THESIS

MOLECULAR ECOLOGY OF FOODBORNE PATHOGENS IN PRIMARY PRODUCTION AGRICULTURE ENVIRONMENTS IN NORTHERN COLORADO AND INVESTIGATIONS IN INTERNALIN A DIVERSITY IN *LISTERIA MONOCYTOGENES* DUP-1039C STRAINS

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Clyde Simmons Manuel

Department of Animal Sciences

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Master's Committee:

Advisor: Kendra Nightingale

Lawrence Goodridge
Marisa Bunning
John Avens

ABSTRACT

MOLECULAR ECOLOGY OF FOODBORNE PATHOGENS IN PRIMARY PRODUCTION AGRICULTURE ENVIRONMENTS IN NORTHERN COLORADO AND INVESTIGATIONS IN INTERNALIN A DIVERSITY IN *LISTERIA MONOCYTOGENES* DUP-1039C STRAINS

Listeria monocytogenes, Salmonella, and Shiga toxin-producing *Escherichia coli* (STEC) represent clinically important human foodborne pathogens. The presence, transmission, and molecular epidemiology of these pathogens in primary agricultural environments (i.e., grazing pastures and produce farms) is understudied and represents a significant knowledge gap. We conducted a two year longitudinal sampling study in Northern Colorado to assess the presence, genetic diversity, and potential transmission patterns of these pathogens in production agriculture environments. Five produce farms and five grazing pastures in Northern Colorado were selected and sampled during the spring, summer, and fall in 2009 and 2010. Microbiological analysis was performed on composite soil (228 samples), composite fecal (105 samples), composite water (80 samples), and individual drag swabs (227 samples) 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, eight (1.25%), eleven (1.7%), and

22 (3.4%) of samples tested positive for *L. monocytogenes, Salmonella,* and STEC, respectively. Thirty-one samples contained *Listeria* spp. other than *L. monocytogenes*, the majority of which were determined to be *Listeria innocua* by *sigB* allelic typing. Additionally, 20 samples were positive for a proposed novel *Listeria* spp. that displayed unique phenotypic traits, i.e., non-motility and impaired growth at 37^{o}C. Pulse field gel electrophoresis analysis indicated isolates obtained in this study to exhibit significant genetic diversity. These results demonstrate a rare presence of foodborne pathogens in agricultural environments in Northern Colorado. Prevalence and molecular subtyping data generated by this study provides important insight into the molecular epidemiology of these pathogens in the pre-harvest agriculture environment.

Listeria monocytogenes uses the key virulence factor internalin A (InlA; encoded by *inlA*) to traverse host cell barriers during establishment of a systemic infection. Multiple naturally occurring mutations leading to a premature stop codon (PMSC) in *inlA* have been reported worldwide, and these mutations have been shown to be virulence attenuating in guinea pig infection models. Ribotype DUP-1039C is a broadly distributed molecular subtype within *L. monocytogenes* that has been commonly isolated along the food continuum. Ribotyope DUP-1039C isolates have been shown to carry three distinct PMSC mutations in *inlA,* including a frameshift mutation in a 5' homopolymeric tract of adenine restudies (PMSC4), a C to T substitution leading to a PMSC at aa 562 (PMSC 7), and a G to A substitution leading to a PMSC at aa 684 (PMSC 12). Additionally, DUP-1039C isolates carry one of three allelic types of a 5' hypermutable region of *inlA*, which may have a role in

phase variation. A selection of DUP-1039C isolates available from the PathogenTracker database (n=368) were screened for known *inlA* PMSCs and the allelic type of the 5' hypermutable region by two multiplex single nucleotide polymorphism (SNP) genotyping assays. SNP genotyping assays included (i) a previously described single nucleotide extension assay and (ii) a newly developed probe-based assay, which uses differentially labeled probes to differentiate between wild type (WT; AAAAAAA), frameshift (FS; AAAAAA), and interrupted HT (IHT; AAGAAAA) 5' allelic types of *inlA*. DUP-1039C isolates carrying a PMSC in *inlA* were not uniformly distributed among isolation sources. *inlA* PMSC type 4 (n=19) and PMSC type 7 (n=25) were associated ($P < 0.05$) with isolation from food and food environments, while WT isolates encoding a full-length InlA (n=184) were associated ($P < 0.05$) with non-food environments and mammalian hosts. Interestingly, all DUP-1039C isolates carrying *inlA* PMSC7 also had the IHT *inlA* allele in the hypermutable 5' region, and these isolates persisted within a single NY state seafood processing plant. The 5' IHT allelic type was frequently encountered among isolates from diverse sources, though at lower frequencies as compared to the WT allele. Together, the results may help to explain the underlying mechanisms behind the ability of lineage II *L. monocytogenes* isolates to adapt to both human and environmental niches.

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AUTOBIOGRAPHY

Clyde Simmons Manuel, more commonly known as “Chip”, was born in Abbeville, South Carolina on January 30, 1986. At a young age, Chip gained an interest in science and math and excelled in these courses throughout middle and high school. After graduating Woodruff High School in 2004, Chip enrolled in the University of South Carolina, where he majored in general engineering. After transferring to Clemson University in 2005, Chip decided engineering was not the right career path for himself, and switched into the Food Science and Technology program. Through opportunities in undergraduate research in Dr. Paul Dawson and Dr. Kay Cooksey’s labs, Chip gained a strong interest in food microbiology and food safety. After earning his Bachelor of Science in Food Science and Technology in Fall 2008, he enrolled in the Master of Science program in the Department of Animal Sciences at Colorado State University under the direction of Dr. Kendra Nightingale, and expects to complete this degree in Fall 2011. In 2011, Chip was accepted into the Department of Food, Bioprocessing and Nutrition Sciences at North Carolina State University, where he will study towards the completion of a Ph.D. in Food Virology. In the future, Chip hopes to manage a food safety research lab where he can make significant contributions towards ensuring that everyone has a healthy and safe food supply.

DEDICATION

I would like to dedicate my thesis to Tripp, Sarah, and Sadie, a young family who reminds me on a daily basis what real happiness truly is. Additionally, I would also like to dedicate my thesis to the memories of Clyde, Lala, and Pappy. I will never forget the life lessons you taught me.

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LITERATURE REVIEW

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Section 1: Overview of Foodborne Illness

1.1 Incidence and Burden.

Foodborne illness represents a considerable public health burden and has significant economic impacts on the United States (U.S.) population. In 2011, researchers with The Centers for Disease Control and Prevention (CDC) published what currently represents the most accurate estimates of the incidence of foodborne illness in the U.S. Using data gathered from various surveillance systems and adjusting for underreporting, Scallan and colleagues (2011) estimated that each year roughly 1 in 6 Americans (or 48 million people) acquire foodborne illness, 128,000 are hospitalized, and 3,000 die of foodborne diseases (207, 208). These estimates represent the total burden from two major groups of foodborne illnesses: known foodborne pathogens (those that are known to cause foodborne illness) and unspecified agents (agents not yet identified but likely cause acute gastroenteritis). Within the group of known foodborne pathogens, 31 agents have been estimated to cause 9.4 million cases of foodborne illness annually, which result in 55,961 hospitalizations and 1,351 deaths. *Listeria monocytogenes*, *Salmonella*, *Escherichia coli* O157:H7 and non-O157 Shiga toxin-producing *Escherichia coli* (STEC) are estimated to be responsible for over a third of domestically acquired bacterial foodborne illnesses attributed to known pathogens. More specifically, *L. monocytogenes* is estimated to annually cause 1,591 cases and 255 deaths; non-typhoidal *Salmonella* is estimated to annually cause 1.03 million cases and 378 deaths; *E. coli* O157:H7 is estimated to annually cause 63,153 cases and 20 deaths; and non-O157 STEC is estimated to annually cause 112,752 cases (data for annual deaths attributed to non-O157 STEC is unavailable due to the skewed nature of this data). Scallan and colleagues (2011) estimated that *L. monocytogenes, Salmonella,* and STEC O157 combined cause approximately 653 deaths out of the 861 (>75%) deaths annually due to domestically acquired bacterial foodborne illness in the U.S. (208).

1.2 Disease Trends.

The study by Scallan and colleagues (2011) used new and revised methodologies to determine estimates of foodborne illness. Because of this, their published estimates cannot be directly compared to previous estimates of foodborne illness (i.e., the study by Mead et al., 1999). Therefore, to determine change in incidence of foodborne illness over time, an active surveillance network must be consulted. The Foodborne Diseases Active Surveillance Network (FoodNet), is an active surveillance network for monitoring of laboratory-confirmed foodborne illnesses in 10 states (California, Colorado, Connecticut, Georgia, Maryland, Minnesota, New Mexico, New York, Oregon, and Tennessee). Since 1995, the goal of FoodNet has been to help public health officials better understand the epidemiology of foodborne diseases in the United States. Additionally, 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, for which FoodNet monitored progress (239). In 2009, FoodNet reported the incidence per 100,000 individuals of *L. monocytogenes, Salmonella, E. coli* O157, and non-O157 STEC as 0.34, 15.19, 0.99, and 0.57 cases, respectively (13). These figures for incidence of disease attributed to *L. monocytogenes, Salmonella, and E. coli* O157 represent a 26%, 10%, and 41%, reduction disease incidence as compared to the 1996-1998 data, respectively; baseline data was not available for non-O157 STEC (13). Of the four foodborne pathogens targeted by Healthy People 2010 (i.e., *Campylobacter*, *Listeria*, *Salmonella*, and STEC O157), only the target of ≤1.0 case per 100,000 individuals for STEC O157 was met in 2009 (13, 239). The Healthy People 2010 target for *L. monocytogenes* infections of ≤0.25 cases per 100,000 individuals was not met; however, the incidence of *L. monocytogenes* infections has declined 26% as compared to the 1996-1998 data (13, 239). The Healthy People 2010 target of ≤6.8 cases per 100,000 individuals for *Salmonella* was not met, and FoodNet surveillance data showed that *Salmonella* infections remained fairly constant. This indicates that significant efforts will be required to further reduce the incidence of salmonellosis among the U.S. population (13, 239).

1.3 Economic Burden.

Foodborne illnesses contribute to major economic losses, even in industrialized nations such as the U.S. In 2010, researchers at Georgetown University estimated that the total annual health related cost of foodborne illness in the U.S. is $152 billion (209). In 2005, Frenzen and colleagues estimated the annual cost of *E. coli* O157 infections to be $405 million (in 2003 dollars), including $370 million for premature deaths, $30 million for medical care (of which hemolytic uremic syndrome [HUS] patients represent the majority), and $5 million in lost productivity (72). Frenzen and colleagues (2010) at the United States Department of Agriculture (USDA) Economic Research Service (ERS) have also published a foodborne illness cost calculator that has been frequently used in regulatory cost-benefit and impact analyses (234). This online tool is currently functional for *Salmonella* and shiga toxin-producing *E. coli* O157; *Campylobacter, Listeria,* and non-O157 shiga toxin-producing *E. coli* are currently in the process of being incorporated into the tool. According to this online tool, the ERS estimates that the total cost estimate for *Salmonella* cases in the U.S. is $2.65 billion dollars (in 2009 dollars), including an average cost of $1,896 per case (234). These estimates are based on previous CDC estimates of the burden of foodborne illness; Frenzen and colleagues are currently working to update the online tool with the updated CDC estimates. Listeriosis has been estimated to total $2.3 billion annually in the U.S. due to medical costs and production losses (235); high hospitalization rates for listeriosis and significant cost of frequent product recalls are major contributing factors to this high economic burden of *L. monocytogenes.*

1.4 Reduction Efforts.

Great strides towards improved control of foodborne pathogens at the processing level have been made by both the meat industry and regulatory agencies, as aggressive control efforts by both parties may be in part responsible for the observed decline in incidence of foodborne illness attributed to *E. coli* O157 and *L. monocytogenes* in recent years (13). One such program is the Hazard Analysis Critical Control Point (HACCP) System. HACCP is a systematic approach to controlling foodborne hazards that places emphasis on the quality of all ingredients and all process steps; it is designed to control organisms at the point of production and preparation (103). The HACCP concept revolves around seven principles: (i) determining the risks and hazards associated with a product, (ii) determining specific critical control points (CCP) required to control the hazards, (iii) establishing critical limits that must be met at each CCP, (iv) monitoring CCPs, (v) establishing corrective actions when a CCP fails, (vi) establishing a verification system to prove that the HACCP system is working correctly, and (vii) establishing an effective HACCP record keeping system (103). HACCP has been widely used by the food industry since the late 1970s, and many food products are required to be processed under a HACCP system. The United States Food and Drug Administration (FDA) issued regulations in 1995 and 2001 that made HACCP mandatory for seafood products and juices, respectively (238). The USDA issued regulations in 1996 that required all large, small, and very small meat and poultry processors to implement a HACCP plan (233). Today, the HACCP program is widely used by multiple processors in diverse food industries, and many consider it to be the "gold standard" for pathogen intervention systems. In summary, aggressive efforts by both the food industry and regulatory agencies have resulted in significant progress in reducing pathogen levels in a post harvest setting. However, there is a critical need to control pathogens at the pre-harvest level to further reduce the incidence of foodborne disease.

The U.S. meat industry, spurred by USDA regulation, has implemented numerous decontamination measures to control pathogens in fresh meat. A series of decontamination technologies, both physical and chemical, are often combined in what is known as hurdle technology. Hurdle technology involves a combination of two or more decontamination strategies (hurdles) at individually sub-lethal levels to improve the microbial quality of a food (127, 128). In 2002, Huffman reviewed current and future decontamination strategies, often used in hurdle technology, that target both pre- and post-harvest segments of the food continuum (97). Some well-characterized examples of post-harvest decontamination techniques include chemical dehairing, hot water rinsing, steam pasteurization, steam vacuum, and chemical rinsing using primarily organic acid solutions, among others (97). The hurdle concept for control of pathogens on meat animal carcasses is well characterized, and numerous studies have demonstrated the utility of this concept (11, 32, 64). Contamination events of *L. monocytogenes* is commonly associated with ready-to-eat foods, due to the organism's association with contamination from the plant environment after the lethality step (202). Therefore, control of *L. monocytogenes* often focuses on ready-to-eat products which have incorporated antimicrobial compounds into their formulations (155). Additionally, *L.*

monocytogenes is often associated with agricultural commodities (i.e., raw milk and cheese products, produce) that receive minimal processing before consumption. In fact, an ongoing 2011 listeriosis outbreak attributed to contaminated cantaloupe has sickened 100 and killed 18 as of October 5, 2011 in what is one of the first documented listeriosis outbreaks attributed to cantaloupe, highlighting a critical research need to better understand this pathogen in a pre-harvest food safety setting (35).

Section 2: Overview of *Listeria monocytogenes*

2.1 *Listeria* spp. taxonomy and evolutionary history.

In a strict sense, the genus *Listeria* currently contains six species, including *L. monocytogenes, L. innocua, L. ivanovii, L. welshimeri, L. seeligeri,* and *L. grayi*. *L. monocytogenes* was first described in 1926 (156), when Murray and colleagues noticed a deadly disease among laboratory research rabbits. *L. monocytogenes* would remain the sole species of *Listeria* for many years, until *Listeria grayi* was added in 1966 (118). *L. innocua, L. ivanovii, L. welshimeri,* and *L. seeligeri* were described in 1981 (215), 1985 (217), 1983 (199), respectively. Only *L. monocytogenes,* and *L. ivanovii* are considered pathogenic, with *L. monocytogenes* representing a pathogen of public health and veterinary health importance. *L. ivanovii* is almost exclusively restricted to causing illness in sheep, though it has caused rare cases of human listeriosis on occasion (86). While the six species above represent recognized species of the genus *Listeria,* two novel species have recently been described in the scientific literature. *L. rocourtiae*, first isolated from lettuce in 2002, was first described in 2010 (121). *L. marthii* was described in 2009 and was isolated from a natural environment in upstate New York (83). The genus *Listeria* could well expand in the coming years, as research emphasizing exploration of the presence of *Listeria* in novel environments continues.

