actA*), and both phospholipases (*plcA* and *plcB*). *Listeria seeligeri* also contains the gene cluster; however, an insertion of *ofrE,* a divergently transcribed open reading frame, disrupts the PrfA-dependent activation of virulence genes (Vázquez-Boland et al., 2001). The *prfA* (positive regulatory factor A)

gene regulates many genes involved in *L. monocytogenes* virulence, including *hly*, *actA*, *plcAB, inlAB, inlC,* and other virulence factors. The major proteins required for adherence to host cells, the internalins, are encoded in a separate location of the genome; however, still belong to the PrfA regulon (Dramsi et al., 1993). Exposure to stressful conditions such as reduced temperature, oxidative stress, carbon starvation, or low pH, activates the transcription of an alternative sigma factor, SigB, which promotes the expression of a set of genes to cope with the stressful environment and virulence genes such as *inlA* (Kim et al., 2004; van Schaik and Abee, 2005).

L. monocytogenes triggers its own uptake by non-professional phagocytoic host cells using invasion proteins that enable attachment and invasion. Adherence to and invasion of host cells requires a variety of mechanisms; however, internalin A (InlA; encoded by *inlA*) and internalin B (InlB; encoded by *inlB*) have been studied most extensively. These surface proteins are sufficient for infection in different cell types and recognize different receptors (Gaillard et al., 1991; Bierne et al., 2007). Upon recognition of E-cadherin, a eukaryotic adhesion protein found in epithelial cells, InlA can bind to intestinal, liver, and dendritic cells along with placental epithelium (Mengaud et al., 1996; Leceut, 2005). Other host receptors include Met, a hepatocyte growth factor tyrosine kinase receptor (Shen et al., 2000), gC1qR, a receptor for the complement protein C1q (Braun et al., 2000), and glycosaminoglycans found on mammalian cell surfaces (Jonquières et al., 2001). Association between the bacterial cell and host receptors initiates to a signal transduction cascade ultimately leading to the active uptake of the bacterial cell (Kathariou, 2002). Studies have also demonstrated passive uptake of *L. monocytogenes* primarily into enterocytes and M cells in Peyer's patches, followed by

the dissemination to the liver and spleen. Surviving bacteria may further infect hepatocytes and cause systemic infection in secondary target organs (central nervous system, placenta, and fetus) (Racz et al., 1972; MacDonald and Carter, 1980; Ireton and Cossart, 1997; Altimira et al., 1999).

Upon internalization of the bacteria by active or passive phagocytosis, *L. monocytogenes* must next escape from the stressful, acidic environment of the vacuole into the cytoplasm. The expression of listeriolysin O (LLO), or hemolysin, enables the escape into the protective environment of the cytoplasm where cells can replicate and evade the host immune system (Tilney and Portnoy, 1989). Encoded by the gene *hly*, LLO is a toxin, which acts at a low pH by forming pores in cell membranes containing cholesterol. Escape from the acidic vacuole into the neutral pH of the cytosol reduces the activity of LLO, thus preventing the host cell membrane from also being lysed. Efficient escape from the vacuole is also dependant on two phospholipases, PlcA (encoded by *plcA*) and PlcB (encoded by *plcB*) (Jenkins and Watson, 1971; Menguad et al., 1991; Vázquez-Boland et al., 2001). Once inside the host cell cytoplasm, *L. monocytogenes* stimulates the polymerization of a eukaryotic cytoskeleton protein called actin. The bacterial protein ActA is responsible for forming this tail like structure, which propels *L. monocytogenes* cells through the cytoplasm, and a long finger like protrusion is formed when bacterial cells reach the host cell membrane (Kocks et al., 1992; Gray and Boor, 2006). These pseudopods are engulfed by the adjacent host cell through phagocytosis, encasing the bacteria in a double layered vacuole. *L. monocytogenes* cells once again utilize phospholipases and LLO to escape into the host cell cytosol and the intracellular cycle continues (Kathariou, 2002).

1.5. *L. monocytogenes* Genetic Diversity and Epidemiology

A variety of subtyping techniques, including both phenotypic and DNA-based methods, have been employed to differentiate *L. monocytogenes* isolates. Phenotypic subtyping by conventional slide agglutination serotyping categorizes *L. monocytogenes* into 13 serotypes: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7 (Seeliger and Hohne, 1979). While all serotypes are capable of causing disease, approximately 98% of strains isolated from humans in both outbreaks and sporadic cases have been linked to a few specific serotypes, including 1/2a, 1/2b, 1/2c, and 4b (Buchrieser, 2007). Based on ribotyping and allelic variation in virulence genes, *L. monocytogenes* can also be classified into four genetic groups referred to as lineages, which correlate with serotypes (Nadon et al., 2001). The population structure can be studied by classifying isolates into the two major (I and II) or two minor lineages (III and IV). Lineage I (serotypes 1/2b, 3b, 3c, and 4b) includes strains known as Epidemic Clones, which have been implicated in the majority of human listeriosis outbreak cases worldwide and are overrepresented among sporadic cases in some countries including the U.S. Lineage II (serotypes 1/2a, 1/2c, and 3a) includes (i) strains have been implicated in human and animal sporadic cases and (ii) strains that are overrepresented among foods but have rarely been linked to human disease (Gray et al., 2004). While isolates belonging to lineage III and IV are capable of eliciting human disease; they are rare in general and thus not typically associated with human illness (Wiedmann et al., 1997; Nightingale, 2010). Most major foodborne outbreaks of listeriosis have been attributed to lineage I serotype 4b strains and more specifically, a number of closely related strains termed Epidemic Clones. Three Epidemic Clone (EC) strains within lineage I, including ECI, ECIa, and ECII, belong to

serotype 4b and have been implicated in one or more epidemics, with ECI involved in outbreaks on multiple continents (Kathariou, 2002). Within lineage II, a serotype 1/2a ECIII strain, caused a sporadic listeriosis case in 1988 and multistate outbreak in 2000, which was shown to persist in the same processing plant for over ten years (Olsen et al., 2005; Orsi et al., 2008).

1.6. *Salmonella* Taxonomy

Salmonella is a Gram-negative, facultative intracellular pathogen that is capable of inducing a variety of diseases in humans and animals, ranging from self-limiting enterocolitis to systemic illness such as typhoid fever (Scherer and Miller, 2001). While the *Salmonella* nomenclature has evolved over the years, the current system used by the CDC divides the genus into two species, *S. enterica* and *S. bongori* (formerly subspecies V). *S. enterica* is further divided into six subspecies including (I) *S. enterica* subsp. *enterica*; (II) *S. enterica* subsp. *salamae*; (IIIa) *S. enterica* subsp. *arizonae*; (IIIb) *S. enterica* subsp. *diarizonae*; (IV) *S. enterica* subsp. *houtenae*; and (VI), *S. enterica* subsp. *indica*. Nearly all mammalian disease is attributed to *Salmonella enterica* subspecies *enterica* with the remaining subspecies and *S. bongori* rarely isolated and more commonly isolated from cold-blooded animals and the environment (Brenner et al., 2000). There are over 2,463 recognized serovars of *Salmonella* (Brenner et al., 2000; Heyndrickx et al., 2005) with Typhimurium and Enteritidis being the most prevalent nontyphoidal *Salmonella* serovars isolated from human salmonellosis cases in the U.S. (Andrews-Polymenis et al., 2009). Predominant serotypes are associated with animals; Montevideo is the most common serotype isolated from ground beef, Derby from market hogs, Kentucky from broilers, and Nima and Pooma from reptiles (Callaway et al., 2007).

1.7. *Salmonella* Presence in Food

Foods commonly linked to *Salmonella*-induced illness include beef, pork, poultry, and egg products; however, more recent outbreaks have been attributed to fruits (Vojdani et al., 2008), vegetables (Klontz et al., 2010), and peanut butter (MMWR, 2009). Cross-contamination has become a major concern in low-moisture foods, where contamination occurs after a lethal processing step and has been traced to poor sanitation practices, poor equipment design, and poor ingredient control (Podolak et al., 2010).

Salmonella has been found in all food animals, although links between salmonellosis in humans and animals is most apparent in poultry. Marcus and colleagues (2007) found significant associations between *Salmonella* Enteritidis infections and eating chicken outside the home, eating undercooked eggs inside the home, and contact with birds and reptiles. A significant increase was found in the number of slaughter establishments with positive *Salmonella* Enteritidis broiler chicken rinses from the year 2000 to 2005 (Altekruse et al., 2006). One study found almost 40% of drag swab samples in a broiler hatchery positive for *Salmonella*. They identified 15 different *Salmonella* serotypes when they collected a variety of sample types through production and processing in two poultry farms. Interestingly, pulsed field gel electrophoresis (PFGE) typing of isolates revealed strains with indistinguishable patterns isolated from one poultry house environment, mice trapped on the farm, and samples taken at a company breeding farm (Liljebjelke et al., 2005). There has been a targeted effort by the U.S. Department of Agriculture: Food Safety Inspection Services to reduce the incidence of *Salmonella* in chickens below 19% (Callaway et al., 2007). *Salmonella* contamination on produce farms has been investigated as well. Two outbreaks of *Salmonella* Newport

were linked to tomatoes grown in Virginia in 2002 and 2005. In 2005, the same PFGE fingerprint from both outbreaks was isolated from pond water used to irrigate the tomato fields (Greene et al., 2008). Multidrug resistant (MDR) *Salmonella* has become a major concern in the food industry as it's associated with more invasive disease and a higher case fatality rate. An increase in MDR *Salmonella* found in foods corresponds to the increased use of antibiotics as growth promotants in food animals (Solghan et al., 2010).

1.8. Non-typhoidal Salmonellosis

The infectious dose of *Salmonella* ranges from 10^6 to 10^9 organisms (Finlay, 1995) and illness is initiated when bacterial cells are ingested via contaminated food or water and begin replicating in the lumen of the small intestine. Contact with the intestinal mucosa of the distal ileum initiates spread to the reticuloendothelial system and opens the avenue for dissemination to the lymph nodes, spleen, liver, and blood (McCormic et al., 1996). There are several mechanisms whereby *Salmonella* may invade host cells; bacterial cells may be taken up by M cells, captured in the lumen by phagocytes, or they may force their entry into non-phagocytic enterocytes (McGhie et al., 2009). Columnar epithelial cells of the intestine appear to be the more prevalent route of invasion. Attachment of non-typhoidal *Salmonella* to the intestinal epithelium triggers neutrophil transmigration, which provokes an intense inflammatory response and manifests itself in the host as diarrhea (McCormic et al., 1996).

1.9. *Salmonella* Virulence

Bacterial cells have adapted to survive and recognize temperature and pH extremes, oxygen limitations, presence of bile salts, digestive enzymes, and competing microflora (McCormick et al., 1996). The hostile environment within the gastrointestinal

tract is not only tolerated by *Salmonella* but serves as a signal to induce transcription of genes required for host cell attachment and invasion. Bacterial mediated endocytosis is dependent upon interactions with the bacterial cell surface and host cells (Finlay, 1994) and through the use of electron microscopy, it was demonstrated that microvilli undergo dramatic changes including membrane ruffling accompanied by cytoskeletal rearrangements at the point of attachment. After internalization, however, the microvilli return to their pre-infected appearance (Takeuchi, 1967). The ability to form membrane ruffles is critical for cell invasion as it creates membrane bound vacuoles that internalize bacterial cells. This forms spacious phagosomes in which bacterial cells survive and replicate. Internalization of *Salmonella* requires a type III secretion system which promotes the translocation of proteins into the host cell (Finlay, 1994; McCormic et al., 1996).