Through DNA/DNA hybridization, 16S rDNA sequencing, protein mapping, and additional methods, researchers have determined that the genus *Listeria* contains two closely related yet distinct lines of descent (202). One of these closely related groups contains *L. grayi*, and the other contains *L. monocytogenes, L. ivanovii, L. innocua, L. welshimeri,* and *L. seeligeri.* Within this second group, further subgrouping can be established, where one group of *L. monocytogenes* and *L. innocua* exist, and another group of *L. ivanovii, L. seeligeri,* and *L. welshimeri* exist (202). Using a comparative genomics approach, den Bakker and colleagues in 2010 proposed that the genome of the genus *Listeria* evolved by limited gene loss and acquisition, and suggested this genus to be an example of a bacteria that has evolved through a loss of virulence rather than acquisition of virulence. Based on this assumption, they proposed that modern *Listeria* spp. diverged from a pathogenic common ancestor and that evolution of this genus is associated with a shift to a saprophytic lifestyle (52).

2.2 *Listeria monocytogenes* in food.

Considered ubiquitous in nature, *Listeria* spp. and *L. monocytogenes* have been isolated from many different environments, including soil (254), water (70, 253), vegetation (254, 255), sewage (2, 253), animal feeds (257, 258), farm environments (67, 159, 161), and food-processing environments (228, 263). Several growth characteristics of *L. monocytogenes* contribute to its unique ability to thrive in harsh settings such as food processing plants, making it a major concern for the food industry. Among these characteristics are its ability to grow at temperatures between -1.5 and 45°C (96, 187), its ability to grow at pH ranges between 4.0-9.6 (187, 188), its ability to grow at water activities as low as 0.90 (164, 261), its ability to tolerate salt concentrations as high as 23.8% (119), and finally, its ability to create bioflms on a variety of surfaces (9, 66, 92, 223). Biofilms are of particular importance, as they allow *Listeria monocytogenes* to persist in the food processing environment, and can become resistant to antimicrobial and sanitizing agents (181).

Though *L. monocytogenes* has been known as a pathogen of veterinary concern since 1926 (156), it was not identified as a foodborne pathogen until 1981, when several human deaths and stillbirths in Nova Scotia were attributed to consumption of contaminated coleslaw (211). Listeriosis cases are often attributed to consumption of contaminated ready-to-eat (RTE) foods, which include foods that do not typically receive additional heat treatment by the consumer prior to consumption and may have an extended shelf-life (236). In addition, other foods that have been shown to be associated with outbreaks of *L. monocytogenes* include raw milk and cheese products (50, 137), pasteurized milk and related cheese products (50, 113), shellfish (129), shrimp (196), butter (139), vegetables (93), and most recently, cantaloupe (34).

L. monocytogenes is readily destroyed through normal cooking temperatures and commercial pasteurization. Given this information, cross contamination of finished product after the lethality step is the primary route in which food becomes contaminated with *L. monocytogenes* (202). Therefore, in order to minimize the risk of *L. monocytogenes* contamination in finish product, proper sanitation of the plant environment and proper handling of product after the lethality step must be ensured.

A significant amount of research has been conducted on the presence, molecular ecology and contamination patterns of *L. monocytogenes* and other *Listeria* spp. in food and food-associated environments. Gombas and colleagues (2003) collected 31,705 samples from various RTE food categories over a 14 month period and found the overall prevalence of *L. monocytogenes* to be 1.82% (82). In addition, this research group found that RTE foods packaged at the point of sale in a market are significantly associated with higher prevalence of *L. monocytogenes* versus RTE foods that are packaged during manufacturing. Sauders and colleagues (2009) conducted a cross-sectional study of *L. monocytogenes* contamination patterns in several retail establishments. In this study, 60% of retail establishments contained at least one sample positive for *L. monocytogenes*, and 2.7% of RTE foods from these establishments were positive for *L. monocytogenes* (206). In addition, this group noted that *L. monocytogenes* in retail environments are genetically diverse and are wide spread due to cross-contamination and dispersal. Finally, Williams and colleagues (2011) conducted a study to investigate prevalence and diversity of *L. monocytogenes* in small and very small RTE meat processing plants, finding that 6.1% environmental samples were positive for *L. monocytogenes*; however, the prevalence of *L. monocytogenes* and other *Listeria* spp. varied widely across plants enrolled in the study (263). Persistence of specific *L. monocytogenes* subtypes for time periods ranging from months to years has been documented for a variety of processing facilities (42, 95, 110, 117, 219, 228). In fact, in 2000, a multi-state outbreak of listeriosis due to contaminated turkey deli meat manufactured at a Texas facility was attributed to the same strain of *L. monocytogenes* that caused a sporadic case of listeriosis 12 years prior by contaminated frankfurters manufactured in the same Texas facility; this particular strain of *L. monocytogenes* persisted in the plant for over 12 years, illustrating *L. monocytogenes'* unique ability to persist in processing plants for long periods of time (175, 178). Persistent strains of *L. monocytogenes* have enhanced adherence properties, including enhanced biofilm formation, over non-persistent strains (20, 134, 135, 167).

2.3 Virulence and Pathogenicity of *Listeria monocytogenes*.

L. monocytogenes is the etiologic agent of listeriosis, a severe invasive human disease that can cause sepsis, meningitis, and stillbirths (202). Listeriosis primarily affects immunocompromised individuals and often results in high hospitalization and case-fatality rates (80). As a facultative intracellular pathogen, *L. monocytogenes* is capable of inducing its own uptake into non-phagocytic cells and spreading from cell to cell in a host (246). Consequently, the virulence determinants of *L. monocytogenes* allowing for its survival and spread within a host have been extensively studied. Molecular determinants of *L. monocytogenes* virulence can be classified into one of two broad functional categories: (i) bacterial adhesion and internalization of host cells and (ii) intracellular spread and proliferation. The proteins internalin A (InlA; encoded by *inlA*) and internalin B (InlB; encoded by *inlB*) were among the first virulence factors identified that mediate *L. monocytogenes* internalization within non-phagocytic cells (58, 74). These proteins belong to a group of proteins known as the internalins, a family of 25 proteins characterized by the presence of leucine-rich repeats (LRR) (26). InlA is an 800-amino-acid (aa) molecule (59) that contains two extended repeat domains: domain A, consisting of 15 leucine rich repeats, and domain B, consisting of 2.5 repeats of about 70 aa each (246). This LRR region of InlA is critical for uptake of *L. monocytogenes* into human epithelial cells (124). In addition, InlA has an N-terminal transport signal motif and a C-terminal LPXTG cell wall anchor motif; this LPXTG motif is responsible for attachment of InlA to the bacterial cell peptidoglycan layer (54). The InlA receptor is E-cadherin (150), an adhesion protein present on the surface of hepatocytes, dendritic cells, and epithelial cells, all of which are potential targets during *L. monocytogenes* infection (79). InlA interacts with the ectodomain of E-cadherin via its LRR region, leading to bacterial adherence. Cellular entry is mediated by interaction of InlA with the intra-cytoplasmic domain of E-cadherin, leading to an actin cytoskeleton rearrangement responsible for bacterial entry (122, 123). InlB is a 630aa internalin that is involved in entry into a broad range of cell types including hepatocytes and nonepithelial cells (58). It consists of a signal sequence on its N-terminal domain which is followed by seven LRR repeats, one inter repeat region, and one B repeat. Instead of binding to the peptidoglycan layer of the bacterial cell via a LPXTG motif, InlB has a C-terminal domain with tandem repeats of 80aa that mediate attachment of InlB to the bacterial cell wall through noncovalent interaction with lipoteichoic acids (107). InlB has three known receptors: gC1qR, a receptor for the complement protein C1q (23), Met, a hepatocyte growth factor tyrosine kinase receptor (220), and glucosaminoglycans

(GAGs), a type of protein found on the surface of mammalian cells (108). In addition to the internalins, other virulence factors contribute to *L. monocytogenes*' ability to adhere and then invade non-phagocytic cells. Ami, a 917aa autolysin, has been shown to promote bacterial adhesion to target cells in Δ*inlAB* strain (152). Also, Listeriolysin O (LLO; encoded by *hly;* discussed below) has been implicated in adhesion (112).

After internalization, *L. monocytogenes* uses several virulence factors to escape the host cell vacuole into the cytoplasm in order to spread and proliferate. LLO is the main factor responsible for bacterial escape from the vacuole (78). LLO is a toxin that forms pores at a low pH in cell membranes containing cholesterol. LLO activity is reduced upon escape from the vacuole given the neutral pH of the host cell cytosol. This prevents LLO from forming pores in the host cell membrane. Escape from the vacuole also relies on two phospholipases: Phospholipase C (PlcA; encoded by *plcA*) and Phospholipase B (PlcB; encoded by *plcB*). These enzymes act synergistically with LLO to lyse the primary and secondary vacuoles (28, 78). Once inside the host cytoplasm, *L. monocytogenes,* using the bacterial protein ActA, induces polymerization of actin filaments on one end of the bacterial cell that promote bacteria intracellular movement and cell-to-cell spread (229). More specifically, bacterial cells are propelled throughout the host cell by actin comet-like tails and form protrusions that become engulfed by neighboring cells in a double-layered vacuole. Once again, *L. monocytogenes* utilizes LLO, PlcA, and PlcB to escape this double-layered vacuole, allowing the intracellular lifecycle to continue (44).

Many of the important virulence factors contributing to the intracellular lifecycle of *L. monocytogenes* are located on a 10 kb gene cluster known as *Listeria* Pathogenicity Island-1 or LIPI-1. This cluster comprises six well-characterized genes, *prfA, plcA, hly, mpl, actA, and plcB,* along with four small open reading frames (ORFs) of unknown function (246). *L. monocytogenes, Listeria ivanovii,* and *L. seeligeri* all contain a functional LIPI-1, contributing to their pathogenicity; however, the nonpathogenic *L. seeligeri* contains an inserted gene, *orfE*, which disrupts the activation of its virulence genes (246). Positive regulatory factor A (*prfA*) regulates genes found on LIPI-1 along with other chromosomal virulence genes (126, 149). Though *inlA* and *inlB* are located outside the LIPI-1 on a different segment of the *L. monocytogenes* genome, it is still a part of the *prfA* regulon (60). Virulence factors regulated by *prfA* are expressed at higher levels at 37°C, which corresponds to the temperature of a human host (125). At temperatures lower than 30°C, *prfA* messenger RNA forms a stable hairpin loop which masks the Shine-Dalgarno sequence, inhibiting translation of *prfA* (105). This thermoregulation allows production of virulence factors to be turned on once *L. monocytogenes* encounters a host and for these genes to be masked in non-host environments.

2.4 *Listeria monocytogenes* genetic diversity.

Various subtyping techniques, both phenotypic and DNA-based, have been used to differentiate *L. monocytogenes* isolates (256). Serotyping based on conventional slide agglutination has been used for many years to subtype *L. monocytogenes* isolates. This method, based on the detection of various surface antigens, groups *Listeria monoctyogenes* into 13 serotypes: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7 (216); consequently, this method has relatively low discriminatory power as compared to molecular subtyping methods (256). Several molecular subtyping and phylogenetic studies have shown that *L. monocytogenes* isolates form a structured population composed of divergent lineages (53, 84, 132, 163, 179, 198, 251, 259). *L. monocytogenes* isolates can be classified into two major lineages (I and II) or 2 minor lineages (III and IV). Lineage I consists of serotypes 1/2b, 3b, 3c, and 4b, has a low genetic diversity, and is overrepresented among human isolates obtained from outbreaks and sporadic cases worldwide (84). Lineage II consists of serotypes 1/2a, 1/2c, and 3a, represents the most genetically diverse of the four lineages, and is overrepresented in food, food-related, and natural environments (84). Lineage III and IV isolates consist of serotypes 4a, 4b, and 4c, are most commonly isolated from ruminants, and have rarely been associated with human disease (198, 249).

2.5 *Listeria monocytogenes* niche adaptation.

Numerous epidemiology and subtyping studies have shown that *L. monocytogenes* serotypes differ in their association with sporadic and epidemic human listeriosis (84, 146, 166, 259). McLauchlin and colleagues (1990) analyzed the serotype distribution of 1,363 *L. monocytogenes* isolates from human clinical cases and found that over 93% of cases were attributed to four serotypes: 4b, 1/2a, 1/2b, and 1/2c (146). Four epidemic clones (ECI, ECIa, ECII, and ECIII) have been described within *L. monocytogenes* and have been linked to a large number of listeriosis outbreaks and sporadic cases more often than other members of the same serotype (110, 250). ECI, ECIa, and ECII are distinct 4b strains that have been responsible for numerous outbreaks in both the U.S. and Europe. ECIII represents a lineage II 1/2a strain that was shown to persist in the plant environment of a turkey processing facility for over 10 years, where it was implicated in a multistate outbreak of listeriosis (110). While these epidemic clones represent a subpopulation of *L. monocytogenes* implicated in numerous repeat outbreaks and sporadic cases worldwide, studies have failed to show these clones to be more virulent than strains from the same serotype. On the other hand, lineage II strains (>30%) represent a subpopulation of *L. monocytogenes* shown to often carry virulence attenuating mutations in *inlA* (101, 162). Over 18 unique mutation types from diverse geographic locations leading to a PMSC in *inlA* have been identified to date (see reference (245) for a summary on the 18 different truncated *inlA* alleles). *L. monocytogenes* strains containing these mutations have been shown to demonstrate attenuated invasion of both human intestinal and hepatic cell lines and attenuated virulence in guinea pig challenge studies (43, 160, 171-173, 201, 244). For example, Nightingale and colleagues (2008) determined that *L. monocytogenes* strains harboring a PMSC in *inlA* displayed a significantly lower infectivity in the liver and small intestine of orally infected guinea pigs as compared to wild type *L. monocytogenes* strains harboring a full length *inlA* (160). *L. monocytogenes* strains carrying a PMSC in *inlA* are common among food and food environment sources while being rare among human clinical sources (101, 162, 201, 243, 245, 249). A SNP genotyping assay (243) was used by Van Stelten and colleagues (2008) to screen a collection of more than 1,000 *L. monocytogenes* isolates sourced from both human clinical samples and RTE food samples (245). Results from this study showed that *inlA* PMSC mutations were overrepresented among *L. monocytogenes* strains isolated from RTE food isolates (45.0%) but underrepresented among isolates from human clinical cases (5.1%). Collectively, these studies suggest the food attribution of listeriosis may be related to the heterogeneous distribution of *L. monocytogenes* isolates carrying a PMSC mutation in *inlA* and EC strains among different RTE food categories.

Section 3: Overview of *Salmonella*

3.1 *Salmonella* taxonomy and nomenclature.

Salmonella is a facultative anaerobic gram-negative rod-shaped bacterium belonging to the family *Enterobacteriacae* and is capable of causing disease in humans and animals. Human *Salmonella* infections can display various clinical conditions, including enteric (typhoid) fever, uncomplicated enterocolitis, and even systemic infection (57). *Salmonella* nomenclature is complex and has progressed through several taxonomic schemes based on biochemical and serological characteristics as well as DNA homology. Currently, combined DNA-DNA hybridization and multilocus enzyme electrophoresis analysis divides the *Salmonella* genus into two species, including *S. enterica* and *S. bongori* (194). The majority of mammalian salmonellosis cases are attributed to *S. enterica,* while *S. bongori* is more commonly found in environmental sources. As recognized by the CDC, *S. enterica* is further divided into six subspecies: (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; S. bongori* itself was formerly subspecies V (24). Serotyping for each species and subspecies of *Salmonella* is done according to the Kauffman-White scheme (190), and currently over 2,463 serovars are recognized, with *S.* Typhimurium and *S.* Enteritidis causing over 50% of human salmonellosis cases in the U.S. (24). *S.* Typhi and *S.* Paratyphi are the causative agents of enteric fever, a severe illness that is estimated to cause 22 million cases of disease and 200,000 deaths each year, primarily in regions with poor sanitation and unclean drinking water (8). Currently, only *S. enterica* subsp. I is given names for its serotypes; subspecies II through VI are recognized by their serotyping code (24). Serotype designations include the following: (i) subspecies designation (subspecies I through VI), (ii) O (somatic) antigens followed by a colon, (iii) H (flagellar) antigens (phase 1) followed by a colon, and (iv) H antigens (phase 2, if present) (for example, *Salmonella* serotype IV 45:g,z_{51}:-.).

3.2 Presence of *Salmonella* in food.

Salmonella are widespread in the environment and can be carried in the intestinal tract of birds, reptiles, farm animals, humans, and occasionally insects (103). *Salmonella* can often be found on other parts of these animals, such as the skin, and are capable of being transmitted to a large number of places. Although *Salmonella* has been implicated in contamination of many different food products, it is most commonly associated with the contamination of poultry and egg products, with *S.* Enteritidis and *S.* Typhimurium being the predominate serotypes in these foods (3, 22, 153, 185). It is estimated that 19% of broiler flocks in the U.S. are currently positive for *Salmonella* (240), and that approximately 1 in every 20,000 eggs is positive for *S.* Enteriditis (62). *Salmonella* is a major concern to the poultry and egg industry, as it can be spread from bird to bird through a fecal-oral route within poultry houses (30, 200). In fact, in 2010, a nationwide outbreak of *S.* Enteriditis triggered the recall of over 500 million eggs from a producer in Iowa in what became one of the largest recalls in history (115). In 2006, USDA-FSIS implemented regulation to reduce the prevalence of *Salmonella* in broilers below 19% (27).

In addition to poultry and egg products, outbreaks of salmonellosis have been associated with a wide variety of food products, including beef (51), pork (81), fish and shellfish (230), milk (176), cheese (4), fruits (36), vegetables (39), pepper (40), and even peanut butter (140). Many of these outbreaks were caused by contamination of the food product after the lethality step, illustrating the importance of proper sanitation practices, equipment design, and ingredient control (189). For example, a nationwide outbreak of *S.* Typhimurium in 2008 and 2009 associated with various peanut products was attributed to improper facility design and equipment, including the presence of large gaps in the roof that allowed for rain water to leak directly onto finished product within the facility (189). This would become one of the largest and most deadly outbreaks in U.S. history. In addition, a multistate outbreak of *S.* Newport linked to tomatoes in 2004 and 2005 was found to be associated with contaminated pond water used to irrigate the fields (85). Given that *Salmonella* is extensively populated throughout the environment and that it is associated with many diverse food products, it is likely that controlling this organism in the food-processing environment will continue to challenge food safety professionals for many years to come.

3.3 *Salmonella* virulence.

Salmonella pathogenesis is a complex process that involves a multitude of virulence factors. After ingestion by a host, *Salmonella* invades the intestinal mucosa by several routes, including passive uptake by M cells and phagocytes, and active entry into non-phagocytic enterocytes (145). Many important factors required for non-typhoidal *Salmonella* invasion of host cells are encoded on five *Salmonella* pathogenicity islands (called SPI-1 through SPI-5) located on the chromosome (5, 213). It has been suggested that these islands were acquired via horizontal transfer through plasmids and/or phage mediated transfer (15). While each pathogenicity island plays a role in establishment of infection, none are as well understood or as important as SPI-1 and SPI-2. It has been shown that between these two islands, over 42 of the approximately 60 genes required for *Salmonella* virulence are present (75). *Salmonella* first initiate infection by attaching to the intestinal mucosa with the aid of fimbrial adhesins encoded by a series of genes on SPI-1 (242). A variety of SPI-1 effector proteins delivered into eukaryotic host cells via a type III secretion system (SPI-1 T3SS) then induce membrane deformation and rearrangement of the actin skeleton, resulting in a phenomena known as "membrane ruffling." This "membrane ruffling" is a critical factor in internalization of *Salmonella* within host vacuoles, as it triggers bacterial uptake by macropinocytosis (145). After internalization, *Salmonella* return the host cell skeleton back to its original state (183). The bacterial cells then remain inside the membrane-bound vacuoles for their entire intracellular lifecycle (68). Survival of the bacteria within the vacuoles is dependent upon another T3SS encoded by SPI-2. This SPI-2 T3SS encodes for various factors that allow for cell invasion and macrophage survival (182). SPI-3 and SPI-5 contains genes required for survival in Mg^{2+} limited environments and macrophage survival, respectively (213). It is not clear what role SPI-4 plays within *Salmonella* infections. Replication of the bacteria within the vacuoles eventually causes the host cell to burst, allowing for the bacteria to spread to other cells and begin the cycle again.

Several additional factors important in *Salmonella* infection have been identified. PhoP and PhoQ are two proteins involved in a two compartment regulatory system that controls over 40 genes critical for *Salmonella* survival within the hostile environment of the macrophage (151). Also, the *inv* locus harbors several genes critical to entry of epithelial cells. A well-characterized example is *invA,* which has been demonstrated to be fully required for *Salmonella* invasion into epithelial cells (76).

Section 4: Overview of Enterohemorrhagic *E. coli*

4.1 *E. coli* taxonomy and pathotype diversity.

Escherichia coli is a Gram-negative, rod-shaped bacterium that is commonly found in the intestines of warm-blooded organisms. *E. coli* display a remarkable amount of both phenotypic and genetic diversity, with an estimated 20% of the genome common to all strains (133). A common subdivision system used to differentiate *E. coli* is antigenic serotyping. Serotyping is based on the presence of various forms of three major surface antigens: O (somatic), H (flageller), and K (capsular). To date, over 174 O antigens, 56 H antigens, and 103 K antigens have been identified (87). However, it is only considered necessary to identify the O and

H antigens when serotyping pathogenic *E. coli*.

While commensal *E. coli* is rarely implicated in disease, there are several strains that have acquired various virulence factors that allow them to cause disease. Pathogenic *E. coli* can be categorized into several pathotypes based on virulence properties and clinical symptoms. *E. coli* pathotypes include enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), diffuse-adhering *E. coli* (DAEC), enteroaggregative *E. coli* (EAEC), and enterohemorrhagic *E. coli* (EHEC) (157). These pathotypes are reviewed intensively by Kaper et al., 2004 (109). EPEC is a major problem in developing countries where it frequently causes severe diarrhea in infants. Humans are important reservoirs of EPEC, and this pathotype has the ability to invade epithelial cells. ETEC are another cause of severe diarrhea in infants primarily in developing countries. It is also an agent often implicated in "travelers' diarrhea." Again, humans are the main reservoir for ETEC, and this pathotype produces an enterotoxin that elicits a diarrheal response in the host. EIEC causes non-bloody diarrhea similar in clinical symptoms to dysentery caused by *Shigella* spp. EIEC invade and multiply within epithelial cells, and this invasive aspect of EIEC is associated with the presence of a large virulence plasmid. DAEC strains are associated with diarrhea in young children between the ages of 1 year to 5 years. These strains produce a unique diffuse-adherent pattern of attachment to HEp-2 cells. EAEC strains differ from other *E. coli* pathotypes in that they produce a unique pattern of aggregative adherence, appearing like a "stack of bricks" in various cell lines. They are the cause of persistent diarrhea in children and infants in several countries worldwide. EHEC elicit the most severe clinical symptoms in the human host. EHEC strains are associated with cases of hemorrhagic colitis as well as hemolytic uremic syndrome (HUS), a severe disorder that is often characterized by renal failure. The presence of shiga toxins 1 and 2 (Stx1 and Stx2, encoded by *stx1* and *stx2*, respectively) are the key virulence factors associated with the severe symptoms of EHEC strains. Many *E. coli* can acquire shiga toxins, leading to the use of the term shiga-toxin producing *E. coli* (STEC) to describe any *E. coli* strain that carries *stx1* and/or *stx2.* The term pathogenic STEC or pSTEC will hereon refer to strains of *E. coli* that carry *stx1* and/or *stx2* and are capable of causing severe disease (i.e., other virulence factors in addition to shiga toxins are present). It is important to note that these pathotype classifications are not always clear-cut. For example, in 2011, a particularly devastating *E. coli* O104:H4 strain carrying virulence factors characteristic of both EHEC and EAEC pathotypes emerged. This strain was responsible for 3,222 cases (810 involving HUS) and 39 deaths (numbers current as of June 18, 2011) in Germany during an outbreak attributed to bean sprouts (148, 210).

4.2 *E. coli* serotype diversity and epidemiology: emergence and importance of non-O157 serotypes.

First associated with human illness in 1982 (197), *E. coli* O157:H7 has since been implicated in numerous outbreaks of foodborne illness worldwide. Following a major outbreak associated with undercooked ground beef from a northwestern retail chain in 1992-1993 (16), the USDA-FSIS declared *E. coli* O157:H7 to be an adulterant in raw ground beef and non-intact beef products, and established a zero-tolerance policy for detection of this organism in these products (63). Since this regulatory change, a primary focus of the food industry and regulatory agencies has been *E. coli* O157:H7, despite emerging evidence that suggests other pSTEC serotypes, called non-O157 STEC, are capable of causing disease as severe as *E. coli* O157:H7 (25). Between 1983 and 2002, the CDC confirmed 940 illnesses related to non-O157 STEC infections, with the majority (>70%) of these isolates being of serotypes O26, O45, O55, O103, O111, O121 and O145 (25). In addition, Scallan and colleagues (2011) estimated that non-O157 STEC causes 112,752 foodborne disease cases annually in the U.S. (208). However, it has been suggested that estimates of non-O157 STEC illness are most likely understated due to several issues, including (i) difficulty in isolation of non-O157 STEC as compared to O157 STEC, leading to an apparent predominance of O157 STEC (204), and (ii) lack of awareness and/or proper diagnostic capabilities among clinical laboratories (17).

Non-O157 STEC presents numerous challenges to researchers, regulatory agencies and the food industry. First and foremost, apart from the presence of toxin encoding genes such as *stx* and *eae*, there are few common virulence factors among pSTEC that allow for simultaneous targeted molecular detection of non-O157 STEC and O157 STEC (63). While *stx* could potentially be used as a universal marker for all pSTEC, it may result in many false positives, as it has been shown that *stx* genes have been found in *E. coli* strains that lack other important virulence factors required for illness (184). Most *E. coli* O157 strains demonstrate delayed fermentation of sorbitol. This has led to the development of a medium, Sorbitol MacConkey agar, which is able to differentiate O157 serotypes from others based on a colorimetric reaction. Unfortunately, no known biochemical markers exist for non-

O157 STEC that could easily be incorporated into a differential medium. In September 2011, USDA-FSIS announced its intention to require routine testing for "the big six" serotypes (O26, O45, O103, O111, O121, and O145) of non-O157 STEC in manufacturing trim and other raw ground beef components (237). The proposed legislation is currently in review, and will be implemented by March 2012.

4.3 STEC reservoirs and distribution.

STEC strains have been isolated from a variety of animals, including cattle (41), goats (18), sheep (18), cats (18), chickens (18), dogs (18), horses (89), pigs (18), deer (12), rabbits (169), pigeons (154), and rats (46); however, it has been shown that STEC are more prevalent in cattle than other animals (98, 191), indicating that cattle are the predominant reservoir for O157 STEC and likely non-O157 STEC. STEC prevalence rates among cattle are widespread and have been reported as high as 60%; however, the majority of studies have found prevalence rates of 10% to 25% (98). Most cattle are transient carriers of STEC, shedding the organism in their feces for periods of time ranging from weeks to months (64). Interestingly, several studies have noted an increased prevalence of O157 STEC isolation during the summer months. For example, one U.S. study identified a significant increase in prevalence of O157:H7 *E. coli* in cattle presented for slaughter in summer months (July through August) than for other seasons (64). This seasonality of STEC prevalence in cattle may be correlated to the observed seasonality of STEC infection in humans, where incidences of disease attributed to STEC peaks during summer months (25). Seasonality of STEC illnesses may also be partly attributed to the increased consumption of ground beef products during the summer months in the U.S., as this is a major food vehicle associated with STEC contamination (57). Factors influencing bovine carriage of STEC are not fully understood, but it is likely that farm management practices (i.e., nutrition, manure handling, etc.) may play an important role in STEC transmission in a herd. Also, it has been shown that gastrointestinal colonization of STEC within cattle is largely a function of age, with calves more likely to become colonized with STEC than cows (47). This is likely due to the fact that the rumen microflora of calves is not as well established as adult cattle.

Unlike other *E. coli* pathotypes, humans are not considered important reservoirs for STEC, and no asymptomatic carriers of STEC have been identified. Most studies have shown that *E. coli* O157:H7 is shed in feces of HUS patients for usually no more than three weeks (57); however, exceptions have been observed. For example, in one instance, a child associated with a STEC outbreak in a daycare facility was shown to shed *E. coli* O157:H7 for over 62 days after onset of illness (177). While humans are not considered reservoirs for STEC, a large number of outbreaks have been associated with person-to-person transmission of STEC by the fecal oral route, emphasizing the importance of proper handling of patients who are infected with STEC (193).

4.4 STEC presence in food.

In a review of outbreaks associated with O157 STEC infection between 1982 and 2003, it was determined that food was the predominant transmission vehicle for O157 STEC, being responsible for 61% of the outbreak-related cases (193). Many of these infections were attributed to consumption of contaminated ground beef, which is considered to be a common food product responsible for foodborne transmission of STEC. Contamination of beef products with STEC occurs primarily during slaughter and subsequent initial processing steps (i.e., stunning, hide removal, and evisceration) (57). In a risk assessment study of *E. coli* O157:H7 in ground beef, the three highest ranked factors associated with increased probability of becoming ill from eating contaminated ground beef were (i) concentration of STEC in animal feces, (ii) host immune status, and (iii) level of carcass contamination, illustrating the need for a “farm to fork” approach when controlling STEC occurrence in the food supply (31). Numerous studies have been conducted to characterize the prevalence of STEC in the U.S. food supply. In a nationwide survey of ground beef samples conducted between 1994 and 1998, USDA researchers found *E. coli* O157:H7 in 23 of 23,900 ground beef samples (103). In addition, another USDA study found 1,006 ground beef samples out of 4,133 samples taken positive for *E. coli* containing *stx*1 and/or *stx*2. However, when these samples were further screened for additional virulence factors (i.e, *eae*), only 10 STEC isolates (0.24% of the total 4,133 samples) represented a significant food safety threat (21).