Many virulence factors are encoded on the five identified *Salmonella* pathogenicity islands (termed SPI-1 through SPI-5) present on the bacterial chromosome. Based on the presence of transposon sequences and the G+C content of these regions, it is hypothesized that they may have been acquired from other species through horizontal gene transfer (Lavigne and Blanc-Potard, 2008). SPI-1 is required for invasion of non-phagocytic cells and consists of approximately 25 genes, including those encoding a type III secretion system (T3SS). This facilitates the transport of bacterial effector proteins into the cytosol, eventually leading to the uptake of the bacterium. SPI-2 is required for *Salmonella* to induce systemic infections; it encodes an additional T3SS enabling bacterial survival in epithelial cells and macrophages. SPI-3 contains genes required for *Salmonella* survival in Mg2+ limiting environments and although SPI-4 has not been

studied in full detail, putative virulence factors have been identified. These include genes that may encode a type I secretion system and a gene required for intra-macrophage survival. SPI-5 encodes SopB and PipB, which are effector proteins for the SPI-1-encoded T3SS and SPI-2-encoded T3SS respectively (Schmidt and Hensel, 2004). A variety of *Salmonella* serotypes also harbor a virulence plasmid encoding the *spv* locus that has been shown to increase virulence in systemic infections by facilitating intracellular replication and host cell apoptosis (Guiney et al., 1994). A two component regulatory system, PhoP and PhoQ, is required for *Salmonella* pathogenesis and controls the expression of over 40 genes (Miller et al., 1989; Scherer and Miller, 2001). A number of other proteins and adhesion factors are required for the invasion of epithelial cells, including InvA; Galan and colleagues (1992) demonstrated *invA* is required for *Salmonella* invasion into epithelial cells.

1.10. *E. coli* Taxonomy

Escherichia coli are a genetically diverse species with the majority of its members being nonpathogenic and part of the natural gut microflora of humans and animals (Eblen, 2008). *E. coli* was not identified as a human pathogen until the early 1940's (Bray and Beavan, 1948). Several different pathotypes of pathogenic *E. coli* have since been categorized based on their virulence properties and disease-causing mechanisms. *E. coli* pathotypes include, (i) Shiga toxin producing *E. coli* (STEC), which are strains that carry genes encoding one or both Shiga toxins (stx1 and stx2) that were acquired from *Shigella* through phage mediated transfer, (ii) enterohemorrhagic *E. coli* (EHEC), which are STEC strains that cause hemorrhagic colitis and hemolytic uremic syndrome, (iii) enteropathogenic *E. coli* (EPEC), which are strains that intimately attach

to intestinal cells and cause severe diarrhea in infants in developing countries, (iv) enterotoxigenic *E. coli* (ETEC) include strains that produce enterotoxins and are the main cause of travelers diarrhea in developing nations, (v) enteroinvasive *E. coli* (EIEC), which are closely related to *Shigella* spp. and produce an inflammatory colitis, (vi) diffuse-adhering *E. coli* (DAEC), which are strains that produce a diffuse pattern of adherence to intestinal cells, and (vii) entero-aggregative *E. coli* (EAggEC), which produce toxins and are named for their auto-aggregative adherence to intestinal cells (Kaper et al., 2004).

E. coli is a Gram-negative, rod-shaped facultative anaerobic bacterium. It is serotyped based on surface antigens, specifically their somatic (O) and flagellar (H) antigens. There are 174 O antigens and 53 H antigens defined in the serotyping scheme; however, non-motile (NM) strains have been identified as well (Gyles, 2007). *E. coli* O157:H7 was the first STEC serotype to be linked to outbreaks of illness involving severe invasive disease in the early 1980s, and now is among hundreds of STEC that have been shown to carry one or both *stx* genes (Eklund et al., 2001, Brooks et al., 2005). More recently, other non-O157 *E. coli* STEC serotypes have been linked to human disease, including a recent 2010 cluster of illnesses in the Northeast attributed to ground beef contaminated by *E. coli* 026 (Khan, 2010). A few specific serotypes, including O26, O45, O55, O103, O111, O121 and O145, appear to be responsibility for the majority (>70%) of human illnesses attributed to non-O157 in the U.S. (Brooks et al., 2005).

1.11. STEC Presence in Foods

Cattle are the primary reservoir of STEC in the U.S., although information is limited regarding the prevalence of non-O157 STEC in the environment (Gyles, 2007).

Various studies have documented the prevalence of STEC in cattle; however, results range from 0% (Wilson et al., 1992) to 71% (Cerqueira et al., 1999) of individual animals. Contamination of meat during harvest may occur if proper sanitation is lacking in the processing environment or contact with the animal hide or gut contents during slaughter. Interventions for reducing microbial counts on carcasses have been implemented in some meat processing plants. One study found significant reductions of *E. coli* O157:H7 by treating carcass hides with one of four antimicrobials (acetic acid, lactic acid, sodium hydroxide, or sodium metasilicate) (Carlson et al., 2008). Contamination of produce is also a concern as it can occur during production, harvest or processing through the use contaminated seeds, water, or untreated animal manure. Sources of human infection have included raw or undercooked meat (primarily ground beef), ready-to-eat sausages, raw milk, cheese, unpasteurized apple cider and juice, lettuce, cantaloupes, alfalfa sprouts, radish sprouts, drinking water, and water for bathing. Contact with animals and person-to-person transmission via the fecal oral route has been documented as well (Gyles, 2007). For example, *E. coli* O157 was linked to petting animals at Colorado's largest stock show in 2009 after the Colorado Department of Health and Environment confirmed over 20 cases of disease (Powell, 2009).

1.12. STEC Virulence

Several virulence characteristics contribute to the pathogenesis of STEC. Acid resistance (Leyer et al., 1995), intestinal colonization (Sherman et al., 1988), and toxin production (Tashiro et al., 1994) are among the factors that enable STEC to elicit disease in humans. The production of Shiga toxins is perhaps the most concerning, as it is directly responsible for the most severe sequelae. STEC infection in humans may result

in no disease, watery or bloody diarrhea, hemorrhagic colitis, thrombotic thrombocytopenic purpura, or hemolytic uremic syndrome (HUS). HUS is characterized by acute renal failure, microangiopathic hemolytic anemia, and thrombocytopenia. Five to 10 percent of individuals infected with STEC O157 develop HUS with children and the elderly being more susceptible (Griffin and Tauxe, 1991). Presence of STEC in food environments is particularly risky as the infectious dose of *E. coli* O157:H7 is only 1-100 CFU (Paton et al., 1998).

Acid resistance adaptation allows STEC to elicit disease by enabling the bacteria to survive the harsh environment of the stomach and eventually colonize the gut and induce disease symptoms. When STEC reach the host intestinal epithelium, a variety of virulence components contribute to the disease process. The locus of enterocyte effacement (LEE) is a pathogenicity island that encodes proteins, including intimin, intimin receptor, effector molecules and a type III secretion system, which are required for adhesion to the intestinal epithelium. Intimin, encoded by the gene *eaeA* and present on LEE, is responsible for the intimate attachment of STEC to the host cell membrane (Donnenberg et al., 1997). Using a type III secretion system, STEC delivers a host epithelial receptor for intimin (encoded by *eae*) called Tir (translocated intimin receptor; encoded by *tir*) into the host cell, along with other secreted proteins (Kenny et al., 1997). This intimate adherence of STEC to the host intestinal epithelium through the Intimin/Tir interaction allows the bacteria to become colonized in the host gastrointestinal tract and leads to the characteristic attaching and effacing (A/E) lesions. These A/E lesions results in loss of enterocyte microvilli and the formation of pedestals from the accumulation of cytoskeletal components (Sherman et al, 1988). At least 16 variants of *eaeA* have been

identified with heterogeneity in the C-terminal end of the molecule, which binds Tir (Garrido et al., 2006). It is generally recognized that intimin is required for STEC pathogenesis; however, alternative forms of adherence have been speculated (Paton et al., 1998; Vidal et al., 2008). Vidal and colleagues (2008) studied adherence to epithelial cells in *eae* negative STEC and found that *saa* and a *psu-int* region participate in the adhesion process. Putative adhesion genes *efa*1 and $lpfA_{O157}$ have also been described (Vidal et al., 2007).

Shiga toxin production is critical in pathogenesis of HUS. The Shiga toxin itself is composed of one A subunit and five B subunits, which form a pentameric ring structure that recognizes mammalian cell targets (Ling et al., 1998). There are two Shiga toxins, including Shiga toxin1 (Stx1; encoded by *stx1*) and Shiga toxin 2 (Stx2; encoded by *stx2*), with Stx2 more commonly associated with HUS. Stx1 is highly conserved with only a few variants, while over 20 variants of Stx2 with differing degrees of toxicity have been described thus far (Nakao et al., 2002). The B subunits bind to globotriaosylceramide (Gb3) receptors on the surface of cells which permit the internalization of the toxin molecule. Protein synthesis is interrupted when the A subunit cleaves a specific adenine base from the 28S rRNA, resulting in apoptosis of the host cell (Ching et al., 2002; Lim et al., 2010).

1.13. STEC Genetic Diversity and Epidemiology

While the evolutionary history of STEC is not entirely understood, it's recognized that the toxin of *Shigella dysenteriae* type I is the earlier source of Stx. The lambda bacteriophage has an ability to infect a variety of bacterial strains and integrate its DNA into the host cell's genome, which is then called a prophage (Recktenwald and Schmidt,

2002; Allison, 2007). The Shiga toxin genes are either encoded on an inducible prophage that can switch into the lytic phase to produce more virus particles, or the genes may be present on prophage remnants in the *E. coli* chromosome (Allison, 2007). Stx phages are widespread in the environment and are more resistant to chlorination, pasteurization, and composting than their bacterial hosts (Muniesa et al., 1999). This poses a problem in food system since phages can survive conditions that are used to control foodborne pathogens. Once in contact with a susceptible host, the phage can then confer the ability to produce Stx. Recombination events coupled with the ability of Stx-phages to infect multiple host cells create an extreme amount of heterogeneity among STEC isolates (Allison, 2007). The disease induced by STEC can be exacerbated if Stx production in the gut is amplified. Evidence suggests that Stx-phages can be transduced to commensal gut flora, escalating the severity of the disease. Antibiotic therapy to treat STEC infections is not recommended because this stress induces Stx-prophages into the lytic cycle, resulting in a surge of Stx production (Karch et al., 2005; Allison, 2007).

E. coli O157:H7 was first associated with human illness in 1982 and has since been linked to numerous outbreaks of foodborne illness (Riley et al., 1983; Uhlich et al., 2008). Following a large foodborne outbreak in 1992-1993, the Food Safety and Inspection Service (FSIS) established strict guidelines including a zero-tolerance policy for *E. coli* O157:H7 and *E. coli* O157 in food products (Naugle et al., 2005). Currently, the focus of STEC monitoring by the U.S. Department of Agriculture: Food Safety Inspection Services (USDA: FSIS) is *E. coli* O157:H7 (Eblen, 2008). Emerging evidence, however, suggests that non-O157 STEC is also present in foods and can induce the same severe disease in humans. Scallan and colleagues (2011) estimated 112,752

total cases of domestically acquired foodborne non-O157 STEC per year; however, the true incidence of non-O157 STEC remains unclear due to the difficulties in distinguishing it from non-pathogenic *E. coli* (Eblen, 2008).

The collaboration between the CDC and other programs to establish FoodNet initiated HUS surveillance in 1997. In 2000, the Council for State and Territorial Epidemiologists passed a resolution which made all Shiga toxin-producing *E. coli* nationally notifiable (Eblen, 2008). Between 1983 and 2002, the CDC confirmed 940 non-O157 STEC isolates, with the incidence peaking in the summer months (Brooks et al., 2005). From 2005 to 2009, the number of non-O157 STEC cases documented in FoodNet sites increased by 55%, while the incidence of *E. coli* O157:H7 decreased slightly (Vigia et al., 2006; Matyas et al., 2010).

1.14. Presence of Key Human Foodborne Pathogens in Nature

There is little data regarding the presence of *Listeria, Salmonella*, and STEC in natural environments that have not been heavily impacted by man and even less data on the molecular characterization of isolates from nature. It is recognized that all of these pathogens can be harbored in a variety of host species asymptomatically (Fsihi et al., 2001; Gyles, 2007; Callaway et al., 2008). Direct contact between humans and wildlife is therefore one route of infection, however indirect exposure to wildlife-associated environments may be an even greater risk. Foodborne pathogens may be transmitted to humans through contact with environmental niches contaminated with pathogens and contamination of the food supply though the interactions between wildlife and crops.

In recent years, outbreaks of foodborne illness have been associated with wildlife. Many organisms can survive for long periods in environment, including O157 STEC,

which has been shown to survive in fecal material in the environment for more than 20 months (Beutin, 2006). In 1998, 157 persons became ill with *E. coli* O157:H7 after being exposed to unchlorinated water in Wyoming contaminated with elk and deer feces (Olsen et al., 2002). Additionally, feral pigs were implicated as a source of *E. coli* O157:H7 that contaminated spinach fields in California. The outbreak was linked to the consumption of fresh, bagged baby spinach in September of 2006, with 205 reported cases and three deaths. Almost fifteen percent of feral swine fecal samples tested were positive for *E. coli* O157:H7, many of which had the same PFGE fingerprint type as the outbreak strain. This PFGE fingerprint was also isolated from soil, sediment and surface water in the same region (Jay et al., 2007). In 2008, eight children became infected with *E. coli* O157:H7 in Evergreen, Colorado. The local Department of Public Health isolated the outbreak strain fingerprint pattern from elk feces collected from a nearby park (Scanlon, 2008). Gilbreath and colleagues (2009) tested fecal pellets from deer and elk in Idaho for the presence of Shiga toxin genes; 19.4% of samples tested were positive for STEC, possibly explained by the large presence of STEC-colonized cattle in the nearby areas.

Weis and colleagues (1975) determined the prevalence of *L. monocytogenes* from soil and plants collected from cornfields (8.4%), grainfields (11.8%), cultivated fields (12.2%), uncultivated fields (44.0%), meadows (9.9%) and forests (15.2%). Additional samples and environments were tested as well including, mud (31.5%), wildlife feeding grounds (23.1%), residues of fodder (27.2%), wildlife feces (15.7%), and birds (17.3%). While the prevalence of *L. monocytogenes* ranged depending on the sample type and the environment, authors noticed an enhanced ability to survive and multiply in moist

environments and a reduced ability in soils with low pH values. Serotypes 1/2b and 4b were found to be most predominant. A more recent study in upstate New York found the prevalence of *L. monocytogenes* in soil, water, and vegetation in natural environments ranging from 0.77% to 2.1% (Sauders et al., 2005). Its distribution in surface waters in Ontario, Canada was determined in 2007, revealing 10% of 314 samples positive for this organism (Lyautey et al., 2007). In Canada, *L. monocytogenes* was isolated from five wildlife fecal samples belonging to deer, moose, otter, and raccoon (Lyautey et al., 2007). The diversity of *L. monocytogenes* isolated from environmental samples was evaluated in two studies. Sauders and colleagues (2005) found that isolates from urban and natural areas are diverse and form distinct but overlapping populations based on *Eco*RI ribotyping and *sigB* allelic profiling. Similarly, Lyautey et al. (2007) found that fecal *L monocytogenes* isolated from different sources belong to overlapping but distinct populations.

In 1975, Thomason and colleagues isolated *Salmonella* from 13% and 25% of environmental water samples from a harsh unfavorable environment and a lush, moisture rich environment respectively, both with high human contact. They noticed a strong correlation between presence of the pathogen in visually wet samples and the absence of the pathogen in dry samples. A variety of *Salmonella* serotypes were isolated from wildlife at two Ohio rehabilitation centers. Eight of 71 (11%) samples tested positive including feces from opossums, squirrels, a woodchuck, a hawk, and a screech owl (Jijon et al, 2007). Additionally, in Norway outbreaks of *Salmonella* Typhimurium were linked to hedgehogs in 1996 and 2000 (Handeland et al., 2002).

1.15. Subtyping of Foodborne Pathogens

Molecular subtyping is commonly used in public health labs to identify and track foodborne pathogens. The CDC PulseNet database contains a large amount of molecular subtyping data from human clinical isolates and food and farm animal isolates; however, limited data is available for environmental isolates. Multiple methods are available to further characterize organisms beyond the species level. These subtyping methods can be grouped into two categories based on the cellular components targeted. Phenotypic subtyping methods target enzymes, proteins, or other metabolites expressed by the cell, while molecular methods probe nucleic acids (Nightingale, 2010).

Several phenotypic methods have been employed to characterize *L. monocytogenes, Salmonella,* and STEC isolates, including serotyping, biotyping, antimicrobial resistance profiling, multilocus enzyme electrophoresis (MEE), and phage typing. Serotyping, for example, is used to characterize many pathogens and is based on the identification of different antigens present on the surface of the bacterial cells. Using antibodies and antisera, the presence of these surface antigens can be detected. *E. coli* O157:H7 represents a single serotype; however, strains can be further differentiated using additional subtyping techniques (Wiedmann 2002).

With increasing availability for DNA sequence data for foodborne pathogens, molecular subtyping methods have been developed to further differentiate organisms beyond the species and phenotypic subtype level. Targeting genetic material, molecular subtyping assays can be grouped into two categories, including band-based and DNA sequence-based methods. Band-based subtyping approaches generate patterns of DNA fragments, also referred to as "DNA fingerprints". Several types of band-based methods are used for the characterization of bacterial isolates, including some PCR based

approaches. These include multiplex PCR, random amplified polymorphic DNA (RAPD), PCR restriction length fragment polymorphism (PCR-RFLP), amplified fragment length polymorphism (AFLP), and repetitive element PCR (rep-PCR) (Carlson and Nightingale, 2009).

Amplified fragment length polymorphism utilizes restriction digests in addition to PCR amplification of fragments. After digestion of the bacterial genome with a frequent cutting restriction enzyme, short adapter sequences are ligated to the ends of the fragments. PCR primers recognize these adapters and through the addition of random nucleotides that interact with unknown nucleotides, a reasonable number of fragments are amplified. Gel electrophoresis is used to separate fragments and the resulting profiles are used to make comparisons (Foley et al., 2009). Torpdahl and Ahrens (2004) used AFLP to study the population structure of *Salmonella.* Their method using two restriction enzymes clearly clustered together strains representing the subgroups of *Salmonella*.

Random amplification of DNA using generic PCR primers is the basis of RAPD-PCR. The combination of short primers and low stringency thermocycling conditions creates multiple amplicons that produce a bacterial fingerprint when separated. A major advantage of this method is that no prior knowledge of the genome sequence is required to identify specific targets, as the targets are random DNA sequences. However, reproducibility can be difficult with this method, as minor changes in the protocol can significantly alter the results (Foley et al., 2009). Intraserotype genetic diversity was detected when RAPD analysis was performed on an assortment of STEC serotypes, including O20:H19, O113:H21, O117:H7, O157:H7, O171:H2 and O174:H21 isolated from cattle feces and bovine meat (Kruger et al., 2006).

Rep-PCR relies on repeated DNA sequence elements that are distributed throughout bacterial genome. PCR primers are designed to recognize specific repeat elements and amplify flanking sequences. PCR products are produced when repeat sequences are close enough together. A banding pattern is produced and the profiles can be used to determine genetic relatedness (Foley et al., 2009). Rasschaert and colleagues (2005) found rep-PCR to be a valuable technique for differentiating *Salmonella enterica* serotypes. They found high reproducibility within a PCR run and can thus be used to limit the number of strains that had to be further characterized by serotyping.

PCR amplification of specific sequences followed by restriction digest of amplicons is known as PCR-RFLP. Sequences with conserved regions flanking variable regions are used as the target and therefore produce different sized fragments after digestion with one or more restriction enzymes (Foley et al., 2009). This method of analysis has been implemented for the characterization of virulence-associated genes in numerous foodborne pathogens including *L. monocytogenes* and STEC (Carlson and Nightingale, 2009). PCR-RFLP analysis of specific virulence genes in *L. monocytogenes* has been able to group isolates into genetic lineages and characterize strains based on their pathogenicity potential (Wiedmann et al., 1997). This method was also used to characterize the allelic diversity within *eae*, which encodes Intimin, and the Shiga toxin genes in STEC isolates (Eklund et al., 2002; Ramachandran et al., 2003). Ramachandran and colleagues (2003) developed a PCR-RFLP assay that reliably differentiates 15 intimin types and found that some intimin types were preferentially associated with specific flagellum types.

Additional band-based subtyping methods do not include PCR, but rather use restriction enzymes to digest total bacterial DNA. The resulting fragments are then separated by size to generate a banding pattern. Both PFGE and ribotyping fall into this category and represent commonly employed macrorestriction subtyping methods to characterize bacterial foodborne isolates (Carlson and Nightingale, 2009). While both methods are used extensively, PFGE is considered the "gold standard" for discriminating different foodborne pathogen subtypes (Silbert et al., 2003). PFGE represents an analysis of total bacterial DNA, including both the bacterial chromosome and plasmid DNA. A rare frequency cutting enzyme is employed to digest DNA into a small number (8 to 25) of large DNA fragments (ranging from 20 Kb to > 1 Mb). In order to protect these large fragments from physical forces such as pipetting, which may cause unwanted shearing, a defined concentration of bacterial cells is first embedded in agarose. These agarose plugs are then subjected to cell lysis through detergents and enzymes such as lysozyme (for Gran-positive organisms only) and proteinase K (for both Gram-positive and Gram-negative organisms). Plugs are then washed extensively to remove any unwanted chemicals or cellular components. At this point, a portion of the plug containing lysed bacterial DNA is digested with a restriction enzyme, which recognizes a specific DNA sequence and cuts the DNA at this site. Following digestion, the plug portion is loaded into an agarose gel and subjected to a unique method of gel electrophoresis. This provides an electric field across the gel and uses alternating currents at 120° angles for the sufficient separation of large sized fragments (Chu et al., 1986). PulseNet: The Molecular Subtyping Network for Foodborne Bacterial Disease Surveillance, a national program created by the CDC, developed standardized PFGE protocols for *Campylobacter*

jejuni, *E. coli* O157:H7, non-O157 STEC, *L. monocytogenes*, *Salmonella*, *Shigella* and *Yersinia pestis*. These protocols specify the lysis conditions, restriction enzymes and gel electrophoresis running conditions that should be employed for each organism. PulseNet, with the collaboration of the CDC and the Association of Public Health Laboratories, provides a searchable electronic database of PFGE patterns for several pathogenic species. Strains isolated from clinical cases and foods can be compared to identify epidemiologically and genetically related strains isolated across the country (Swaminathan et al., 2001).

An alternative band-based subtyping method known as ribotyping relies on genetic variations that accumulate in the genes that code for ribosomal RNA. Genes encoding 5S, 16S, and 23S rRNAs are essential for all bacteria to survive and are thus highly conserved (Stull et al., 1988). Multiple rRNA operons are contained within a bacterial chromosome, permitting discrimination within and between species based on this genetic information coded on the chromosome. Total bacterial DNA is released by cell lysis followed by digestion by a frequent cutting restriction enzyme that yields hundreds of DNA fragments ranging from 1 to 30 kb in size (Wiedmann, 2002). Fragments are subjected to agarose gel electrophoresis for size separation and then transferred to a nylon membrane. A chemically labeled rRNA probe is then hybridized to the fragments containing rRNA genes and only rRNA fragments are visualized in banding patterns (Baloga et al., 1991; Wiedmann, 2002). New commercially available technology has enabled automated ribotyping, which requires minimal labor and has improved reproducibility among different labs (Hahm et al., 2003).

DNA sequence-based molecular subtyping could potentially provide additional discriminatory power as sequence data is less ambiguous than banding patterns and thus easier to interpret. DNA sequence-based methods that have been used to characterize foodborne pathogens include multilocus sequence typing (MLST), multiple-locus variable-number tandem repeat analysis (MLVA), single nucleotide polymorphism (SNP) typing and full genome sequencing (Nightingale, 2010).

Repetitive DNA elements are found throughout bacterial genomes and often contain tandem repeat sequences. MLVA is a PCR based approach that characterizes bacterial strains based on their tandem repeat sequences across multiple loci. These regions are typically the most highly variable regions in the genome as polymerase errors and recombination events are more likely to occur (Lindstedt, 2005). This can lead to differences in the number of repeat sequences, referred to as variable-number tandem repeats (VNTRs). The identification of VNTRs typically requires a full genome sequence and then primers flanking VNTR sequences can be developed for the MLVA assay. The size of the VNTR amplicon is visualized via gel electrophoresis (Foley et al., 2009; Carlson and Nightingale, 2009). Chiou et al. (2010) identified 16 VNTR loci in 40 diverse *Salmonella* Typhimurium isolates and evaluated their MLVA assay for allelic diversity, variability and stability, and compared it to the discriminatory power of PFGE. They found that MLVA based on four or five highly variable VNTRs is comparable to PFGE for outbreak investigations and the use of a larger set of VNTR loci can aid in the understanding of *S.* Typhimurium phylogeny.