4.5 STEC pathogenesis and virulence.

The spectrum of illness that O157 STEC and non-O157 STEC can cause includes non-bloody diarrhea, hemorrhagic colitis (bloody diarrhea), and hemolytic uremic syndrome (HUS), considered the most severe form of illness (87). HUS seems to affect approximately 6% of patients, and the overall case fatality rate for an EHEC infection is around 1% (87). The majority of HUS patients are children under the age of 10 years, and O157:H7 infection represents the leading cause of HUS in this demographic (147). The infectious dose for STEC is unknown, but is thought to be less than 100 cells based on numerous retrospective analyses of outbreaks (231).

A key characteristic of *E. coli* O157:H7 contributing to its pathogenicity is its ability to tolerate acidic environments (61, 114, 141, 224). This allows the bacterium to easily pass through the low pH environment of the human stomach and enter into the intestinal tract where it can elicit disease. It is unknown whether non-O157 STEC strains contain similar mechanisms of acid tolerance; this is an important knowledge gap that warrants further research.

Once STEC reaches the host intestinal epithelium, it recruits a variety of virulence factors that contribute to the onset of disease. STEC strains have evolved to be pathogenic mostly through acquisition of mobile elements such as prophages, transposons, plasmids, and genomic islands (120). One of the most important factors involved with the pathogenesis of STEC infection are the Shiga toxins (Stx). Within STEC, Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2) are both encoded on temperate bacteriophages that have integrated within the *E. coli* chromosome (212). Stx1 is very homologous to the Shiga toxin produced by *Shigella dysenteriae*, as it only differs by one amino acid (212). Stx2 on the other hand, is much more genetically diverse, and over 20 genotypes of Stx2 have been identified to date (218). In addition, there has been evidence that Stx2 producing STEC strains are more virulent and result in more HUS cases than strains that produce Stx1 only (180). Shiga toxins are composed of a single A-polypeptide and a B-pentamer. The B-pentamer binds to the eukaryotic cell receptor, globotriaosylceramide, expressed on both epithelial and endothelial cells, and leads to internalization of the Shiga toxin (205). Once internalized, the A subunit is cleaved into two active subunits which leads to removal of a single adenine residue from the 28S rRNA of eukaryotic ribosomes. This mutation results in inhibition of protein synthesis within the host cell since the removal of the adenine residue leads to the inability of the aminoacyl-tRNA to bind to the ribosome, resulting in cell death and apoptosis (102).

Both non-O157 pSTEC and O157 STEC produce characteristic A/E (attaching and effacing) lesions resulting from the assembly of pedestal-like actin structures during bacterial adhesion to intestinal epithelial cells (77). The locus of enterocyte effacement (LEE) is a pathogenicity island found in many STEC strains. Nearly all proteins produced by the genes located on the LEE are important for adhesion of the bacteria to the epithelial cells. Several important products of the LEE include a type III secretion system (T3SS), an outer membrane adhesin (intimin, encoded by *eae*), a translocated intimin receptor (Tir), and finally, a variety of effector proteins (55, 186). Using the T3SS, Tir is delivered into the host cell then intimin can bind to the Tir proteins, creating an intimate adherence between the bacteria and the host cell, leading to the formation of A/E lesions (55, 186). This intimin/Tir attachment allows for pSTEC to colonize the host gastrointestinal tract, leading to the onset of illness. It has long been thought that intimin is a required virulence factor required for pSTEC infection; however, this has been challenged recently, as emerging evidence indicates the existence of additional adhesion factors. For example, Vidal and colleagues (2007) studied numerous strains of intimin negative STEC and identified several important putative adhesion factors required for host cell attachment (247).

Section 5: Prevalence and ecology of foodborne pathogens in primary production agricultural environments.

5.1 Importance of subtyping.

Efficient foodborne disease surveillance, outbreak detection, and source tracking relies on sound epidemiologic studies combined with rapid and precise molecular subtying methods (256). Large subtyping databases such as the CDC PulseNet database are key in facilitating both outbreak detections and investigations (225). The PulseNet database contains a significant amount of molecular subtyping data for foodborne pathogens isolated from (i) human clinical cases, (ii) foods, and (iii) farm animals; however, environmental isolate subtype data is lacking. The inclusion of environmental isolate molecular subtype data from production agriculture areas (i.e., grazing pastures and produce fields) into subtyping databases could provide important insight into diversity of foodborne pathogens from farm to fork. Further emphasizing the need for this data is a recent outbreak of *E. coli* O157:H7 that was attributed to fecal contamination of spinach fields in California by feral hogs (104). Consumption of the contaminated spinach product resulted in 205 reported cases and three deaths. Nearly fifteen percent of feral swine fecal samples in the area tested positive for the outbreak strain of *E. coli* O157:H7, and soil, water, and sediment samples were also positive in the same area. Additionally, the outbreak strain was identified on a nearby cattle operation, and feral hogs were implicated as a likely source of transmission of the strain onto the spinach fields (104). This outbreak illustrates the potential for environmental sources of foodborne pathogens to be implicated in outbreaks of human foodborne illness. Given the impact that environmental contamination can have on food destined for human consumption, it is clear that a critical need exists for increased molecular subtyping of environmental isolates of foodborne pathogens.

5.2 Prevalence and ecology of foodborne pathogens in beef production environments.

Beef cattle production in the United States generally consists of three industry segments: cow-calf, stocker, and feedlot. Cow-calf and stocker cattle, as well as some dairy cattle, are reared primarily in range- or pasture-based environments; therefore, grazing of livestock on pastures represents an important step in the beef and milk production process. While numerous studies have focused on the intensively managed beef feedlot and dairy industries, few studies have focused on the presence and molecular ecology of foodborne pathogens in beef cattle on pasture. The majority of these studies focused on *E. coli* O157:H7, as it is widely recognized that cattle feces is the primary source of contamination in foodborne outbreaks of *E. coli* O157:H7 (10). In 2002, Renter and colleagues determined the diversity, frequency, and persistence of *E. coli* O157 strains from cattle, wildlife, and water sources over an 11-month period within range cattle production environments (195). These researchers analyzed 9,643 fecal samples from cattle and wildlife that were present on the range. In addition, they analyzed 4,083 water samples from sources that animals had access to. Using a latex agglutination assay followed by multiplex PCR (detecting presence of *eae, stx1,* and *stx2*) and PFGE, the researchers determined the presence of *E. coli* O157 in cattle, water, and wildlife to be 0.90%, 0.24%, and 0.20%, respectively (195). The researchers concluded that their findings were in agreement with other prevalence studies of *E. coli* O157 in range environments. Interestingly, studies investigating presence of *E. coli* O157 in feedlot settings have reported much higher prevalence rates, indicating that animal density may be an important factor in presence and transmission of this organism in beef production settings (64). Renter and colleagues also noted that potential mechanisms for maintenance and dissemination of *E. coli* O157 strains might be quite different in range production environments from intensive feedlot settings due to the observed diversity in subtypes between the two environments (195).

The ability for *E. coli* O157:H7 to survive for extended periods of time within environmental samples is well documented and represents an additional factor that may allow for this pathogen to be transmitted to food via production agriculture environments. In 1996, Wang and colleagues documented survival of *E. coli* O157:H7 in fecal slurries under laboratory conditions and discovered that the pathogen is able to survive for 56 days at 22°C and low moisture content (248). Other researchers found similar results for non-O157 STECs in fecal slurries, where Fukushima and colleagues (1999) observed survival of *E. coli* O26, O111, and O157 in fecal slurries ranging from 3 to 12 weeks at 25°C (73). Long-term survival of *E. coli* O157 has also been documented in soil samples. Ogden and colleagues (2002) observed long term survival of *E. coli* O157 in loamy sand samples naturally contaminated with the pathogen for 15 weeks at 25°C (170). Together, these results illustrate the need to consider a variety of environmental sample types when conducting an epidemiologic investigation of a foodborne outbreak.

Studies investigating the presence and diversity of *Salmonella* and *L.*

monocytogenes in cattle production environments also exist. In a large case-control study conducted by Nightingale and colleagues in 2004, 24 case farms and 28 matched control farms were investigated for presence of *L. monocytogenes* in order to elucidate the prevalence, ecology, and genetic diversity of *L. monocytogenes* in the farm environment (161). The researchers collected 2,056 samples, including 528 fecal, 504 soil, 516 feedstuff, and 508 water samples. They encountered 414 positive samples, including 107 fecal, 120 soil, 87 feedstuff, and 100 water samples; these results indicated an overall *L. monocytogenes* prevalence of 20.1% (161). Interestingly, small ruminant case farms had significantly higher prevalence of *L. monocytogenes* in fecal, soil, water, and animal feed samples than small ruminant control farms, indicating that these samples may represent important routes through which farm animals may become contaminated. The researchers concluded that (i) cattle represent a major reservoir for *L. monocytogenes* and may contribute to the dispersal of the organism within the farm environment, (ii) bovine farms have higher incidence of *L. monocytogenes* subtypes linked to human disease, and (iii) *L. monocytogenes* subtypes may differ in their abilities to infect animals and to survive in farm environments (161). *Salmonella* presence in environmental farm samples from cattle production operations has also been investigated. In 2006, Rodriguez and colleagues collected 2,496 environmental farm samples from diverse farms (i.e., poultry, dairy, beef cattle, and swine farms) in order to determine the presence of *Salmonella* in diverse farm environments (200). These researchers determined the prevalence of *Salmonella* on beef and dairy farms to be 8.5% and 17.9%, respectively. On each farm setting, soil samples yielded the highest prevalence, indicating a need to consider this environmental sample type as a potential route for *Salmonella* transmission in farm settings (200).

5.3 Prevalence and ecology of foodborne pathogens in produce production environments.

In response to an increase in per capita consumption of fresh produce, the proportion of outbreak-associated cases linked to fresh produce increased from $<$ 1% to 12% between 1973 and 1997, reflecting the increasing ability of these food products to serve as vehicles for major foodborne outbreaks (221). Contamination of fruits and vegetables with foodborne pathogens can occur at the pre-harvest stage (i.e., planting, irrigation, and soil amending), as has been indicated by several recent multi-state outbreaks of foodborne illness (36-38). Recent studies suggest that contamination of these products with foodborne pathogens is much more complex than simple passive transfer (232). For example, upon flooding with contaminated irrigation water, it has been shown that *E. coli* O157 can be taken up by lettuce in greenhouse settings, and young tomato plants may take up *Salmonella* of certain serotypes; in both cases, the pathogens can be incorporated into the plant tissues at levels higher than 10^3 CFU/g (136). Contamination of produce in a pre-harvest setting is complex and not well characterized; however, numerous routes have been described. During harvest, it is thought that human handlers in the field may serve as an important route of transmission (143). In addition, wildlife and irrigation water have been implicated as important potential sources for foodborne pathogen contamination of pre-harvest produce (48, 104). Studies investigating surface water sources used for irrigation have recognized these as potential vehicles for pathogen spread in a farm environment (136). Pathogen loading of surface waters is greatest after periods of heavy rainfall, as natural runoff can leech contaminants into source water (49). The method of irrigation (i.e., flood, sprinkler, or drip) used on a field can also have an impact on the distribution of contaminated irrigation waters, with flood and sprinkler systems being correlated with spread of enteric organisms on a farm environment (91).

Given the potential for contaminated environmental samples to be an important route for transmission of pathogens on produce farms, it is clear that a need to study the ecology and presence of foodborne pathogens in these pre-harvest agriculture environments exists. Studies investigating the presence of foodborne pathogens on fresh produce have been focused primarily at the retail level; very few surveys have been conducted at the pre-harvest farm level. A general theme amongst these studies is that the incidence of foodborne pathogens was often between 0 and <10% of all samples tested (90). For example, of >3,800 samples of ready-to-eat salad mixes from retail markets sold in the U.K., only 0.2% were positive for *Salmonella* and an additional 0.5% had presence of *E. coli* or *L. monocytogenes* at >100 CFU per g of product (203). Though the incidence of foodborne pathogens present on fresh produce at retail markets may be low, it does not necessarily make these products less dangerous, as they are often consumed raw with little to no processing before consumption. In 2004, Castillo and colleagues conducted a study investigating the occurrence of *Salmonella* in cantaloupe and environmental samples from farms in Texas and Mexico (33). Of a total of 1,735 samples collected, 31 (1.8%) tested positive for *Salmonella*. These researchers noted a significantly higher rate of *Salmonella* isolation from irrigation water than cantaloupe samples, further emphasizing the potential for environmental samples to contribute to pathogen dissemination on a field. Additional studies investigating the genetic diversity of foodborne pathogens among these agricultural environments are needed to fill important knowledge gaps concerning the presence, transmission, and potential for disease that these isolates carry.

Section 6: Review of Phenotypic and Molecular Subtyping Methods

Subtyping of bacterial pathogens allows for classification of isolates beyond the species level (256) and is used for determining variability within populations, defining clonal lineages, and describing changes within a population over time (71). When combined with epidemiological data, the utility of subtyping for microbial source tracking purposes has proved to be an extremely valuable tool to mitigate the public health impact of foodborne illness outbreaks. Subtyping methods can be divided into two broad categories: (i) phenotypic subtyping methods, which target enzymes, proteins, or other metabolites expressed by the cell, and (ii) molecular subtyping methods, which target nucleic acids (158). Molecular subtyping methods can further be divided into two categories: (i) band based methods (also known as DNA fingerprinting), and (ii) sequence based methods (256).

6.1 Phenotypic subtyping methods overview.

Several common phenotypic methods used to subtype foodborne pathogens include serotyping, phage typing, and multi-locus enzyme electrophoresis (MLEE). Phenotypic methods are still widely used to subtype foodborne pathogens because information gained from phenotypic subtyping is often of clinical relevance (256).

Serotyping is one of the oldest subtyping methods and involves the detection of various forms of three major surface antigens: O (somatic), H (flageller), and K (capsular). Detection of these antigens is accomplished via an agglutination reaction with antiserum of known specificity. Serotyping is commonly used to subtype several important foodborne pathogens, including *Salmonella, E. coli*, *L. monocytogenes*, and *Campylobacter*. Though the method is somewhat dated, serotyping still has utility, particularly in subtyping *Salmonella* and *E. coli*. *Salmonella* is a diverse genus with well over 2,400 unique serotypes (24). Because of this, *Salmonella* isolates are typically characterized initially by serotyping. In some cases, serotyping can detect outbreaks of rare *Salmonella* serotypes, especially when disease clusters of these serotypes are encountered (174). Within *E. coli*, serotyping is often used as an initial screen of isolates implicated in disease, as it has been shown that specific serotypes often correlate to the different pathotypes of *E. coli* (157). Though serotyping has proven useful for subtyping of *Salmonella* and *E. coli*, it is a relatively insensitive tool for subtyping of *L. monocytogenes* (202).

Phage typing characterizes bacterial isolates by their susceptibility to lysis by a standard set of phages (256). Due to the rapid nature of this method, phage typing has proven itself useful when screening a large number of sporadic isolates for cluster detection. Within the realm of foodborne pathogens, phage typing has perhaps been most extensively used to characterize *Salmonella* isolates beyond the serovar level (6, 7, 144, 214), and has successfully been used in outbreak investigations (88). Despite the rapidity of phage typing, the method suffers from considerable experimental variability, thereby making it extremely difficult to standardize phage typing between laboratories (202).

MLEE differentiates bacterial strains by assessing the charge differences in various alleles of constitutive enzymes. Cell extracts containing the soluble enzymes are separated based on size in non-denaturing starch gels, and enzyme activities are determined by color-generating substrates (256). MLEE has been used to assess genetic relationships among foodborne pathogens. For example, MLEE was used to assess the relationships between various pathotypes of *E. coli*. Using MLEE, the researchers concluded that pathogenic strains of *E. coli* had arisen not by a single evolutionary event, but through multiple independent events (192). A drawback of MLEE is the considerable difficulty in standardizing the method between laboratories. Additionally, MLEE has poor discriminatory power for outbreak investigations (202).