MLST relies on sequencing gene fragments (450-600 bp long) from five to seven loci across the chromosome. The DNA sequence at each locus is determined and the

unique combination of polymorphisms within a particular gene is given an allelic type. The unique combination of allelic types is used to assign sequence types to each isolate. Online databases, such as the Institut Pasteur MLST Databases (http://www.pasteur.fr/recherche/genopole/PF8/mlst), EcMLST database (www.shigatox.net/mlst) and MLST.net (http://www.mlst.net), provide a means to share and compare MLST types among labs across the globe (Enright and Spratt, 1999; Urwin and Maiden, 2003; Qi et al., 2004; Maiden, 2006, Nightingale, 2010). Alcaine and colleagues (2006) employed a three gene MLST scheme to characterize human and bovine clinical *Salmonella* isolates. They found some sequence types associated with one particular source, however numerous common sequence types were found among human and bovine sources over a widespread geographical location. They were also able to differentiate 335 *Salmonella* isolates representing 52 serotypes into 72 unique sequence types.

Single nucleotide polymorphisms (SNPs) are sites in the genome in which more than one nucleotide variation is found (Foley et al., 2009). Probing these variations can provide information regarding strain diversity and relatedness. SNPs can be located across the genome and the comparison of sequence data alignments can identify targets for the development of assays. SNP typing assays have been developed to directly target discriminatory nucleotides that identify unique allelic types. Multiple SNP typing methodologies have been used to characterize foodborne isolates. An assay that targets virulence attenuating mutations leading to a premature stop codon in the key *L. monocytogenes* virulence factor InlA was developed using single base pair extension chemistry (Van Stelten and Nightingale, 2008). SNP typing of *Salmonella* has primarily

focused on polymorphisms in genes encoding quinolone resistance (Esaki et al., 2004) and flagellar antigens (Mortimer et al., 2004). 906 different SNPs were identified in *E. coli* O157:H7 chromosomes that may be utilized in future genotyping assays (Zhang et al., 2006). While SNP analysis has primarily been employed as a research tool, these assays have the potential to be used in routine surveillance and outbreak investigations (Gerner-Smidt et al., 2006; Nightingale, 2010).

Next generation sequencing technologies have significantly reduced the amount of labor involved by sequencing clonally amplified DNA molecules in parallel (Voelkerding et al., 2009). Several sequencing projects for *L. monocytogenes, S. enterica* subsp. *enterica,* and *E. coli* have been completed with several more projects underway (Milillo et al., 2011). Whole genome sequencing may provide insight into highly clonal PFGE types that may be masked through the limitations of existing subtyping methodologies. The improvement of genomic technologies and cost reduction will facilitate the sequencing of bacterial genomes for epidemiological studies (Nightingale, 2010). Recently, high-throughput genome sequencing was used to investigate two *L. monocytogenes* isolates with similar, but distinct PFGE patterns that were implicated in a foodborne outbreak in Canada. Sequence analysis uncovered the extent of genome similarity and revealed how next generation sequence technology can be applied to foodborne outbreak investigations (Gilmore et al., 2010).

The development of online databases to share information on a global scale will enhance our ability to incorporate data regarding foodborne pathogens. The national database for foodborne bacterial subtypes, on the other hand, is highly standardized and accessible only to select institutions. PulseNet USA, developed in 1996, is the molecular

surveillance network for foodborne pathogens in the United States. It includes PFGE data for organisms such as *E. coli* O157, non-O157 STEC, *Salmonella enterica, L. monocytogenes, Shigella* spp., and *Campylobacter* spp. and primarily utilizes PFGE as the subtyping method. Laboratories participating in PulseNet must be certified before they can submit or have access to subtyping data and the database is thus not publically searchable (Gerner-Smidt et al., 2006). Through the collection of subtype data of organisms isolated from a variety of environments, the molecular ecology and epidemiology of foodborne pathogens may be better understood. PathogenTracker, a publically available online database, has been developed as a tool for the exchange of information pertaining to bacterial subtypes and strains for the understanding of the biodiversity of bacterial strains (www.pathogentracker.net). Development of this database was initiated in 2001 and currently contains several thousand bacterial isolates and molecular subtyping data.

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CHAPTER II

Molecular Ecology of *Listeria* spp., *Salmonella, Escherichia coli* O157:H7, and non-O157 Shiga Toxin Producing *E. coli* in Northern Colorado Wilderness Areas

Abstract

Listeria monocytogenes, Salmonella, and Shiga toxin-producing *Escherichia coli* (STEC) represent clinically important human foodborne pathogens. While molecular subtyping methods are used in foodborne disease surveillance, there is a knowledge gap regarding the presence, transmission and molecular ecology of these pathogens in non-food associated environments. The objective of this study was to collect foodborne pathogen isolates from pristine environments to aid in interpretation of a shared subtype between clinical and food isolates during an outbreak. Five wilderness locations in Colorado were selected and sampled during the spring, summer, and fall in 2009 and 2010. Soil, water, sediment, surface soil and fecal samples were microbiologically analyzed to detect *Listeria* (i.e., *L. monocytogenes* and other *Listeria* spp.), *Salmonella*, *E. coli* O157:H7, and non-O157 STEC. Samples were evaluated to detect each target organism using modified versions of the U.S. Food and Drug Administration Bacteriological Analytical Manual and presumptive colonies for each target organism were confirmed by PCR. Overall, three samples tested positive for *L. monocytogenes*. Nineteen samples contained *Listeria* spp. other than *L. monocytogenes*, 14 of which were determined to be *Listeria welshimeri* by *sigB* allelic typing. The remaining five other *Listeria* spp. isolates were

identified as being closely related (99% identity) to *Listeria rocourtiae* sp. nov. by 16s rDNA sequencing. *Salmonella* was isolated from two samples, five samples contained non-O157 STEC and *E. coli* O157:H7 was not detected in this study. Pulse field gel electrophoresis results indicated three unique DNA fingerprints among the *L. monocytogenes* isolates. All five non-O157 STEC- and both *Salmonella*-positive samples had unique DNA fingerprints. These results demonstrate a rare presence of foodborne pathogens in pristine environments. Molecular comparisons of these isolates will provide insight into epidemiological associations between isolates in nature and clinical isolates.

Introduction

Listeria monocytogenes, Salmonella, Escherichia coli O157:H7 and non-O157 Shiga toxin-producing *E. coli* (STEC) are four key foodborne pathogens of public health significance in the US. Recent literature estimated 9.4 million episodes of domestically acquired foodborne illness are caused by 31 major pathogens each year (Scallan et al., 2011). More specifically, *L. monocytogenes* causes 1,591 cases of domestically acquired foodborne disease, including 255 deaths, annually in the U.S. Non-typhoidal *Salmonella* was estimated to cause 1.03 million foodborne disease cases, including 378 deaths, while *E. coli* O157:H7 was estimated to cause 63,153 foodborne illness cases, including 20 deaths. Non-O157 STEC was estimated to cause 112,752 foodborne illnesses annually, although the number of non-O157 STEC related deaths has not been determined. Collectively, these four pathogens are estimated to be responsible for over 75% of deaths attributed to major bacterial pathogens each year in the US. The number of deaths

attributed to non-O157 STEC was reported as zero due to extremely skewed data (Scallan et al., 2011).

The Foodborne Diseases Active Surveillance Network (FoodNet) identifies the actual number of laboratory-confirmed infections of foodborne pathogens through population-based surveillance in 10 sites. In 2009, the incidence per 100,000 of foodborne disease attributed to *L. monocytogenes, Salmonella,* STEC O157, and non-O157 STEC was 0.34, 15.19, 0.99, and 0.57 respectively (CDC, 2010). When compared to baseline data from 1996-1998, the incidence of foodborne disease caused by *L. monocytogenes*, *Salmonella* and *E. coli* O157:H7 decreased by 26%, 10% and 41%, respectively (baseline data were not available for non-O157 STEC) (CDC, 2010). The goal to reduce foodborne illness attributed to key pathogens by 50% outlined in the Healthy People 2010 (U.S. Department of Health and Human Services, 2010) was met in 2009 for *E. coli* O157:H7, though still fell short for *L. monocytogenes* and *Salmonella*; supporting the critical need for additional efforts to control these pathogens along the food continuum and to probe the role of the presence of these pathogens non-food environments on the burden of human foodborne disease.

Due to the importance of these pathogens in the food system, substantial effort has been made to understand the transmission, distribution, and epidemiology of these organisms in humans, animals, foods, and food processing environments. While there is some data on the presence of *Listeria, Salmonella,* and STEC in various environments, limited information is available regarding the distribution of these organisms in non-food environments over time. Previous studies found that the prevalence of *L. monocytogenes* in natural areas (i.e., those that have had limited impact by man such as national parks

and wildlife areas) ranged from 6% to 15.7% in wildlife fecal samples (Weis and Seeliger, 1975; Lyautey et al., 2007); 1.3% to 15.2% in soil samples (Weis and Seeliger, 1975; Sauders et al., 2006); and 1.3% in water samples (Sauders et al., 2006). *Salmonella* has been isolated in 11% of wildlife fecal samples tested (Jijon et al., 2007); however, the prevalence in soil and water in natural environments is unknown. The prevalence of *E. coli* O157:H7 is highly variable in fecal samples from wildlife, ranging from 0.3% to 23.4% (Dunn et al., 2004; Jay et al., 2007) and the prevalence of this human foodborne pathogen in soil and water from natural environments remains unknown. There is an even more significant lack of knowledge regarding the prevalence of non-O157 STEC in most environments, including natural environments.

L. monocytogenes, Salmonella, and STEC (i.e., serotype O157:H7 and non-O157 serotypes) can asymptomatically colonize the gastrointestinal tract of a variety of species of animals and birds (Callaway et al., 2008; Fsihi et al., 2001; Gyles, 2007). In recent years, outbreaks of foodborne illness have been associated with wildlife, illuminating the need to evaluate the molecular ecology of foodborne pathogens in natural environments. In 1998 for example, 157 persons became ill after being exposed to *E. coli* O157:H7 in unchlorinated water in Wyoming that was contaminated with elk and deer feces (Olsen et al., 2002). In 2006, an outbreak of foodborne illness was caused by *E. coli* O157:H7, where the consumption of fresh, bagged baby spinach was linked to 205 reported cases and three deaths (Jay et al., 2007). A follow-up epidemiological investigation implicated feral pigs as one potential source of *E. coli* O157:H7 that contaminated spinach fields. Most recently, identical *E. coli* O157:H7 molecular subtypes (PFGE patterns) were

isolated from elk feces collected in a local park and eight children who became ill in Evergreen, Colorado (Scanlon, 2008).

While some research has initially probed the presence of these pathogens in nature, there is a lack of studies that developed a sampling plan that was adequate to determine the prevalence of foodborne pathogens present in nature with a defined region, and minimal data concerning the molecular ecology of foodborne pathogens in wildlife and natural environments exist. Many previous studies were based on sampling plans of convenience that were restricted to collection of samples during a single visit to one specific site and many studies were conducted before molecular subtyping methods were available (e.g., Weis and Seeliger, 1975). Molecular subtyping techniques are routinely used to characterize foodborne pathogen isolates from human clinical cases but isolates from other sources (e.g., animal clinical cases, food, and food environment) are now becoming increasing characterized by laboratories certified to participate in the PulseNet system. PulseNet is a database where certified laboratories deposit molecular subtype patterns; periodic searches of the database are performed to identify clusters of epidemiologically related foodborne illness cases to facilitate early detection of outbreaks (Gerner-Smidt et al., 2006). PathogenTracker, on the other hand, represents a publically accessible online database that contains epidemiological and molecular subtyping data for foodborne pathogens from human and animal clinical cases, foods and a variety of food-associated environments (e.g., processing plants and retail) and non-food associated environments (e.g., urban and natural environments) (Fugett et al., 2006).

Some molecular subtypes appear to be broadly distributed in general, which can potentially confound the meaning of a shared molecular subtype between human clinical

isolates and food isolates during an outbreak investigation (Fugett et al., 2007). The current study was conducted to (i) determine the prevalence of four key foodborne pathogens, including *L. monocytogenes*, *Salmonella*, *E. coli* O157:H7 and non-O157 STEC, in natural environments located in Northern Colorado, (ii) characterize isolates from natural environments by molecular subtyping and compare subtypes to temporally and geographically associated human clinical isolates and (iii) populate the PathogenTracker database with epidemiological and subtyping data for foodborne pathogen isolates from natural environments in Northern Colorado. Populating databases (e.g., PulseNet and PathogenTracker) with molecular subtype data for isolates from non-food associated environments is critical to gain a more comprehensive understanding of the molecular ecology and transmission dynamics of these pathogens.

Materials and Methods

Sample collection. Five wilderness sites (Cache la Poudre, Comanche Peak, Rawah, Never Summer, and Mount Evans wilderness areas) were selected in the state of Colorado and each was sampled three times (spring, summer, and autumn) in 2009 and 2010, with the exception of early snow preventing access to the fifth wilderness site in the fall of 2009. In replacement, a sixth wilderness site was sampled in the fall of 2009. Five soil, five water, up to five fecal, and one surface soil (drag swab) samples were collected from three areas (approximately $100m^2$) within each site during each visit and sediment samples were collected during select visits to each site. A hand-held global positioning satellite (GPS) device (Magellan MobileMapper CX, Santa Clara, California) was used to record GPS coordinates for each sample location so that locations could be

recorded and mapped (TopoUSA 8.0; Delorme, Yarmouth, Maine) over the duration of the project. Fecal and soil samples were collected with clean latex gloves and sterile scoops, while surface soil samples were collected by dragging pre-soaked sterile gauze attached to a string along the ground in a figure-eight pattern for several minutes (Uesugi et al., 2007). Individual fecal, soil, sediment and drag swab samples were placed into sterile Whirl-pak bags (Nasco, Fort Atkinson, WI) and water samples were collected directly into Whirl-pak bags. Samples were stored in coolers with ice packs and were processed within 24 hours of collection.

Bacterial isolation. The five soil, five sediment and five water samples from each area were manually homogenized and used to prepare composite samples to represent each area. Composite 25g soil (5g from each sample), composite 25g sediment (5g from each sample) and individual 10g fecal material were prepared in duplicate sterile filter whril-pak bags to first non-selectively enrich for gram-positive or gram-negative target pathogens. A 50 ml aliquot of each of the five water samples from a given area was passed over the same filter (0.45 μm) (Nalgene, Rochester, NY), which was then aspetcially divided into three equal sections and transferred into three sterile filter Whirl-Pak bags. Similarly, drag swab samples were homogenized and a 10 ml aliquot of the phosphate buffered tryptic soy broth (pTSB; Becton, Dickinson and Company, Sparks, MD; Fisher) used to dampen drag swab samples was transferred into three bags. Phosphate buffered tryptic soy broth was added to one soil and sediment bag and buffered *Listeria* Enrichment Broth (BLEB, Difco, Sparks, MD) was added to the other soil and sediment bag to create a 1:10 dilution. Phosphate buffered tryptic soy broth (90 mls) was added to one fecal, two water filter and two drag swab bags and the same

amount of BLEB was added to each of the remaining bags to create a 1:10 dilution. Sample bags were homogenized manually for one minute. Samples containing BLEB were incubated for four hours at 30°C. Bags containing pTSB were incubated at room temperature for two hours to allow for the recovery of injured cells. Soil, sediment and fecal bags were then divided, transferring half of the contents to a new bag to facilitate selective enrichment for *Salmonella* and *E. coli*. Three bags for each sample were then further processed using selective incubation times and enrichments for *Listeria*, *Salmonella*, and STEC (i.e., separate protocols for *E. coli* O157:H7 and non-O157 STEC). Positive and negative control samples were included each time samples were processed, with one colony from each positive control each added to their own positive control bag. Negative controls were each enrichment media without any inoculums. Positive control strains included *Salmonella* Typhimurium and *E. coli* O157:H7, each with green fluorescent protein inserts, (Noah et al., 2005), non-O157 STEC (Michigan State STEC Reference Center), and *L. monocytogenes* (Bishop and Hinrichs, 1987).

Isolation of *Listeria.* Isolation of *L. monocytogenes* and other *Listeria* spp. from environmental samples was performed as detailed in previous studies (Nightingale et al., 2004; Sauders et al., 2006). Selective agents (acriflavin, sodium nalidixate, cycloheximide, Oxoid) were added to BLEB bags after the initial four hour incubation. Samples were then incubated for 48 hours at 30°. After 24 and 48 hours of enrichment, 100 μl of each sample was streaked onto modified Oxford plates (MOX; Difco) and *L. monocytogenes* plating medium (LMPM; Biosynth Biochemica & Synthetica). MOX plates were incubated at 30°C for 48 hours and LMPM plates were incubated at 35°C for 24 hours. Up to four colonies displaying typical *Listeria* morphology on MOX were

substreaked onto LMPM. Up to two colonies with typical *Listeria* spp. morphology on LMPM (white colonies) and up to four colonies displaying characteristic *L. monocytogenes* morphology on LMPM (turquoise-blue color) were substreaked onto BHI (BHI; Difco, Sparks, MD).

Isolation of *Salmonella.* *Salmonella* isolation was performed using modified versions of the procedures outlined in the US Food and Drug Administration Bacteriological Analytical Manual (FDA, 2001). After the two hour room temperature incubation, pTSB bags were incubated at 35°C for six hours. Methods were evaluated after the first year (data not shown) and a longer incubation time of 24 hours for pre-enrichment was implemented for the second year to improve *Salmonella* detection. A 0.1 ml aliquot of pre-enriched samples was added to 10 ml Rappaport Vassiliadis (RV; Oxoid; Fisher; Acros Organics, Belgium) broth and a 1.0 ml aliquot of pre-enrichments was transferred to 10 ml of tetrathionate (TT; Oxoid) broth and incubated at 42^{o}C for 24 hours. A 100 µl aliquot of each enrichment was plated onto xylose desoxycholate (XLD; Neogen) agar. Additional plating onto CHROMagar-*Salmonella* (CHROMagar, Paris, France) was added in the second year. XLD and ChromAgar plates were incubated at 35^{o}C for 24 hours and up to four colonies with typical *Salmonella* morphology were sub-streaked onto BHI agar plates.

Isolation of *E. coli* O157:H7. *E. coli* O157:H7 isolation was performed as described previously (Barkocy-Gallagher, 2002, 2005) and as implemented by our group (Carlson et al., 2006). Following the two hour room temperature incubation, pTSB samples were incubated at 42°C for six hours. Methods were evaluated after the first year (data not shown) and a longer incubation time of 24 hours was implemented. Aliquot of each pre-

enriched samples was then subjected to immunomagnetic separation (IMS) to concentrate *E. coli* O157 cells. A 50 µl aliquot of washed IMS beads (Invitrogen) were plated onto modified soribitol MacConkey agar (mSMAC) (Becton, Dickinson and Company, Sparks, MD) and in the second year mSMAC and CHROMagar-O157 (CHROMagar, Paris, France), both supplemented with 20 mg/L of novobiocin (Sigma-Aldrich, St. Louis, MO) and 2.5 mg/L of potassium tellurite (Sigma). Plates were incubated for 36 hours at 37°C and up to four individual colonies with typical *E. coli* O157:H7 phenotypes were sub-streaked onto BHI.

Isolation of non-O157 STEC. Following the two hour room temperature incubation, pTSB samples were incubated at 35°C for six hours the first year and 24 hours the second year. A 1 ml aliquot of each enrichment was added to 9 ml *E. coli* broth (EC broth; Oxoid) and incubated at 37°C for 24 hours. A 100 µl aliquot of enrichment was plated onto washed sheep blood agar (Becton Dickson; Remel, Lenexa, KS) (WMBA) containing $CaCl_2$ (10 mM) (Acros Organics, Belgium) and 0.5 µg/ml mitomycin C (Fisher). WBMA plates were incubated a 35˚C and evaluated for enterohemolysis at six and 24 hours (Sugiyama et al., 2001). Up to 20 colonies that demonstrated enterohemolysis were sub-streaked onto sorbitol MacConkey agar and up to ten colonies that rapidly ferment sorbitol were then sub-streaked onto BHI.

Soil analysis. The pH of each pooled soil sample was measured by combining 0.01 M $CaCl_2$ to samples to create a 1:2 dilution and subsequently measured using a calibrated pH meter (Beckman) (USDA, 1995). Pooled soil samples from the summer collection of 2010 were analyzed for organic matter content and particle size analysis (Soil Testing Laboratory, Colorado State University).

Phenotypic confirmation. Biochemical tests were performed to further describe less well characterized organisms including non-O157 STEC and atypical *Listeria* isolates. API-20E (BioMerieux) test strips were used to confirm non-O157 STEC isolates as *E. coli*. Atypical *Listeria* strains were tested with API-*Listeria* (BioMerieux) test strips as well as catalase, oxidase, Gram stain and motility tests.

PCR confirmation. Up to four presumptive colonies representing each targeted organism were confirmed using PCR assays that detect a sequence unique to each organism. Gene fragments with s*igB* and *hly* were the genes targeted to confirm *Listeria* spp. and *L. monocytogenes*, respectively, as detailed previously (Nightingale et al., 2005; Norton et al., 2001). Presumptive *Salmonella* colonies were confirmed by amplifying *invA* (Nucera et al., 2006) and all STEC isolates were confirmed using a multiplex PCR assay with six targets (*hly, eaeA, stx-II, stx-I, flic*$_{h7}$*, rfbE*) (Carlson et al., 2006).

Serotyping. Confirmed *Salmonella* and STEC isolates were shipped to the National Veterinary Service Laboratory (Iames, Iowa) or the *E. coli* Reference Center (Pennsylvania State University; State College, PA), respectively, for serotyping by conventional slide agglutination. Confirmed *L. monocytogenes* isolates were grouped into one of four molecular serogroups each containing one of the four most common serotypes associated with human listeirosis (i.e., 1/2a, 1/2b, 1/2c and lineage I 4b) as detailed previously (Doumith et al., 2004).

DNA sequencing. Confirmed *Listeria* spp. isolates were speciated by DNA sequencing and allelic typing of *sigB* using previously described primer sequences and reaction conditions (Nightingale et al., 2005). Amplification and DNA sequencing of additional genes, *gap* (Nightingale et al., 2005), *prs* (Graves et al., 2009), and 16S rRNA

(Wiedmann et al., 1997), were performed on atypical *Listeria* isolates. PCR amplification and DNA sequencing of *mdh* was used to confirm presumptive non-O157 STEC isolates as *E. coli* (Boyd et al., 1994). Purification of PCR products and DNA sequencing was performed as described previously (Nightingale et al., 2005). Sequences were assembled and proofread using Seqman (DNAStar, Madison, WI), aligned in Megalign (DNAStar) using the Clustal W algorithm and multiple sequence alignments were trimmed to consistent lengths. DnaSP software (www.ub.edu/dnasp/) was used to assign allelic types in which sequences with one or more nucleotide difference were considered different allelic types. *mdh* sequences were compared to allelic types in the EcMLST database (STEC reference center, Michigan State University). BLAST searches were performed for each *sigB* sequence to determine the species of each *Listeria* spp. isolate. Sequencing *sigB* and allelic typing was previously shown to be concordant with API *Listeria* test strips for *Listeria* identification purposes (Sauders, 2005). Phylogenetic analysis for *sigB* allelic types was performed using a single isolate representing each unique allelic type. Phylogenetic trees were created in PAUP* (Swofford, 2003) using the neighbor joining method (Felsenstein, 2004).