6.2 Molecular subtyping methods overview.

Molecular subtyping methods often provide improved discriminatory power as compared to phenotypic methods, and also offer a higher level of standardization between laboratories (256). As mentioned previously, molecular subtyping methods are divided into two categories: (i) band based methods and (ii) sequence based methods. Common band based methods include multiplex PCR, random amplified polymorphic DNA (RAPD), repetitive element PCR (rep-PCR), amplified fragment length polymorphism (AFLP), PCR-restriction fragment length polymorphism (PCR-RFLP), Pulsed field gel electrophoresis (PFGE), and ribotyping.

Multiplex PCR is a basic band based molecular subtyping method that involves the simultaneous amplification of multiple gene targets within a single PCR reaction (29). Multiplex PCR is a rapid, cost efficient, and repeatable method that can be used to efficiently screen for numerous genetic targets within the same sample. For example, Kawasaki and colleagues developed a sensitive multiplex PCR assay that allowed for simultaneous detection of *L. monocytogenes*, *S. enterica*, and *E. coli* O157:H7 in food enrichments (111). Multiplex PCR has also been successfully used to characterize isolates into their respective serogroups. For example, Doumith and colleagues developed a multiplex PCR based system that successfully identifies isolates of *L. monocytogenes* belonging to groups that differentiate the major human disease associated serotypes, including 1/2a, 1/2b, 1/2c, and 4b (56).

Random amplified polymorphic DNA (RAPD) is a PCR based method used to subtype bacterial isolates. However, RAPD differs from conventional PCR in that (i) short (usually 9- or 10-mers), arbitrarily designed oligonucleotides are used and (ii) relatively low annealing temperatures are used (262). This combination allows for amplification of random sections of DNA throughout the bacterial chromosome. The banding patterns can be viewed by gel electrophoresis and analyzed using computer software. RAPD has the advantage of being a very rapid technique that requires minimal equipment; all that is needed is simple PCR and gel imaging equipment that most molecular biology labs have access too. The main disadvantage of RAPD is the difficulty in reproducibility of the method. RAPD has been used in several outbreak investigations, including a 1996 outbreak of *E. coli* O157:H7 in Japanese primary schools (252).

Various repetitive DNA sequence elements are found universally in bacterial genomes. These repetitive elements are the target in subtyping methods based on rep-PCR. Primers homologous to these repetitive elements are designed, and amplification occurs when the elements are close together. The banding patterns produced can help infer genetic relatedness between strains. Rep-PCR is inexpensive and requires very basic equipment that most molecular biology labs have access to; however, it requires prior DNA sequence knowledge of an organism, due to the requirement of designing specific PCR primers. Rep-PCR has been successfully used to characterize a hospital outbreak of *S.* Infantis (106), as well as for subtyping *Vibrio parahemolyticus* strains that caused an outbreak associated with oysters in Canada (142).

Amplified fragment length polymorphism (AFLP) is a method of subtyping that combines restriction digestion of total genomic DNA with selective PCR to characterize bacterial isolates (19). The procedure involves the restriction of purified genomic DNA by using one or more endonucleases of varying cutting frequency. Special double stranded oligonucleotide adapters are then ligated to the digested DNA fragments, and a small number of PCR amplification rounds are performed. PCR primers are then added to the mixture that recognizes the adapter sequences. After PCR amplification, a number of fragments are generated, and visualization of banding patterns is typically performed using polyacrylamide gel electrophoresis. AFLP is useful in that it is broadly applicable, provides high discriminatory power, permits whole genome polymorphism analysis, and does not require prior knowledge of sequence information (29). AFLP has been successfully used to characterize a number of foodborne pathogens, including *L. monocytogenes*, for which an automated, highly discriminatory method using two enzymes was developed (1).

PCR followed by restriction fragment length polymorphism analysis (PCR-RFLP) is a useful subtyping tool that involves PCR amplification of a gene product followed by endonuclease digestion of the amplicons with one or more enzymes (71). The discriminatory power of PCR-RFLP depends on (i) the site chosen for PCR amplification (i.e., how variable the gene is) and (ii) the enzymes and number of enzymes chosen for restriction digest (29, 71). PCR-RFLP has proven very useful for subtyping of foodborne pathogens. For example, PCR-RFLP was used to group *L. monocytogenes* isolates into specific lineages and also grouped the isolates based on their pathogenic potential (259). PCR-RFLP analysis of *stx* genes carried by STEC has also been used for epidemiological investigations (264). PCR-RFLP has two main disadvantages, including (i) the need for prior DNA sequence knowledge of the organism of interest, and (ii) the inability of the method to interrogate the whole genome (29).

Pulsed field gel electrophoresis (PFGE) has often been cited as the "gold standard" in subtyping methods due to its high discriminatory power (94). The utility of the method was first demonstrated during the investigation of a large outbreak of *E. coil* O157:H7 in ground beef in the U.S. Pacific Northwest (14). After this investigation, the CDC chose PFGE as the subtyping method for PulseNet, the national network for surveillance of foodborne diseases (225). PFGE methods are designed to restrict total DNA (i.e., both chromosomal and plasmid DNA) using rare cutting enzymes that produce 10 to 20 bands between 10 to 800kb (71). Because large fragments of chromosomal DNA are susceptible to shearing and fragmentation, the DNA is often embedded in an agarose gel “plug” which confers protection against shearing. Once embedded within the plug, enzymes such as lysozyme and proteinase-K are used in conjunction with detergents to remove unwanted cellular constituents other than DNA. The agarose plugs are then subjected to restriction digest using rare cutting enzymes such as *Xba*I, *Asc*I, and *Apa*I. Because the sizes of the DNA fragments are too large to separate in standard agarose gel electrophoresis, PFGE depends on periodic inversion of the electric field to allow for migration of the large fragments of DNA. The most common method of electric field inversion in use today is the contour-clamped homogeneous electric field electrophoresis (CHEF) method, which uses a hexagonal array of electrodes that generate electric fields at 120° to each other (45). This allows for DNA fragments to move in a straight line with no distortion. Resulting patterns produced by PFGE are analyzed by computer software. Generally, patterns that differ by 3 or fewer bands are considered genetically related (227), although sound epidemiological evidence needs to in-place before drawing conclusions about a causal relationship between a PFGE type and an outbreak of foodborne illness (256). Standardized and validated PulseNet protocols for various foodborne pathogens have been developed, including *Campylobacter jejuni*, *E. coli* O157:H7, non-0157 STEC, *L. monocytogenes*, *Salmonella*, *Shigella,* and *Yersinia pestis*. PulseNet is perhaps the greatest example of the utility of PFGE, as it allows for public health labs and federal agencies to deposit and rapidly compare isolates from human patients in order to detect nationwide foodborne outbreaks and to trace the outbreak to a specific food product during a subsequent outbreak investigation

(225). Drawbacks of PFGE include the relative high cost of equipment, the need for specially trained personnel, and the laborious and time-consuming nature of the method (29).

Ribotyping is a method of subtyping that interrogates the ribosomal RNA subunits (rRNA) of bacteria. In prokaryotes, the genes encoding the rRNA subunits of the ribosome (5S, 16S, and 23S) are highly conserved and are clustered together in 5-8 operons throughout the genome (116). Because the ribosome is indispensible for cell survival, rRNA genes are stable and evolve stably over time, making them an excellent target for phylogenetic and subtyping analyses in prokaryotes. In ribotyping, bacterial DNA is initially restricted using one or more frequent cutting enzymes (e.g., *Eco*RI), which digest the DNA into many (>300) fragments ranging in size from 2 to 20kb (256). The fragments are separated by size by agarose gel electrophoresis and transferred to a nylon membrane. Next labeled rRNA probes from *E. coli* are hybridized to the digested fragments carrying rRNA genetic material from the isolate being characterized and a resultant banding pattern image is captured. The automated Riboprinter system marketed by DuPont Qualicon is a popular subtyping system used when high throughput is needed, as the system is capable of subtyping over 24 isolates in under 24h (256). Ribotyping has been used to identify and subtype a variety of foodborne pathogens, including *C. jejuni*, *Salmonella* serotypes, *Shigella*, *L. monocytogenes,* and *E. coli* (71).

Fragment pattern ambiguity encountered in many methods of band-based subtyping can make interpretation of results difficult and ambiguous. DNA sequence based subtyping methods may offer increased discriminatory power and lessened ambiguity versus band-based methods thanks to its ease of interpretation. Common DNA sequence based methods used to subtype foodborne pathogens include multi locus sequence typing (MLST), multi locus variable number tandem repeat analysis (MLVA), genomic sequencing, microarrays, and single nucleotide polymorphism (SNP) typing.

Multi-locus sequence typing (MLST) is a DNA sequence typing method that is very similar in concept to MLEE; however, unlike MLEE, MLST assigns allelic types based on gene sequence data rather than electrophoretic mobility of enzymes (138). In MLST, internal fragments of about 450-600bp in five to 10 genes are amplified and sequenced. The unique combination of polymorphisms in each gene is used to assign distinct alleles or allelic types, and each unique combination of allelic types is used to assign multi locus sequence types (138). Housekeeping genes are often chosen for MLST schemes since these genes evolve slowly over time primarily by point mutations, thus providing reliable information on genetic relatedness between isolates (65). Since it is based on nucleotide sequencing, MLST typically exhibits much greater discriminatory power than is provided by band based methods such as PFGE (65). Another advantage of MLST is the ease of which the data generated can be shared via the Internet. Online databases such as the Institut Pasteur MLST Database (http://www.pasteur.fr/recherche/genopole/PF8/mlst), EcMLST database (www.shigatox.net/mlst) and MLST.net (http://www.mlst.net) are currently used and help facilitate worldwide collaboration between laboratories (65). The discriminatory power of MLST can be increased through the inclusion of virulence genes, as these genetic elements evolve more rapidly than housekeeping genes. A disadvantage of MLST is that it does not provide good discriminatory power for recently emerged and thus highly clonal organisms, such as *E. coli* O157:H7 (165).

Bacterial chromosomes often contain DNA elements that are repeated in tandem (226). These tandem repeat regions, called variable nucleotide tandem repeats (VNTRs), are the target for multi locus variable number tandem repeat analysis (MLVA) (99). During several genomic sequencing projects, researchers noticed that different strains carried different copy numbers of VNTRs. DNA regions containing VNTRs are highly variable as recombination events and polymerase errors are likely to occur in these regions (100). MLVA uses PCR to amplify these VNTRs located throughout a bacterial genome. Visualization via gel electrophoresis allows for analysis of the size of the VNTR amplicon, which provides the discriminatory power of the method. MLVA is particularly suited for subtyping of highly clonal organisms, such as *E. coli* O157:H7 (130), since the method interrogates highly variable genetic elements. MLVA has been shown to provide higher discriminatory power than standard *Xba*I PFGE analysis of *S.* Typhimurium isolates (131). MLVA has also been used to genotype *C. jejuni* isolates (241). A drawback of MLVA is that it requires genomic sequence data to identify and design primers for VNTRs. However given the explosion of genomic sequencing projects within the previous five years, it is expected that MLVA may be more involved with outbreak investigations in the future.

The next generation sequencing technologies that have emerged in recent years have significantly reduced the amount of labor required to sequence a full bacterial genome and drastically increased the throughput and amount of data generated from an individual sequencing run (265). Since the data generated from these technologies represent the entire DNA coding sequence of an organism, these technologies represent the ultimate tool for genetic analysis. The utility of next generation sequencing to the realm of bacterial subtyping is vast; not only will genome sequencing help to create new and improved subtyping technologies, it may also find utility as a highly discriminatory subtyping method for clonal organisms (265). As the cost of next generation sequencing technologies continues to drop, the ability to use these methods for routine epidemiological studies will become feasible, thus allowing us to use the full power of these methods for outbreak studies (158). Multiple genomes for several foodborne pathogens have already been sequenced, including *E. coli* O157:H7, *L. monocytogenes*, and *S. enterica*; many other foodborne pathogens are currently the focus of sequencing projects (260). Whole genome sequencing was recently used to characterize an outbreak foodborne illness attributed to consumption of bean sprouts contaminated by *E. coli* O104:H4 in Germany (148, 210). The authors of this study used next generation sequencing technology to characterize both an outbreak strain of O104:H4 and a historic strain; thus the characterization represents one of the first documented instances of whole genome sequencing used as a tool during an outbreak investigation (148). The use of genomic sequencing as an investigative tool in this instance allowed researchers to identify genetic targets for diagnostic assays that helped narrow down cases associated with this outbreak. The authors concluded that due to stepwise gain and loss of chromosomal and plasmid-encoded virulence factors, a highly pathogenic hybrid of EAEC and EHEC emerged as the outbreak clone in Germany (148).

A single nucleotide polymorphism (SNP) is a DNA sequence variation that occurs when a single nucleotide within a genetic element differs between taxa of interest (69). Since SNPs can provide useful information regarding the genetic relatedness of strains, these sites are often targeted for development of subtyping assays. Various SNP typing methodologies have been developed, and an in depth review by Sorbrino and colleagues discuss these at length (222). SNP typing methods have been used to characterize many foodborne pathogens. For example, Van Stelten and colleagues developed a SNP typing assay using single base pair extension chemistry to detect premature stop codons in *L. monocytogenes* virulence protein InlA (243). In addition, Octavia and colleagues developed an SNP typing method based on PCR-restriction enzyme digestion that is able to provide useful discriminatory power for the clonal *Salmonella enterica* serovar Typhi (168). Though primarily used in academic settings, SNP typing is beginning to be used more often in outbreak investigations and epidemiologic studies.

Though subtying data has value to an individual researcher, this value can be greatly enhanced when data can be shared among a large number of entities in a timely and efficient manner. Online databases achieve these goals and greatly enhance the utility of subtyping. To be useful, these databases need to provide a standardized analytic framework from which data can be stored and shared. PulseNet, the molecular surveillance network for foodborne pathogens in the United States, is a highly standardized surveillance database and is known for its optimization and national standardization of PFGE for detection of foodborne pathogen outbreaks (225). The database includes PFGE data from standardized protocols for many foodborne pathogens, including *C. jejuni*, *E. coli* O157:H7 and non-O157 pathogenic *E. coli*, *C. botulinum, L. monocytogenes, Y. pestis, V. cholera,* and *V. parahemolyticus.* PulseNet is highly standardized and laboratories are required to be certified before they can submit samples and access the database (225). PulseNet has become indispensible as it has the capacity to quickly detect nationwide foodborne pathogen outbreaks. PathogenTracker (www.pathogentracker.net) is another commonly used database. However, unlike PulseNet, PathogenTracker is a publicly available database for use to researchers, the food industry and public health agencies and contains over 34,000 isolate depositions to date. PathogenTracker allows for confidential isolate submission by entities that wish to remain anonymous. The goal of PathogenTracker is to encourage worldwide exchange of subtype data for strain collections between laboratories in order to facilitate studies on biodiversity of bacterial species.