Pulse field gel electrophoresis. All confirmed isolates from each sample positive for targeted pathogens were subjected to PFGE typing by the standardized CDC PulseNet protocol for *L. monocytogenes, Salmonella* and non-O157 STEC (Ribot et al., 2006; Halpin et al., 2010). Briefly, bacterial isolates were grown on BHI agar plates at 37°C for 18 hours, imbedded in 1% SeaKem Gold® agarose (Cambrex Bio Science), lysed, and washed. *L. monocytogenes* isolates were digested separately with *AcsI* and *ApaI*. *Salmonella* and *E. coli* cells were digested with *XbaI.* Restricted agarose plugs were then

placed into 1% agarose gels and electrophoresed on a CHEF Mapper XA (BioRad Laboratories) for 19 hours. Switch times were 4.0s to 40.0s for *L. monocytogenes*, 6.76s to 35.38s for non-O157 STEC, and 2.16s to 35.38s for *Salmonella*. *XbaI* digested *Salmonella* ser. Braenderup (H9812) DNA was used as a reference size standard in each gel (Hunter et al., 2005). Agarose gels were stained in ethidium bromide for 40 minutes, destained in ultrapure water for two hours, and resultant images were captured with a Fotodyne. PFGE patterns were analyzed and compared using BioNumerics (Applied Maths, version 5.10, Saint-Matins-Latem, Belgium) software. Similarity clustering analyses was performed with BioNumerics software using the unweighted pair group-matching algorithm and the Dice correlation coefficient as detailed previously (Hunter et al., 2005). PFGE bundle files were sent to the Colorado State Department of Public Health and Environment for comparison to available human clinical isolate patterns.

Results

Isolation and detection of bacterial isolates. Altogether 1,292 samples collected were collected over this two-year longitudinal study, where 90, 90, and 27 composite soil, water and sediment samples along with 275 individual fecal and 90 individual drag-swab samples were microbiologically analyzed to detect four key foodborne pathogens, including *L. monocytogenes*, *Salmonella*, *E. coli* O157 and non-O157 STEC. All samples were also microbiologically analyzed to detect *Listeria* spp. other than *L. monocytogenes*. Three samples tested positive for *L. monocytogenes*, including one fecal collected from Mt. Evans wilderness during the summer of 2009, a second fecal sample collected in the fall of 2010 from the same area in Mt. Evans, and one water sample

collected in the fall of 2009 from Cache la Poudre wilderness (Table 1; Figure 1). Nineteen samples tested positive for *Listeria* spp. other than *L. monocytogenes*, with almost 75% isolated from a single wilderness location (Rawah). *Salmonella* was detected in two samples, including one water sample collected in the summer of 2010 from Never Summer and one fecal collected in the spring of 2010 from Cache la Poudre . Five samples tested positive for non-O157 STEC, including in one fecal sample collected in the fall of 2010 from Mt. Evans, one sediment sample collected in the fall of 2010 from Cache la Poudre, one water sample from the spring of 2009 in Comanche Peak, one water sample collected in the summer of 2010 in Mt. Evans, and one water sample collected in the fall of 2010 in Cache la Poudre. *E. coli* O157:H7 was not detected in any samples collected from the five wilderness locations enrolled in the project.

Soil pH and composition. The average pH of soil samples was 5.19 ± 0.51. pH measurements were very similar among areas within a wilderness location and remained consistent throughout the study (Table 2). The average soil pH ranged from 4.53 in Never Summer to 5.78 in Cache la Poudre. The organic matter content for areas representing a wilderness location averaged at 9.1%, 11.0%, 16.9%, 14.0%, and 21.1% for Cache la Poudre, Comanche Peak, Rawah, Never Summer, and Mt. Evans respectively. Particle size analysis determined the texture of all samples to be sand with sand contents of 95.3%, 98.0%, 92.0%, 97.3% and 96.0%, for Cache la Poudre, Comanche Peak, Rawah, Never Summer, and Mt. Evans respectively.

Phenotypic characterization of presumptive non-O157 STEC and atypical *Listeria*-like isolates. A series of basic biochemical tests were performed to further describe less well-characterized organisms, including presumptive non-O157 STEC, which can display

a wide variety of phenotypic characteristics, and isolates with ambiguous *Listeria*-like characteristics. Five isolates with ambiguous *Listeria*-like characteristics, such as weak *sigB* amplicons that were larger than the positive control or had no *sigB* amplicon at all but looked like *Listeria* on MOX and *Listeria* spp. other than *L. monocytogenes* on LMPM agar plates were determined to be short Gram-positive rods exhibiting tumbling motility at 30˚C, as determined by a wet mount. Furthermore, these isolates were catalase-positive and oxidase-negative, as expected for a member of the genus *Listeria.* The isolates were non-hemolytic when they were streaked onto Tryptic Soy Agar supplemented with 5% washed sheep's blood. All five isolates had the same API-*Listeria* test strip results (octal code 2210); however, this octal code does not match any previously described *Listeria* spp. The biochemical profile of all presumptive non-O157 STEC isolates from each putative-positive sample were characterized by an API-20E test to confirm them as *E. coli* before further phenotypic and molecular subtyping. Results from API-20E strips confirmed all presumptive non-O157 STEC isolates to belong to *E. coli* above 80% identity.

Serotyping. Conventional O antigen serotyping of STEC isolates by slide agglutination revealed that isolates could be grouped into three O antigen types, including O8, O20, and O174. One isolate was untypable and one did not react with standard antisera. *Salmonella* isolated from a fecal sample clustered with other *Salmonella* Typhimurium, while the isolates from the water sample that tested positive for *Salmonella* clustered with *Salmonella* 4,5[12]:i:,-, based on cluster analyses with PFGE patterns in a database containing >5,000 *Salmonella* isolates (www.pathogentracker.com). The molecular serotyping multiplex PCR assay previously described by Doumith et al. (2004) showed

that two *L. monocytogenes* isolates (from a water sample in Cache la Poudre and a fecal sample in Mt. Evans wilderness) could be grouped into the molecular serogroup containing seortypes 1/2b, 3b and 7, and two isolates (from fecal samples in Mt. Evans wilderness in 2009 and 2010) were grouped into the molecular serogroup containing serotypes 1/2a and 3a (Table 3).

DNA sequencing analyses. Amplification of *sigB* was used to confirm isolates presumptively belonging to *Listeria* spp. other than *L. monocytogenes* and *sigB* sequencing was used to speciate isolates as detailed previously (Sauders et al., 2005). Since *sigB* is a key regulator of the general stress response in Gram-positive bacteria including *Listeria*, *Staphylococcus* and *Bacillus*; *sigB* is expected to be present in all *Listeria* spp. (Hain et al., 2006). Fourteen presumptive *Listeria* spp. isolates from 14 samples were determined to be *L. welshimeri* via *sigB* sequencing and allelic typing. The PCR primers, reaction conditions and thermocycling parameters; however, failed to amplify *sigB* in one of the remaining five isolates and BLAST results were inconsistent for the other four isolates and we thus deemed these five isolates as atypical *Listeria*. To better characterize these five atypical *Listeria* isolates, the full 16S rRNA gene was amplified by a previously described 16S rDNA PCR reaction (Wiedmann et al., 1997). All five isolates were most closely related to *Listeria rocourtiae* sp. nov. (99% identity) according to BLAST searches. A *gap* fragment was successfully amplified in all five atypical *Listeria* isolates; however, multiple bands were present, thus preventing a clear interpretation and downstream sequence characterization. A *prs* fragment was successfully amplified in two isolates in which PCR products were sequenced; a BLAST search did not match any high scoring (above 90% identity) Genbank sequences.

Pulsed field gel electrophoresis (PFGE) typing. Results from PFGE typing revealed three unique DNA fingerprints among samples testing positive for *L. monocytogenes*. Two unique PFGE types were isolated from the same fecal sample in Mt. Evans wilderness in the fall of 2010; while one of those PFGE types was also isolated from a fecal sample within the Mt. Evans wilderness in the summer of 2009. *Salmonella* and STEC positive samples contained isolates that had unique DNA fingerprints and two unique STEC patterns were isolated from a single sediment sample (Figure 2). One STEC isolate, which did not react with standard antisera, was also untypable by PFGE. *Salmonella* isolated in this study matched human clinical isolates from patients in Colorado in 2002, 2004, and 2005 and had similar patterns to other human isolates. Additionally, two *L. monocytogenes* isolates digested with *Asc*I matched clinical isolates in Colorado from 2008.

Discussion

Testing nearly 1,300 environmental samples from natural environments in Northern Colorado through a two-year longitudinal study allowed us to assess the prevalence and molecular ecology of *Listeria*, *Salmonella,* and STEC (i.e., *E. coli* O157 and non-O57 STEC) in non-food associated environments. Water, sediment, soil, surface soil (drag swab), and fecal samples were collected in five pristine locations during the spring, summer and fall from 2009 through 2010 and microbiologically analyzed for four target pathogens, including *L. monoctogenes*, *Salmonella*, *E. coli* O157:H7 and non-O157 STEC. Our data indicate that (i) there is a rare presence of these key human foodborne pathogens in natural environments in Northern Colorado, (ii) there is genetic diversity

between organisms isolated within each wilderness location and between wilderness locations, and (iii) the unique climate and topography found in Northern Colorado may be associated with the low prevalence of these organisms.

There is a rare presence of *Listeria, Salmonella,* and STEC in pristine environments in Colorado. Results from this study revealed a low prevalence of *Listeria, Salmonella* and STEC in natural environments located in Northern Colorado. We observed a notably reduced presence of *Listeria* as compared to other studies designed to probe the presence of organism in nature. More specifically, Lyautey et al. (2007) identified *L. monocytogenes* in 3.73% of wildlife fecal samples in Ontario, Canada and Weis and Seeliger (1973) recorded 15.7% of fecal samples positive in wildlife feeding ground in Germany; however, we only found *L. monocytogenes* 0.73% of fecal samples from wildlife feeding areas. Previously, *L. monocytogenes* was isolated from 1.3% of soil samples collected in natural areas (Sauders et al., 2006) and 15.2% (Weis and Seeliger, 1975) of forest soil samples; whereas we did not find *L. monocytogenes* in any soil samples in this study. We isolated *L. monocytogenes* in 1.11% of pooled water samples, which is similar to a prevalence of 1.3% in water samples from natural areas reported by Sauders et al. (2006). Prevalence data for *Salmonella* in natural environments has only been described in wildlife fecal samples. More specifically, Jijon and colleagues (2007) detected *Salmonella* in 11% of fecal samples from two wildlife rehabilitation centers in Ohio. In the current study, *Salmonella* was only detected in 0.36% of wildlife fecal and 1.11% of water samples. Previous reports of STEC in wildlife fecal samples are highly variable with nearly all focusing on the detection of *E. coli* O157:H7 (Dunn et al., 2004; Jay et al., 2007). While prevalence of STEC belonging to serotype O157 ranged from

0.3% in white-tailed deer feces (Dunn et al., 2004) to 15.7% in wildlife feces (Weis and Seeliger, 1975), we did not detect this organism in any fecal or other environmental samples in the current study. However, we did isolate non-O157 STEC from fecal, water and sediment samples in this study, with a prevalence of 0.36%, 3.33%, and 3.70% in each sample category, respectively. While there is a low prevalence of these key human foodborne pathogens in natural environments in Colorado, these pathogens are present and molecular characterization of these isolates is needed in order to make informed statements about the ecology and transmission of these organisms in a broader scope.

There is diversity among *Listeria, Salmonella,* and STEC found within and between wilderness locations. We assessed the diversity of organisms isolated in natural areas through the use of phenotypic and molecular subtyping. There were multiple subtypes detected both within a single wilderness location and between locations. More specifically, two unique STEC strains and two unique *L. monocytogenes* strains were isolated from Mt. Evans wilderness. A total of five samples tested positive for STEC, all with unique PFGE fingerprints and *mdh* allelic types. A sediment sample from Cache la Poudre wilderness contained two distinct STEC PFGE fingerprints. The two *Salmonella* isolates had distinct PFGE fingerprints; however, *Salmonella* isolated from a fecal sample matched human clinical isolates in Colorado from previous years. In concordance with our results, a previous report by Sauders et al. (2005) described different *L monocytogenes* subtypes isolated from different natural environments.