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CHAPTER II

Molecular Epidemiology of *Listeria, Salmonella, Escherichia coli* O157:H7, and non-O157 Shiga Toxin Producing *E. coli* in Northern Colorado Primary Production Agricultural Environments

Chapter II Summary

Listeria monocytogenes, Salmonella, and Shiga toxin-producing *Escherichia coli* (STEC) represent clinically important human foodborne pathogens. The presence, transmission, and molecular epidemiology of these pathogens in primary agricultural environments (i.e., grazing pastures and produce farms) is understudied and represents a significant knowledge gap. We conducted a two year longitudinal sampling study in Northern Colorado to assess the presence, genetic diversity, and potential transmission patterns of these pathogens in production agriculture environments. Five produce farms and five grazing pastures in Northern Colorado were selected and sampled during the spring, summer, and fall in 2009 and 2010. Microbiological analysis was performed on composite soil (228 samples), composite fecal (105 samples), composite water (80 samples), and individual drag swabs (227 samples) 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 microorganism using modified versions of the U.S. Food and Drug

Administration *Bacteriological Analytical Manual* and presumptive colonies for each target organism were confirmed by polymerase chain reaction (PCR). Overall, eight (1.25%), eleven (1.7%), and 22 (3.4%) of samples tested positive for *L. monocytogenes, Salmonella,* and STEC, respectively. Thirty-one (4.8%) samples contained *Listeria* spp. other than *L. monocytogenes*, the majority of which were determined to be *Listeria innocua* by *sigB* allelic typing. Additionally, 20 samples were positive for a proposed novel *Listeria* spp. that displayed unique phenotypic traits, i.e., non-motility and impaired growth at 37°C. Pulse field gel electrophoresis analysis indicated isolates obtained in this study to exhibit significant genetic diversity. These results demonstrate a rare presence of foodborne pathogens in agricultural environments in Northern Colorado. Prevalence and molecular subtyping data generated in this study provides important insight into the molecular epidemiology of these pathogens in the pre-harvest agriculture environment.

Introduction

Foodborne illness places a considerable public health and economic burden on the United States (U.S.) population. Recent studies published by The Centers for Disease Control and Prevention (CDC) estimate 1 in 6 Americans (or 48 million people) become ill from consuming contaminated food, 128,000 are hospitalized, and 3,000 die of foodborne diseases caused by both known pathogens and unspecified agents each year (77, 78). The economic burden of foodborne illness is also great, with one recent estimate suggesting the total annual health related cost of foodborne illness in the U.S. to be as high as $152 billion (79). Estimates of bacterial foodborne illness suggest that *Listeria monocytogenes*, *Salmonella*, *Escherichia coli* O157:H7 and non-O157 Shiga toxin-producing *Escherichia coli* (STEC) are responsible for over 75% of domestically acquired bacterial foodborne illnesses and deaths (77). More specifically, *L. monocytogenes* annually causes an estimated 1,591 foodborne disease cases and 255 deaths; Non-typhoidal *Salmonella* annually causes an estimated 1.03 million foodborne disease cases and 378 deaths; *E. coli* O157:H7 annually causes an estimated 63,153 foodborne disease cases and 20 deaths; and non-O157 STEC annually causes an estimated 112,752 foodborne disease cases (data for annual deaths attributed to non-O157 STEC is unavailable) (77).

In response to the significant burden caused by these microorganisms, aggressive efforts to reduce their entry into the U.S. food supply have been made. For example, the Hazard Analysis Critical Control Point (HACCP) program, a systematic approach to controlling foodborne pathogens at the processing level, is required by The United States Food and Drug Administration (FDA) and The United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) for producers of numerous food products, including seafood, juices, meat, and poultry (93, 95). Since 1996, The Foodborne Diseases Active Surveillance Network (FoodNet) has monitored the public health impact of the 1996 USDA HACCP regulations (6). FoodNet monitors laboratory-confirmed cases of major foodborne bacterial and parasitic pathogens through active surveillance in ten states. Data generated by FoodNet showed an initial decline in illness by major bacterial foodborne pathogens (i.e., *Salmonella, L. monocytogenes,* and *E. coli* O157) after implementation of the 1996 USDA regulations. However, this steady decline has since halted in more recent years (6). In addition, in the CDC FoodNet annual report on foodborne illness for 2008, the most recent year for which the information is complete, *L. monocytogenes, Salmonella,* and STEC cases had increased in 2008 compared to data from 2003 to 2007 (17). These data suggest that additional research is needed to further reduce the entry of these microorganisms into the U.S. food supply.

Molecular subtyping methods such as pulsed field gel electrophoresis (PFGE) are important tools in determining the molecular epidemiology of pathogens. Large subtyping databases such as the CDC PulseNet (87) and PathogenTracker (www.pathogentracker.net) help facilitate outbreak detection and investigations, as well as contribute to increased understanding of the complex nature of foodborne pathogens. These databases contain a large amount of molecular subtyping data for foodborne pathogens isolated from human clinical cases, foods, and farm animals. However, environmental isolate subtype data is underrepresented in these databases. The routine inclusion of molecular subtype data for environmental isolates could greatly enhance understanding of the transmission dynamics and molecular ecology of foodborne pathogens facilitating the utility of subtyping for microbial source tracking.

Production agriculture environments (i.e., produce fields and cattle grazing pastures) are an important component of the human food continuum. A 2006 multistate outbreak of *E. coli* O157:H7 attributed to fecal contamination of spinach fields in California by feral hogs resulted in 205 reported cases of foodborne illness and three deaths (43). Nearly fifteen percent of swine fecal samples in the area tested positive for the outbreak clone of *E. coli* O157:H7. Also, soil, water, and sediment samples were positive in the same area (43). In addition, a 2008 U.S. outbreak of *Salmonella* Saintpaul linked to raw jalapeno peppers sickened over 1,500 individuals in 43 states (10). The outbreak strain was detected in agricultural water and serrano peppers on a Mexican farm, suggesting an environmental source of product contamination (10). Together, these outbreaks emphasize the potential for foodborne pathogens in production agriculture environments to contribute to human illness.

Numerous studies have investigated the environmental presence of STEC, *L. monocytogenes* and *Salmonella* in livestock production environments (7, 22, 24, 25, 29, 39, 45, 46, 57, 66, 68, 91). However, many of these studies have been limited in scope. Given the fact that cattle feces have been implicated as a major source of pathogenic *E. coli* (28), nearly all studies investigating foodborne pathogens in livestock production systems have focused on enteric pathogens such as *E. coli* O157:H7 (23, 29, 33, 48), non-O157 STEC (7, 39, 91) or *Salmonella* (22, 25); few studies have investigated *L. monocytogenes* (57) in these environments. Furthermore, subtype specific studies of beef production systems have almost exclusively focused on intensively managed beef feedlot and dairy operations (24, 29, 45, 68); few studies have investigated extensively managed range or pastured cattle operations (45, 46, 66). Overall, molecular subtyping studies examining these four bacterial pathogens using longitudinal sampling schemes (i.e., repeated samplings over time) in livestock pasture settings are scarce, resulting in a considerable knowledge gap in the understanding of these bacterial pathogens in the food production continuum.

In recent decades, foodborne outbreaks associated with consumption of fresh produce in the United States have increased and is likely associated with an increased per capita consumption these foods than in previous years (81). Produce contamination probabilities are highest during three stages of production: pre-harvest (on the farm), initial processing, and consumer preparation and handling (51). The interaction among pathogens and produce in farm environments are complex, and contamination from a variety of sources have been documented, including wildlife feces (43), contaminated irrigation water (84), and contaminated compost (41). Studies investigating the presence, distribution, and diversity of bacterial foodborne pathogens in pre-harvest produce environments primarily focus on *E. coli* O157:H7 (19) and *Salmonella enterica* (30). Studies examining non-O157 STEC or *L. monocytogenes* in pre-harvest produce environments are minimal, despite recent outbreaks being attributed to these pathogens in produce (15, 16). Thus, a knowledge gap concerning the presence, transmission, distribution, and diversity of these two important pathogens in pre-harvest produce environments exists.

To address this knowledge gap concerning foodborne pathogens in production agriculture environments, the objectives of this study were to (i) conduct a two year longitudinal sampling study to probe the presence and distribution of *L. monocytogenes*, *Salmonella*, *E. coli* O157:H7, and non-O157 STEC in the environment of grazing pastures and produce farms in Northern Colorado, (ii) characterize isolates from these environments by molecular subtyping, and (iii) contribute to the PathogenTracker database by providing molecular epidemiology data for foodborne pathogen isolates from production agriculture environments in Northern Colorado. Results from this study further elucidate our understanding of prevalence, genetic diversity, and potential for transmission of these pathogens in production agriculture environments.

Materials and Methods

Sample collection. Five produce farms (coded PF1 through PF5) and five grazing pastures (coded GP1 through GP6) in northern Colorado were used as sampling locations in a two-year longitudinal sampling study (Tables 2.1 and 2.2). Each location was sampled during spring, summer, and fall of 2009 and 2010 for a total of six samplings per location. One location (GP1) containing a Colorado State University research herd was removed from the study after summer 2009 due to cattle being transported out of state for an extended period of time. A replacement (GP6) location was identified for the 2010 season. Sampling collection areas were selected to represent geographically distinct areas (i.e, separate fields on produce farms or separate fenced areas on livestock grazing pastures) of approximately 1,000m^2. During each sample collection, five soil, up to five water, five fecal, and one surface soil (drag swab) were collected from each of the four areas in a given location. A hand-held global position satellite (GPS) device (Magellan MobileMapper CX, Santa Clara, California) was used to record GPS coordinates so that each sampling site could be resampled (within the sensitivity limits of the GPS unit, or <1m) during subsequent visits. Fecal samples were not collected from produce farms, as no samples of suitable size for collection and analysis were available. Soil and fecal samples were collected into sterile Whirl-Pak bags (Nasco, Fort Atkinson, WI) using sterile plastic scoops (Fisher Scientific, Hampton, New Hampshire). Surface soil samples were collected using the methods of Uesugi et al. (92), where sterile gauze pads pre-soaked in sterile phosphate buffered tryptic soy broth (pTSB; Becton, Dickinson and Company, Sparks, MD; Fisher) media were dragged along the ground in a figure-eight pattern for five to ten minutes, and then placed into sterile Whirl-Pak bags. Water samples were collected directly into sterile Whirl-Pak bags. All sample bags were stored in coolers with ice packs for transport. Samples were processed within 24 hours of collection.

Sample processing and non-selective pre-enrichment. Composite samples created by pooling the five samples taken from an individual area were prepared. Specifically, composite soil and fecal (25 g; 5 g from each respective sample type collected from the same area) were combined into duplicate sterile filter Whirl-Pak bags for non-selective enrichment of either gram-positive or gram-negative target microorganisms. Composite water samples were prepared by passing a 50 ml aliquot of each of the five water samples collected from an area over the same filter (0.45 μm; Nalgene, Rochester, NY). This filter was then aseptically divided into three equal sections and transferred into three sterile filter Whirl-Pak bags. Drag swab samples were homogenized by hand and a 10 ml aliquot of the pTSB media used to presoak drag swab samples was transferred into each of three sterile filter Whirl-Pak bags. Composite soil and composite fecal samples were combined with 225 ml (1:10 dilution) of either pTSB (for gram-negative isolation) or buffered *Listeria*

Enrichment Broth without antimicrobials (BLEB, Difco, Sparks, MD; for gram positive isolation) for pre-enrichment. Two of the Whirl-Pak bags containing a water filter section received 90 ml of pTSB, while the remaining bag received 90 ml of BLEB. Similarly, two of the Whirl-Pak bags containing 10ml pTSB aliquots of drag swab samples received 90 ml of pTSB, while the remaining bag received 90 ml of BLEB, each creating a 1:10 dilution. All bags were homogenized by hand for one minute after addition of enrichment broth in order to minimize the risk of bag puncture.

Sample bags containing pTSB samples were incubated at 25°C for two hours to allow for recovery of injured cells. After two hour incubation, composite soil and composite fecal samples were homogenized by hand and divided by transferring half of the contents of each bag (based on enrichment weight) into a new sterile Whirl-Pak filter bag for selective enrichment of either *Salmonella* or STEC. After division, pTSB bags were transferred into either a 35°C (for *Salmonella* and non-O157 STEC) or a 42°C (for *E. coli* O157:H7) incubator for a six hour non-selective pre-enrichment step prior to transfer into selective media. These methods were evaluated after the first year (data not shown) and incubation time for pre-enrichment were each increased to 24 hours during the 2010 sampling in order to facilitate *Salmonella* and STEC detection. Sample bags containing BLEB were subjected to a pre-enrichment step by incubating for four hours at 30°C. For each sample, this initial processing resulted in three bags for further processing using selective incubation times and enrichments for *Listeria, Salmonella,* and STEC (i.e., separate protocols for *E. coli* O157:H7 and non-O157 STEC). One colony from positive control strains was inoculated into an enrichment bag for each protocol and processed in parallel with sample bags. Positive control strains included *Salmonella* Typhimurium and *E. coli* O157:H7, each with green fluorescent protein inserts (59), an O111 serotype non-O157 STEC (Michigan State STEC Reference Center), and *L. monocytogenes* (11). Negative controls were simply sterile enrichment media that were processed in parallel with sample bags.

Detection and Isolation of *Listeria*. Isolation of *L. monocytogenes* and other *Listeria* spp. from samples was performed as previously described (57, 75). After incubation for four hours at 30°C, *Listeria* Selective Enrichment Supplement containing acriflavin (22.5mg/L), sodium nalidixate (90mg/L), and cycloheximide (112.5mg/L) (Oxoid Ltd, Cambridge, England) was added to BLEB bags. Samples were then incubated for 48 hours at 30°C. After 24 and 48 hours of selective BLEB enrichment, 100 µl of each sample was streaked onto modified Oxford agar (MOX; BD Difco, Franklin Lakes, NJ) and *L. monocytogenes* plating medium (LMPM; Biosynth International, Itasca, IL). MOX plates were incubated at 30°C for 48 hours, while LMPM plates were incubated at 35°C for 24 hours. Up to four colonies displaying *Listeria* morphology on MOX plates (grey-green crater-like colonies with black halo) were sub-streaked onto LMPM plates and incubated at 35°C for 24 hours. Up to four colonies displaying typical *L. monocytogenes* morphology on LMPM plates (blue colonies) and up to two colonies displaying typical *Listeria* spp. morphology on LMPM plates (white colonies) were sub-streaked onto brain heart infusion agar (BHI; BD Difco, Franklin Lakes, NJ) prior to phenotypic and PCR confirmation.

Detection and Isolation of *Salmonella*. Isolation of *Salmonella* was performed using a modified version of the procedures outlined in the US Food and Drug Administration *Bacteriological Analytical Manual* (94). After non-selective pre-enrichment, a 0.1 ml and 1.0 ml aliquot of pre-enriched samples was added to 10 ml Rappaport Vassiliadis (RV; Oxoid; Fisher; Acros Organic, Belgium) broth and 10 ml tetrathionate (TT; Oxoid) broth, respectively. RV and TT broth tubes were both incubated at 42°C for 24 hours in a water bath, after which a 100 µl aliquot of each selective enrichment was plated onto xylose desoxycholate (XLD; Neogen, Lansing, MI) agar. During the second year, selective enrichments were also plated onto CHROMagar *Salmonella* agar (CHROMagar, Paris, France), in addition to XLD agar. Both XLD and CHROMagar *Salmonella* plates were incubated at 35°C for 24 hours. Up to four colonies representing typical *Salmonella* morphology on XLD (black precipitate with red background) and CHROMagar *Salmonella* (purple colonies) were sub-streaked onto BHI agar prior to phenotypic and PCR confirmation.

Detection and Isolation of *E. coli* O157:H7. Isolation of *E. coli* O157:H7 from samples was performed as previously described (8, 9) and as implemented by our group (14). After non-selective pre-enrichment, a 1ml aliquot of pTSB was used for immunomagnetic bead separation (IMS) as previously described (8), ultimately resulting in 50 µl aliquots of each sample plated onto modified sorbitol MacConkey agar (mSMAC; Becton, Dickinson and Company, Sparks, MD) and also CHROMagar O157 (second year only; CHROMagar Paris, France). Both mSMAC and CHROMagar O157 were supplemented with 20 mg/L of novobiocin (Sigma-Aldrich, St. Louis, MO) and 2.5 mg/L of potassium tellurite (Sigma). Both mSMAC and CHROMagar

O157 plates were incubated at 37°C for 36 hours. Up to four colonies representing typical *E. coli* O157:H7 morphology on mSMAC (round with no pink halo) and CHROMagar O157 (purple colonies) were sub-streaked onto BHI agar prior to phenotypic and PCR confirmation.

Detection and Isolation of non-O157 STEC. After the initial pre-enrichment step, a 1 ml aliquot of each enrichment broth was added to 9 ml *E. coli* broth (EC broth; Oxoid) tubes and incubated at 37°C for 24 hours in a water bath. After incubation, a 100 µl aliquot of EC broth was plated onto washed sheep's blood agar (WBMA; Becton, Dickinson and Company; Remel, Lenexa, KS) supplemented with $CaCl_2$ (10 mM) (Acros Organics, Belgium) and 0.5 µg/ml mitomycin C (Fisher). This media is designed to enhance enterohemolysin production in STEC strains, thus making it easier to identify presumptive non-O157 STEC (86). WBMA plates were incubated at 35°C and evaluated for enterohemolysis at six and 24 hours. After evaluation, up to 20 colonies that demonstrated enterohemolysis were sub-streaked onto sorbitol MacConkey agar (SMAC; Becton, Dickinson and Company), and up to 8 colonies that rapidly ferment sorbitol on SMAC were sub-streaked onto BHI agar prior to phenotypic and PCR confirmation.

PCR confirmation. For each target microorganism, up to four colonies representing presumptive positives were selected for PCR confirmation using assays designed to detect unique DNA sequences in each microorganism (table 2.3). To facilitate high throughput screening of presumptive target organisms, the colony PCR method, which uses intact cells as a source of DNA template, was used (80). A small portion of each colony to be screened was placed into the bottom of either a sterile PCR reaction tube or a sterile 96 well plate with a sterile toothpick. Plates or tubes were then microwaved at 1000W for either 30 seconds for gram-negative organisms, or four minutes for gram-positive organisms to facilitate release of DNA from the cell.

Confirmation of *Listeria* spp. and *L. monocytogenes* was performed using previously described primers and thermocycling conditions targeting fragments of the genes *sigB* and *hly*, respectively (58, 60). Confirmation of presumptive *Salmonella* colonies was performed using previously described primers and thermocycling conditions targeting fragments of the *invA* gene from *S. enterica* (44). We defined a STEC isolate as an isolate that was biochemically confirmed to be *E. coli* and contained one or more Shiga toxin genes. Confirmation of STEC (both O157 and non-O157) isolates was performed using a six-gene multiplex PCR based off the methods of Hu et al (36). The original PCR assay, which contains 5 gene targets (*eaeA, stx-II, stx-I, flich7, rfbE*), was modified through the addition of primers 0.2 μM targeting *hlyA* (See Table 2.3). Additionally, all presumptive non-O157 STEC isolates (i.e., those that carried one or both Shiga toxin genes but not *eaeA*, which is specific to O157) were also screened using both a previously described PCR assay to detect a 541 bp fragment of *eae* conserved among non-O157 STEC (Online protocol at http://www.shigatox.net/stec/cgi-bin/stx-eae ,Thomas S. Whittam STEC Center, Michigan State University, East Lansing, MI) and a previously described PCR assay to detect a 932 fragment of *mdh* (Online protocol at http://mlst.ucc.ie/mlst, University College, Cork, Ireland). For each PCR, a 25 μl PCR reaction mixture containing primers, 1x GoTaq Green Master Mix, and nuclease-free water was prepared and dispensed into tubes or a 96 well plate containing DNA template. After thermocycling, PCR products were separated by gel electrophoresis in either 1.5% agarose gels (*invA, sigB,* and *hly*) or 2.0% agarose gels (six gene *E. coli* multiplex PCR), stained with ethidium bromide, and visualized using UV illumination. Gel images were captured and recorded using a FOTO/Analyst Investigator system (FOTODYNE, Inc., Hartland, WI).

Phenotypic confirmation. Due to wide genetic diversity of Non-O157 STEC isolates, biochemical confirmation using the API-20E (bioMérieux, Marcy-l'Etoile, France) system according to manufacturer's instructions was performed before further molecular characterization of these isolates. Results from API-20E strips confirmed all presumptive non-O157 STEC isolates to belong to *E. coli* above 80% identity.

Serotyping. Conventional slide agglutination was performed by the National Veterinary Service Laboratory (Ames, Iowa) or the *E. coli* Reference Center (Pennsylvania State University; State College, PA) on confirmed *Salmonella* and non-O157 STEC isolates, respectively. Confirmed *L. monocytogenes* isolates were grouped into one of four serogroupes containing one of four serotypes commonly associated with human listeriosis (i.e., 1/2a, 1/2b, 1/2c and lineage I 4b) as detailed by Doumith et al. (21).

DNA sequencing. Identification of *Listeria* isolates at the species level was performed by *sigB* sequencing and allelic typing using previously described primer sets and reaction conditions (58). In addition, comparison of *sigB* sequences with nucleotide sequences available in the GenBank database using the BLASTN program available through the National Center for Biotechnology Information server was performed (2). These methods have been shown to be concordant with API *Listeria* strips (bioMérieux) for *Listeria* identification (74, 97). For non-O157 STEC, PCR amplification and DNA sequencing of the *mdh* gene with previously described primers and conditions (13) was performed. BLASTN comparison of *mdh* DNA sequences with those available in the GenBank database served as an additional means of confirmation for presumptive non-O157 STEC isolates. Purification of all PCR products prior to DNA sequencing was performed using either the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA) or the GenElute PCR purification Kit (Sigma-Aldrich, St. Louis, MO) according to manufacturer's instructions. DNA sequencing was performed using Big Dye Terminator chemistry and AmpliTaq-FS DNA polymerase at Colorado State University's Proteomics and Metabolomics Facility (Fort Collins, CO). Sequences were assembled and proofread using SeqMan (Lasergene 7; DNAStar, Madison, WI) and aligned using MegAlign (Lasergene 7; DNAStar) using the Clustal W algorithm. Individual *sigB* sequences were trimmed to 660 nucleotides before further analysis. DnaSP software (70) was used to assign allelic types in sequences with one or more polymorphisms. A single isolate representing each unique *sigB* allelic type was used for phylogenetic analysis. Phylogeny was inferred using the Neighbor-Joining method with 100 bootstrap replicates (72). The Tajima-Nei model for nucleotide substitution was chosen and was modeled with a gamma distribution (88). The analysis involved 177 total *sigB* sequences with 660 nucleotide positions each. All evolutionary analyses were conducted using MEGA5 (89).

Pulsed field gel electrophoresis. PFGE typing using the standardized CDC

PulseNet protocols (32, 67) for *L. monocytogenes, Salmonella, E. coli* O157:H7, and non-O157 STEC was performed on all confirmed isolates from each positive sample. Briefly, bacterial isolates were grown on BHI agar plates at 37°C for 18 hours, embedded 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 up to 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* and *E. coli* O157:H7*. XbaI* digested *Salmonella* ser. Braenderup (H9812) DNA was used as a reference size standard in each gel (37). Agarose gels were stained in ethidium bromide for 40 minutes, destained in ultrapure water for a minimum of two hours, and resultant PFGE images were captured. Analysis of PFGE data was performed using BioNumerics (Applied Maths, version 5.10, Saint-Matins-Latem, Belgium) software. Similarity clustering analyses was performed using the unweighted pair group-matching algorithm and the Dice correlation coefficient as detailed previously (37). Isolates were considered genetically related if the number of observed band differences was three or less (90).

Genomic sequencing of novel *Listeria* isolates and phylogenetic analysis. The full genome sequence of a single representative isolate of the novel *Listeria* spp. was determined using ABI SOLiD™ 4 instrumentation located in the Colorado State University Infectious Disease Research Center Next Generation Sequencing Core (Fort Collins, CO). Sequence assembly was performed using SOLiD de novo pipeline software (Life Technologies, Inc., Carlsbad CA). Automated annotation of assembled sequences was performed using the Rapid Annotations using Subsystems Technology (RAST) automated service (5). The evolutionary position of these novel *Listeria* isolates within the phylum Firmicutes was determined by creating an alignment of 31 concatenated amino acid sequences (totaling 7,149 aa) of housekeeping genes from 48 representative Firmicutes in MacClade 4.08 (53, 98). RAXML was used to perform maximum likelihood phylogenetic analysis using 100 bootstrap replicates (83).

Data analysis and statistical methods. Prevalence estimates were calculated by dividing the number of samples with positive results for each pathogen by the total number of samples tested. Differences in prevalence across month of isolation were determined by the use of logistic regression. All statistical analyses were conducted with Statistical Analysis Systems software (version 9.2, SAS Institute, Cary, N.C.). The significance level for all statistical tests was $P < 0.05$, and marginally significant was $P < 0.10$.

Results

***L. monocytogenes* prevalence, distribution, and genetic diversity.** Overall, *L. monocytogenes* was detected in eight (1.25%) samples in this study, yielding a total of 27 isolates for our collection. The prevalence of *L. monocytogenes* did not significantly differ across sampling seasons ($P = 0.22$; Figure 2.12). Characterization by PFGE using *AscI* and *ApaI* yielded 8 distinct patterns among 26 typeable isolates (one isolate was deemed untypable; Figure 2.13). Only one PFGE subtype was present in each *L. monocytogenes* positive sample.

On produce farms, two composite soil samples (1.7%), two composite water samples (5.1%), and two drag swab samples (1.7%) were positive for *L. monocytogenes* (Table 2.4). The two composite soil samples yielding *L. monocytogenes* were from two different produce farms (i.e., PF1 and PF4), where the two positive drag swab samples were also obtained from the same area on each of these farms (Figures 2.1 and 2.4). However, PFGE determined these isolates to be genetically unrelated as each PFGE pattern differed by more than 3 bands (Figure 2.13). *L. monocytogenes* positive composite water samples were identified on farms PF4 and PF5 that were located within 6 miles of each other and share the same water source (Fulton canal) for irrigation (Figures 2.4 and 2.5). Isolates from these two water samples were determined to be closely related (differed by 3 bands) based on PFGE typing (Figure 2.13).

On grazing pastures, *L. monocytogenes* was isolated from two composite water samples (4.9%; Table 2.4) collected from a creek located on pasture GP2 and a pond located on pasture GP4 (Figures 2.7 and 2.9). Isolates from these two water samples were unrelated by PFGE typing (Figure 2.13).

sigB allelic typing and molecular serotyping was performed on all *L. monocytogenes* isolates. *L. monocytogenes* isolates grouped into 4 *sigB* allelic types (Table 2.7). Phylogenetic analysis using a large data set of *sigB* sequences showed these allelic types to cluster well with existing *L. monocytogenes*, with isolates from this study closely matching *sigB* sequences of lineage I, II, and IIIA *L. monocytogenes* isolates (Figure 2.17). Molecular serotyping based on the methods of Doumith (21) failed to produce amplicons outside of the internal positive control gene *prs*, meaning that *L. monocytogenes* isolates from this study align with serotypes 4a and 4c, both lineage III serotypes.

In addition to the four pathogens of interest, all samples were microbiologically analyzed to detect *Listeria* spp. other than *L. monocytogenes*. Overall, other *Listeria* spp. were isolated from 31 total samples, including 6.8% of samples analyzed from produce farms and 3.9% of samples analyzed from grazing pastures (excluding a proposed novel *Listeria* spp.; Table 2.5). *Listeria* spp. were isolated from all sample types, with water samples having the highest isolation rates (Table 2.5). *Listeria* spp. isolated included *L. innocua, L. welshimeri, L. seeligeri,* and *L. ivanovii* (Table 2.7).

Identification and characterization of a novel *Listeria* sp. During this study, 20 samples (3.1%; Table 2.5), including five soil, six fecal, two water, and seven drag swabs, were presumptively positive for *Listeria* spp. based on morphological characteristics on selective and differential media; however, *sigB* was not amplified in these isolates (Table 2.3). A series of basic phenotypic tests were then performed to further characterize the isolates, where these isolates were described as short rod shaped bacteria that were non-motile on a wet mount slide after overnight growth in a nutrient rich broth (BHI) at 25-30˚C. They were Gram positive, catalase positive, and oxidase negative, all expected results from the genus *Listeria.* The isolates were non-hemolytic when plated and observed on Tryptic Soy Agar supplemented with 5% washed sheep's blood. Additionally, API *Listeria* biochemical tests (bioMérieux, Marcy-l'Etoile, France) showed these isolates to hydrolyze esculin, and acidify arabitol, xylose, rhamnose, methyl-D-glucoside, and ribose (data not shown).

Amplification of a portion of the 16S rRNA gene was performed using the MicroSeq500 kit from Applied Biosystems (Life Technologies). PCR products were sequenced and a BLASTN search was performed. The top match for all 8 isolates from Genbank was listed as "*Listeria* sp. 102 16S ribosomal RNA gene, partial sequence", with a score of 630 and a matching identify of 99.7%. No other *Listeria* spp. were reported in the top 100 hits from the BLAST search.

As these isolates displayed phenotypic characteristics typical of *Listeria* but did not fall into an existing *Listeria* spp. based on a combination of these characteristics, we sequenced the whole genome of a single representative isolate. The genome of this isolate displayed several characteristics unknown in the genus *Listeria*, including absence of genetic motility factors, existence of a nitrate reduction pathway, and presence of metaloproteases (Enhancin) as well as other putative adhesion factors (large repetitive proteins). A maximum likelihood tree showed that the presumptive novel *Listeria* sps. belongs to the family Firmicutes. The clade containing the *Listeria* genus also contains the novel *Listeria* spp. isolate; however, the longer branch lengths indicate that this novel species is genetically divergent on the nucleotide level from the genus *Listeria* (Figure 2.18), a characteristic shared with *Listeria grayi*, indicating that these novel *Listeria* sp., along with *L. grayi,* may actually represent a new genus within the family Firmicutes.

Salmonella* prevalence, distribution, and genetic diversity. Salmonella* was detected in eleven (1.7%) samples across both agricultural areas, yielding a total of 44 isolates. PFGE typing using *XbaI* generated 11 unique patterns (Figure 2.14), where only one *XbaI* PFGE type per *Salmonella* positive sample was observed. The prevalence of *Salmonella* marginally differed between sampling seasons (*P* = 0.08; Figure 2.12), with percentage being highest in the month of August.

On produce farms, *Salmonella* was isolated from three composite water samples (7.7%; Table 2.4). One *Salmonella* positive composite water sample originated from a pond on farm PF2 (Figure 2.2), while the remaining two originated from irrigation ditches on farms PF4 and PF5 (figures 2.4 and 2.5). Farms PF4 and PF5 are within 6 miles of each other and use the same water source for irrigation of fields. Upon analysis of PFGE data, it was determined that these three patterns were unrelated (Figure 2.14). In addition, serotyping of these three isolates revealed three different serovars, including III 41:z4,z23:-, III 50:r:z, and Rough O:d:1,7 (Figure 2.14).