L. monocytogenes was isolated from two different wilderness locations. The same strain was isolated from fecal samples in one location collected over one year apart. This demonstrates the ability for a single strain to persist in an environment over an

extended period of time. Multiple *sigB* allelic types were isolated from wilderness locations where *Listeria* was isolated at least two times. This assessment indicates *sigB* allelic diversity among *Listeria* species in natural environments and an overrepresentation of *L. welshimeri* isolates.

Five atypical *Listeria* isolates were further characterized using additional biochemical and genetic tools. Species identification was dependant on the method used, as different techniques yielded different results. While the strains group closest to *L. rocourtiae* sp. nov. via 16S and *sigB* sequencing, our isolates have unique phenotypic and genetic properties that make them distinct from these and previously described species. API-*Listeria* results for *L. rocourtiae* sp. nov. displayed a positive DIM and Mannosidase reaction, while our isolates were negative for those tests; *L. rocourtiae* sp. nov. acidified L-Rhamnos and D-Ribose, while our strains did not (Leclercq et al., 2010).

PFGE fingerprints from our environmental isolates were compared with human clinical strains from the Colorado State Department of Public Health. Identical patterns between human clinical isolates and strains isolated from pristine environments suggest the distribution of these pathogens may be widespread. Sauders and colleagues (2006) found that while *L. monocytogenes* subtypes isolated from urban and natural environments overlap, they differ significantly in their associations with different environments. Lyautey et al. (2007) similarly found that fecal *L monocytogenes* isolated from different sources belong to overlapping but distinct populations. Molecular characterization of foodborne pathogens found in natural environments is crucial in order to appropriately evaluate the meaning of subtype matches between human and food

isolates. This data also aids in the identification of subtypes associated with natural environments to help identify environmental sources of contamination.

The unique Colorado environment may impact the ability for organisms to survive. Numerous aspects of the Colorado environment, including low moisture, high solar radiation and acidic soils exert extra stress on bacterial communities (McCambridge and McMeekin, 1981; Xu et al., 2004; Rousk et al., 2009). The five wilderness locations chosen in this study are situated at different altitudes. Samples were collected in Cache la Poudre wilderness at approximately 2,300 meters, while samples collected in Mount Evans wilderness were located at approximately 3,175 meters above sea level. The remaining three sampling locations had altitudes of 2,600 meters (Comanche Peak), 2,650 meters (Rawah), and 2,750 meters (Never Summer) meters. The thin atmosphere at high altitudes allows greater penetration of solar radiation (Doesken et al., 2003), which can stress and reduce the growth and survivability of microorganisms (McCambridge and McMeekin, 1981). Colorado's unique climate also includes large seasonal swings in temperature and extensive day to night changes (Doesken et al., 2003). The annual precipitation in Colorado is 17 inches and is highly variable within the state (Doesken et al., 2003).

We measured the soil pH, composition and organic matter content from each wilderness location. We found the average pH of soils sampled to be 5.19, which may be explained by sampling coniferous forest soil overlaid by decomposed acidic needles (Alfredsson et al., 1998). Soils with a high sand content, such as those sampled in this study, have a lower water-holding capacity and fewer microaggreagtes. These properties are less conducive for microbial growth than soils with a closer to neutral pH and higher

proportions of clay and silt (Monrozier et al., 1991). Multiple environmental stresses in Northern Colorado may reduce the ability for organisms to survive and further characterization of organisms found in this study may reveal genetic adaptations that allow them to survive in this type of climate.

Conclusions

We assessed the prevalence and molecular ecology of four major foodborne pathogens in natural environments in Northern Colorado through a two-year longitudinal study. Our results demonstrate a rare prevalence of *Listeria, Salmonella*, and STEC in these locations, where isolates from different locations appeared to be genetically distinct. While subtyping techniques revealed genetic relatedness between organisms, additional genetic and phenotypic assessments can evaluate the relatedness of these environmental strains. Understanding the distribution of molecular subtypes in a variety of environments may provide evidence and guidance during outbreak investigations through source tracking and by evaluating how common a given subtype is in the environment. Additional comparisons among strains isolated from non-food and non-human associated environments with clinical and foodborne isolates will provide additional insight into molecular epidemiology of these organisms.

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Figures and Tables

Table 2.1 Number of positive samples and percent positive for *L. monocytogenes,* other *Listeria* species, *Salmonella, E. coli* O157:H7, and non-O157 STEC.

	Fecal	**Soil**	**Water**	**Drag**	**Sediment**	**total**
L. monocytogenes	2(0.73%)	0(0%)	1(1.11%)	0(0%)	0(0%)	3(0.23%)
Listeria spp.	7(2.55%)	4(4.44%)	5(5.56%)	2(2.22%)	1(3.70%)	19(1.47%)
Salmonella	1(0.36%)	0(0%)	1(1.1%)	0(0%)	0(0%)	2(0.16%)
E. coli O157	0(0%)	0(0%)	0(0%)	0(0%)	0 (0%)	0(0%)
non-O157 STEC	1(0.36%)	0(0%)	3(3.33%)	0(0%)	1(3.70%)	5(0.39%)
total	11(4.00%)	4(4.44%)	11(12.22%)	2(2.22%)	2(7.41%)	29(2.25%)

Table 2.2. Soil pH values

Soil pH						
	2009			2010		
Area	Spring	Summer	Fall	Spring	Summer	Fall
W1A1	5.21	7.14	5.81	5.84	5.77	5.95
W1A2	6.16	5.62	5.76	5.55	5.58	5.44
W1A3	5.65	5.82	5.93	5.59	5.65	5.59
W2A1	4.92	4.99	5.55	4.93	5.24	5.04
W2A2	5.35	5.29	5.6	5.29	5.47	4.88
W2A3	5.21	5.60	5.85	6.57	6.40	5.06
W3A1	5.00	5.24	5.21	5.52	5.53	5.28
W3A2	5.55	5.17	4.71	5.06	5.67	5.14
W3A3	5.84	5.59	4.82	5.71	5.27	5.08
W4A1	4.52	4.04	4.36	4.36	4.29	4.22
W4A2	4.37	5.51	4.31	4.16	4.25	4.82
W4A3	4.89	4.58	4.83	4.57	4.73	4.77
W5A1	4.14	5.39	5.25	4.75	4.79	5.03
W5A2	4.50	4.78	5.15	5.29	5.30	5.3
W5A3	4.74	4.78	5.02	5.57	4.77	4.51

W1-W5 correspond to Cache la Poudre, Comanche Peak, Rawah, Never Summer, and Mount Evans wilderness locations respectively. Analysis was done on composite soil samples from each area in the spring, summer and fall in 2009 and 2010.

Table 2.3 Soil analysis of organic matter content and particle size analysis.

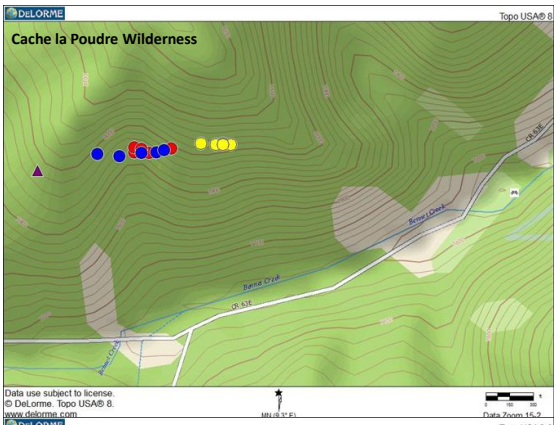
Sample ID #	% Organic Matter	----------%---------- Sand	Silt	Clay	Texture
W1A1	8.3	92	8	0	Sand
W1A2	10.6	94	6	0	Sand
W1A3	8.4	100	0	0	Sand
W2A1	15.5	100	0	0	Sand
W2A2	13.5	98	2	0	Sand
W2A3	3.3	96	4	0	Sand
W3A1	15.2	92	4	4	Sand
W3A2	12.9	92	4	4	Sand
W3A3	22.5	92	2	6	Sand
W4A1	10.6	92	4	4	Sand
W4A2	14.9	100	0	0	Sand
W4A3	16.5	100	0	0	Sand
W5A1	29.5	96	2	2	Sand
W5A2	17.0	96	4	0	Sand
W5A3	16.8	96	4	0	Sand

W1-W5 correspond to Cache la Poudre, Comanche Peak, Rawah, Never Summer, and Mount Evans wilderness locations respectively. Analysis was done on composite soil samples from each area in the summer of 2010.

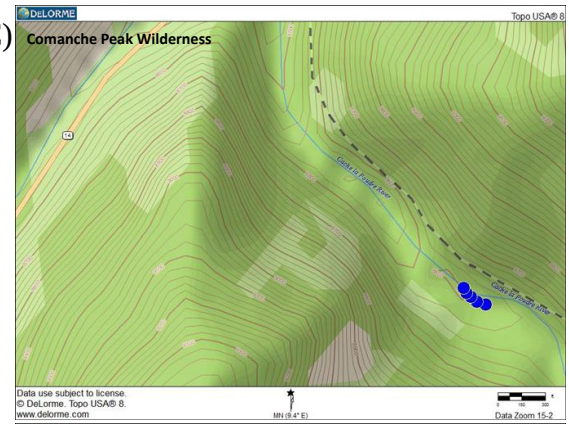
Figure 2.1. Geographic distribution of positive samples

A)

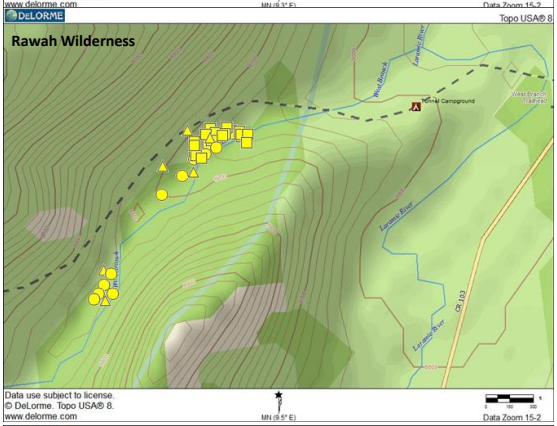
B)



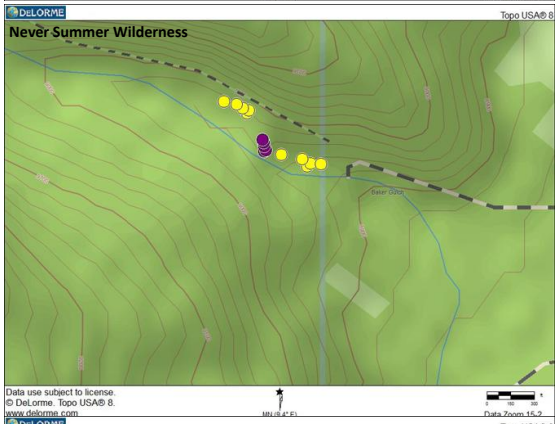
C)



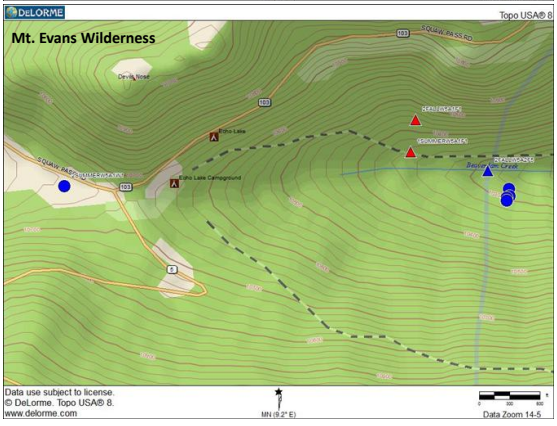
D)



E)



F)



G)

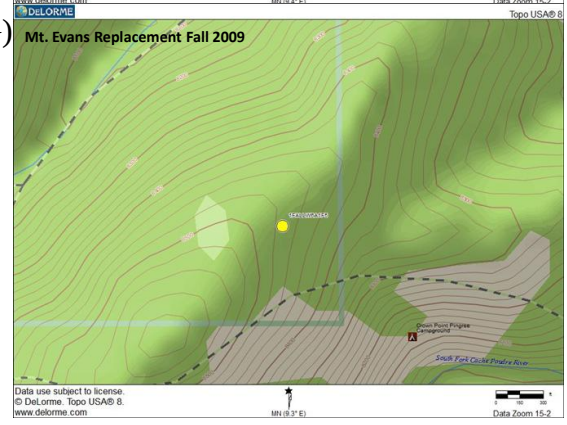


Figure 2.1. Map of wilderness locations across Colorado (A) and positive sample locations within Cache la Poudre, Comanche Peak, Rawah, Never Summer, Mount Evans, and the Mount Evans replacement (fall of 2009) (B-G). Geographic distribution of positive sample locations for *L. monocytogenes* (red), other *Listeria* spp. (yellow), *Salmonella* (purple), and non-O157 STEC (blue). Sample types include soil (square), water (circle), and fecal (triangle). Distance scale (m) as indicated.

Figure 2.2. Dendrograms *L. monocytogenes*, non-O157 STEC, and *Salmonella* isolates

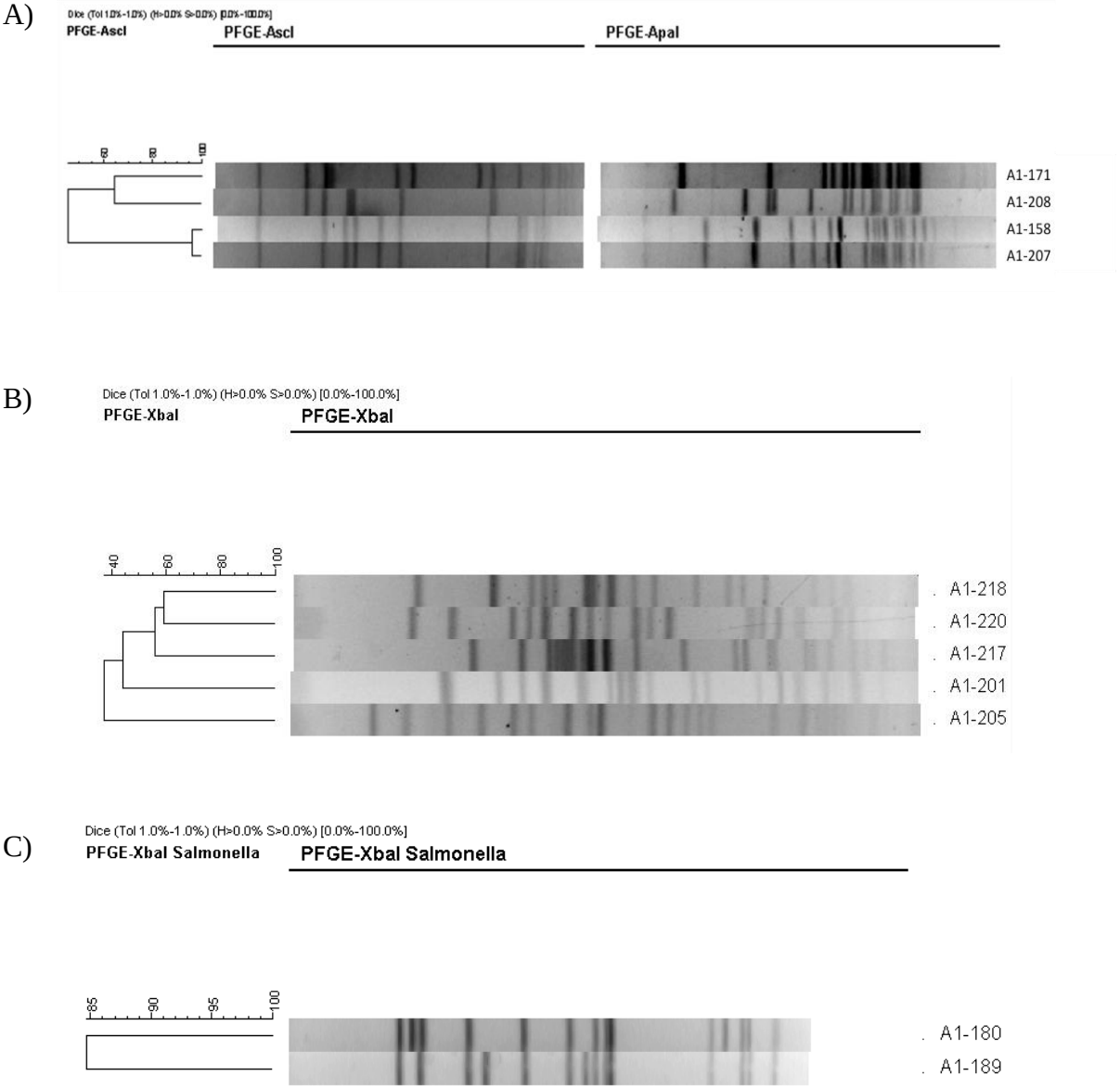


Figure 2.2. Dendrograms created using the unweighted pair group matching algorithm and the Dice correlation coefficient as implemented using BioNumerics software to visualize similarity of A) *L. monocytogenes,* B) non-O157 STEC, and C) *Salmonella* isolates.

Figure 2.3 Phylogentic tree of unique *Listeria sigB* allelic types

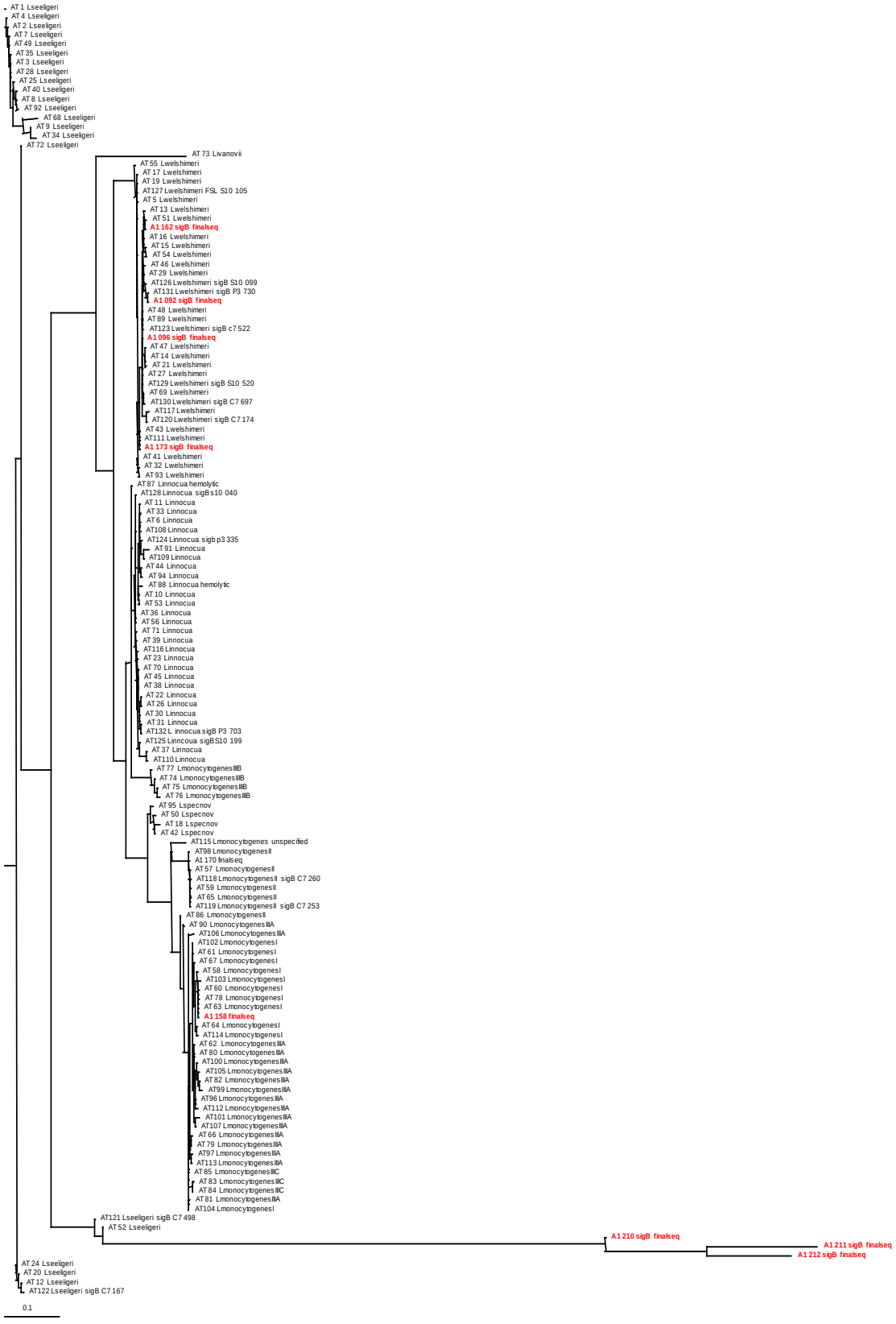


Figure 2.3 Neighbor joining tree of unique *Listeria sigB* allelic types. Strains collected in this study are colored red.

APPENDIX

Source description of *L. monocytogenes, Salmonella,* and non-O157 STEC isolates

ID	**Organism**	**Wilderness**	**Source**	**Season**	**Year**	**serotype**
A1-091	*E. coli*	Comanche Peak	water	spring	2009	-
A1-201	*E. coli*	Mt. Evans	water	summer	2010	untypable
A1-202	*E. coli*	Mt. Evans	fecal 5	fall	2010	O174
A1-217	*E. coli*	Cache la Poudre	sediment	fall	2010	O8
A1-218	*E. coli*	Cache la Poudre	sediment	fall	2010	O20
A1-220	*E. coli*	Cache la Poudre	water	fall	2010	O20
A1-158	*L. monocytogenes*	Mt. Evans	fecal 1	summer	2009	1/2b
A1-170	*L. monocytogenes*	Cache la Poudre	water	fall	2009	1/2a
A1-206	*L. monocytogenes*	Mt. Evans	fecal 1	fall	2010	1/2b
A1-208	*L. monocytogenes*	Mt. Evans	fecal 1	fall	2010	1/2a
A1-177	*Salmonella*	Cache la Poudre	fecal 3	spring	2010	Typhimurium
A1-189	*Salmonella*	Never Summer	water	spring	2010	4,5[12]:i:-

Source description of *Listeria* spp. other than *L. monocytogenes*

ID	Species	Wilderness	Source	Season	Year	*sigB* allele
A1-210	*Listeria* spp.	Rawah	water	fall	2010	5
A1-211	*Listeria* spp.	Rawah	water	fall	2010	6
A1-212	*Listeria* spp.	Never Summer	water	fall	2010	7
A1-214	*Listeria* spp.	Never Summer	water	fall	2010	7
A1-221	*Listeria* spp.	Mt. Evans	sediment	fall	2010	-
A1-092	*welshimeri*	Rawah	fecal 5	spring	2009	2
A1-096	*welshimeri*	Rawah	fecal 1	spring	2009	1
A1-141	*welshimeri*	Cache la Poudre	water	summer	2009	2
A1-145	*welshimeri*	Rawah	soil	summer	2009	1
A1-148	*welshimeri*	Rawah	soil	summer	2009	1
A1-149	*welshimeri*	Rawah	soil	summer	2009	2
A1-162	*welshimeri*	Rawah	fecal 2	fall	2009	4
A1-163	*welshimeri*	Rawah	fecal 2	fall	2009	1
A1-166	*welshimeri*	Rawah	soil	fall	2009	1
A1-173	*welshimeri*	W6 - Comanche Peak	fecal 5	fall	2009	3
A1-181	*welshimeri*	Rawah	soil	spring	2010	1
A1-185	*welshimeri*	Rawah	fecal 1	spring	2010	4
A1-193	*welshimeri*	Rawah	drag	summer	2010	1
A1-195	*welshimeri*	Rawah	drag	summer	2010	2
A1-197	*welshimeri*	Rawah	fecal 1	summer	2010	2
A1-199	*welshimeri*	Rawah	fecal 2	summer	2010	1