On grazing pastures, four composite fecal samples (3.8%; Table 2.4), including one positive sample each from pastures GP1, GP2, GP4 and GP5, were positive for *Salmonella* (Figures 2.6-7, 2.9-10). Additionally, three composite water samples were positive for *Salmonella* (7.3%; Table 2.4). Two of these samples were obtained from a single pond on GP4, which cattle had access to, during separate sample collections (Figure 2.9). Subsequent PFGE analysis of these isolates determined that these isolates were unrelated (Figure 2.14). In addition to water samples, 1 individual drag swab (0.9%) originating from pasture GP4 was positive for *Salmonella* (Table 2.4; Figure 2.9). Serotyping of isolates from grazing pastures identified 6 serotypes, including *Salmonella* Oranienburg, III 61:-:1,5,7, IV 50:z52:z, 8:c:e,n,z15, III 38:(k):z35, and 4,12:i:-. In addition, two isolates sourced from one pond and one fecal sample from grazing pastures did not react with the standard antisera (Figure 2.14).

STEC prevalence, distribution, and genetic diversity. Prevalence of all STEC (both *E. coli* O157:H7 and Non-O157 STEC) significantly differed between month of sampling (P = 0.02; Figure 2.12), with percentages highest during the month of August. Sampling of produce farms did not yield any *E. coli* O157:H7 isolates. However, *E. coli* O157:H7 was detected in one soil sample (0.9%) and one fecal sample (1.0%) from grazing pastures GP2 and GP3, respectively, yielding an overall prevalence of 0.3% from agricultural areas (Figures 2.7-8; Table 2.4). Six isolates were collected from these samples and subsequent PFGE analysis using *XbaI* yielded identical patterns among all isolates (Figure 2.15). Pastures GP2 and GP3 are in close proximity (less than 1 mile apart) yet represent unique cattle operations.

Non-O157 STEC was detected in 20 (3.1%) samples across both agricultural areas, yielding a total of 41 isolates. Additionally, a multiplex PCR was used to determine presence of key virulence genes in confirmed STEC isolates (see materials and methods). Of the confirmed STEC isolates, 83.3% were positive for *hlyA*, 30.0% for *eae*, 20.0% for *stx1* and *stx2*, 33.3% for *stx2* only, and 33.3% for *stx1* only (Table 2.6). A total of 28 unique *XbaI* PFGE patterns were identified from the 41 isolates (Figure 2.16; one isolate was not typable by *XbaI).* Seven non-O157 STEC positive samples yielded multiple *XbaI* PFGE subtypes, with three subtypes recovered from a single sample and two subtypes recovered from the remaining six samples. No recurring Non-O157 *Xba*I PFGE subtypes were identified (i.e., identified from multiple samplings of a location).

On produce farms, non-O157 STEC was isolated in one composite soil sample from PF3 (0.8%; Table 2.4; Figure 2.3). On grazing pastures, three composite soil samples (2.8%) from pastures GP2, GP5, and GP6 were positive for non-O157 STEC (Table 2.4; Figures 2.7, and 2.10-11). Subtyping by PFGE identified four unique *XbaI* patterns from these three composite soil samples (Figure 2.16). Additionally, 12 composite fecal samples (11.4%) from pastures GP2, GP4, GP5, and GP6 were positive for non-O157 STEC (Table 2.4; Figures 2.7 and 2.9-11). Subtyping by PFGE identified 16 unique *XbaI* patterns from these 12 composite fecal samples (Figure 2.16). Finally, two composite water samples (4.9%), and two individual drag swabs (1.9%) were positive for non-O157 STEC (Table 2.4). Upon PFGE analysis, composite water samples and individual drag swabs yielded 4 and 3 unique *XbaI* patterns, respectively (Figure 2.16).

Despite not identifying recurring identical *XbaI* PFGE subtypes of confirmed non-O157 STEC isolates on any of the enrolled locations, several identical subtypes present in multiple samples during a single visit were identified. Isolates CSU-M1-112 and CSU-M1-115 were recovered from soil and water samples, respectively, from pasture GP6 during a single visit (Figure 2.11). PFGE typing revealed these isolates to differ by only a single band (Figure 2.16), indicating a high degree of genetic relatedness. In addition, isolates CSU-M1-108 and CSU-M1-109 were recovered from drag swab and fecal samples, respectively, from pasture GP5 during a single visit (Figure 2.10). Again, *XbaI* patterns of these two isolates differed by only one band, suggesting these isolates to be genetically related (Figure 2.16).

In addition to PFGE analysis, conventional O antigen serotyping of STEC isolates by slide agglutination was performed after API 20E characterization confirmed isolates to be *E. coli* (score of 80% or higher). Serotyping revealed that STEC isolates could be grouped into 11 O-antigen types, including O3, O5, O8, O15, O20, OX25, O84, O113, O157, O168, and O174, with O8 being the most frequently encountered serotype (Table 2.6). Nine isolates were untypable as they did not react with the standard antisera (Table 2.6).

Discussion

Analysis of nearly 2,300 environmental samples from agricultural areas through a two-year longitudinal study allowed us to assess the prevalence, distribution, and genetic diversity of *Listeria, Salmonella,* and STEC (i.e., *E. coli* O157 and non-O57 STEC) in production agriculture environments in Northern Colorado. Our data indicate (i) a rare presence of these key human foodborne pathogens in agricultural environments in Northern Colorado, (ii) significant genetic diversity between organisms isolated within each agricultural environment and between agricultural environments, indicating the molecular epidemiology of these foodborne pathogens in production agricultural environments to be complex, and (iii) a presumptive novel non-motile *Listeria* spp. was identified and may represent a new species within the genus *Listeria.*

There is a rare presence of human foodborne pathogens in agricultural environments in Northern Colorado. There was a low prevalence of *L. monocytogenes, Salmonella, E. coli* O157:H7, and non-O157 STEC in environmental samples (i.e., soil, fecal, water, drag swab) sourced from agricultural environments in Northern Colorado. In this study, 2.2% (6/279) and 0.6% (2/361) of samples from produce farms and grazing pastures were positive for *L. monocytogenes,* respectively (Table 2.4). Weis and Seeliger (1975) identified *L. monocytogenes* in 20.3% of soil and plant samples from fallow fields and 9.9% of pasture samples (96). However, these authors used mice models of infectivity to confirm *L. monocytogenes* isolates. More recent studies reported a prevalence of *L. monocytogenes* in samples not associated with animal production to be much lower. Fenlon and colleagues (1996) failed to identify any *L. monocytogenes* isolates from 25g samples of soil, grass, stems, leaves, and roots from production fields before harvesting (26). Additionally, several studies described low prevalence of *L. monocytogenes* in soil samples from areas not heavily impacted by animal agriculture, including 0.7% in garden soil samples from Great Britain (52) and 1.3% in pristine environment (i.e., locations with little human contact) soil samples in New York state (75). It has been suggested that moist surface soil coupled with the presence of decaying vegetation provides a suitable environment for *L. monocytogenes* growth and proliferation (76). Given that Northern Colorado is a dry environment receiving an average of 17 inches of rainfall annually (20), soil from this area may be less suitable for *L. monocytogenes* compared to soil from areas with greater rainfall, which may account for the low prevalence of *L. monocytogenes* in soil samples encountered in this study (42).

L. monocytogenes was isolated from 5.1 and 4.9% of composite water samples tested on produce farms and grazing pastures, respectively (Table 2.4). These results are similar to findings reported by Arvanitidou et al., where 3.9%

(5/128) of water samples from four rivers and one lake yielded *L. monocytogenes* (4). However, the prevalence reported of *L. monocytogenes* in fresh water samples varies greatly, ranging from 1.3% in pristine environments (75) to 53% in agricultural environments (27). Variation of *L. monocytogenes* prevalence in water samples could be to a variety of factors, including presence of wildlife, proximity to populated areas, and unsanitary conditions of water being tested. Additional research into the ecology *L. monocytogenes* in water sources is needed to better understand these factors that may influence contamination.

Recent studies suggest *L. monocytogenes* prevalence to be greater in farm environments associated with animal production than other environments (76). For example, Fenlon et al. isolated no *L. monocytogenes* in soil samples associated with vegetable production, yet isolated *L. monocytogenes* in soil samples collected from fields where cattle and sheep were kept (26). In addition, Nightingale et al. (57) isolated *L.monocytogenes* in 20.1% of 2,056 ruminant farm samples collected, noting a much higher prevalence rate as compared to a study investigating urban (7.3%) and pristine environments (1.3%) by Sauders et al. (75) during the same time frame and in the same geographical region. Results from these studies and others suggest ruminant farms to be an important natural reservoir for *L. monocytogenes* (40, 57, 82). Thus, we expected to encounter a higher prevalence of *L. monocytogenes* in soil and fecal samples collected from grazing pastures than produce farms in Northern Colorado. However, we did not isolate *L. monocytogenes* from any soil or fecal samples collected on grazing pastures (Table 2.4). Grazing pastures in Colorado (and other western states) generally have a much lower density of cattle than pastures located in areas having higher rainfall totals and more vegetation. This may help explain the low prevalence of *L. monocytogenes* isolated from livestock operations in this study as compared to previous studies (40, 57). Thus, the low prevalence rates of *L. monocytogenes* encountered on produce farms and grazing pastures in Northern Colorado may be influenced by the climate and ranching practices unique to the region.

Salmonella was identified in 1.1 and 2.2% of samples collected from produce farms and grazing pastures, respectively (Table 2.4). No composite soil samples analyzed in this study was positive for *Salmonella* and only 1 drag swab sample was positive (Table 2.4). Gorski and colleagues (2011) investigated the prevalence of *Salmonella* in the environment of a major produce production region of California (30). The authors identified *Salmonella* in 2.6% of the 617 soil samples analyzed, indicating a low prevalence of *Salmonella* in the soil from this area (30). Additionally, Rodriguez and colleagues (2006) examined the distribution of *Salmonella* across a variety of animal production farm types, regions, and sample types (69). The authors encountered a range of *Salmonella* prevalence across farm types, ranging from 3.4% in beef pasture soil samples to 9.4% of soil samples from swine farms (69). This suggests that prevalence of *Salmonella* in soil samples from Northern Colorado agricultural areas is low, and this may in part due to geographical factors unique to Colorado.

A total of 3.8% of the 105 composite fecal samples analyzed in this study were positive for *Salmonella* (Figure 2.4). Numerous surveys of cattle fecal samples for *Salmonella* have reported various prevalence rates ranging from 4.4% (7) to

23% (85); however, the majority of these studies have focused on confined cattle (i.e., feedlots). Few studies have focused on *Salmonella* presence in pasture based cattle production systems. Gorski and colleagues (2011) reported a prevalence of 0.13% (1/795) in cattle fecal samples obtained from a pasture environment in California (30). Additional studies utilizing long term sampling plans in pasture environments from geographically diverse locations are needed to assess both the true prevalence of *Salmonella* in environmental fecal samples and the role of these samples in transmission of *Salmonella* in pre-harvest agricultural environments.

Water samples analyzed had the highest percentage of positive samples for *Salmonella* (Table 2.4). Gorski and colleagues (2011) observed the same trend, identifying water samples as having the highest percentage of samples positive among all types of samples (i.e, soil, water, fecal) analyzed to detect *Salmonella* in a produce region in California (30). They also found similar prevalence results for *Salmonella* from standing water and irrigation ditches, with 7.1% samples positive as compared to 7.7% in the current study.

Cattle are considered the major reservoir of STEC, and, in general, the prevalence of STEC in livestock production environments displays tremendous variability (38). Prevalence rates for *E. coli* O157 in cattle fecal samples range from 0.3 to 19.7% in the feedlot to 0.7 to 27.3% on the pasture. Additionally, prevalence rates for fecal samples positive for non-O157 STEC range from 4.6 to 55.9% in the feedlot to 4.7 to 44.8% on the pasture (38). In the current study, isolation of both *E. coli* O157 and non-O157 STEC was almost exclusively restricted to livestock environments. The percent composite fecal positives in the current study for *E. coli*

O157 (1.0%) and non-O157 STEC (11.4%) were within ranges reported elsewhere (Table 2.4). Several previous studies also identified a low prevalence for *E. coli* O157:H7 in fecal samples from pasture environments. These studies report a low prevalence ranging from 0.9 to 1.3% of *E. coli* O157:H7 from fecal samples located in pasture environments (65, 66, 73). While it is often difficult to compare results from studies due to factors such as differences in methodologies, environmental factors, and production factors, these particular studies included similar geographic locations, sampling plans and microbiological methodologies as compared to the current study (65, 66, 73). Additionally, we encountered a seasonal trend in STEC isolation, with frequency of isolation highest during August (Figure 2.12). These findings are consistent with several studies in which the largest number of STEC positive samples from livestock environments was identified during warmer months (7, 18, 45). However, several studies have reported no apparent seasonality of STEC isolation (73).

In this study, soil samples from grazing pastures had higher rates of STEC (both O157 and non-O157) isolation (3.7%) compared to soil samples from produce farms (0.8%; Table 2.4). Although outbreaks attributed to *E. coli* O157:H7 contaminated soil exposure have been documented (35), they are rare and typically occur in locations that are in close proximity to livestock operations. Thus, contamination of soil on produce farms is closely related to the proximity of the farm to livestock who shed the pathogen, an aspect noted in a recent study surveying for *E. coli* O157:H7 in a large produce production area in California after a series of major outbreaks (19). Considering that none of the produce farms enrolled in the current study were adjacent to any livestock operation, the low prevalence of STEC in these produce farms may be in part due to the absence of potential sources for transmission of the pathogen onto the produce farm, an observation consistent with the absence of any noticeable fecal depositions encountered on produce farms.

Studies have described an association between increased prevalence of STEC in cattle when herds have access to contaminated water sources (47, 64). We identified non-O157 STEC from 4.9% of composite water samples tested on grazing pastures (Table 2.4). Reports on the prevalence of STEC in water sources on pasture environments vary widely in the literature, likely due to differences in methodologies, environmental factors, and various interpretations of what defines STEC. Water prevalence estimates of 0.34% have been reported for *E. coli* O157 (66) and 6.8% have been reported for non-O157 STEC (64) in pasture based livestock systems. Despite the varying prevalence estimates of STEC in water sources in the literature, it is clear that water may play an important role in the epidemiology of STEC in cattle environments.

Listeria, Salmonella, **and STEC isolated within each agricultural environment and between agricultural environments are genetically diverse.** The genetic diversity of *Listeria* spp., *Salmonella*, and STEC isolates identified in this study was determined using a combination of phenotypic and molecular methods. In general, multiple subtypes of each organism within and among agricultural areas sampled were identified, including multiple subtypes of non-O157 STEC within individual samples. These results indicate a high level of genetic diversity among foodborne pathogens in these environments.

L. monocytogenes diversity was assessed using a combination of PFGE, *sigB* allelic typing, and molecular serotyping. Eight unique *Apa*I and *Asc*I PFGE patterns were identified from isolates obtained in this study (Figure 2.13). We did not encounter any identical *Apa*I or *Asc*I restriction patterns, indicating an absence of persistent strains of *L. monocytogenes* in our study areas. However, of the eight unique PFGE patterns identified in this study, two patterns from an identical irrigation water source on separate produce farms (PF4 and PF5) during the same day in June 2009 differed by three bands, indicating these isolates were genetically related. While similar PFGE patterns do not provide conclusive evidence for persistence of *L. monocytogenes* in farm irrigation water, it does suggest that the two isolates are closely related to one another.

In addition to *L. monocytogenes*, non-pathogenic *Listeria* spp. genetic diversity was determined using *sigB* allelic typing. A total of 21 unique *sigB* allelic types were identified among *Listeria* spp. other than *L. monocytogenes* (Table 2.7). *L. innocua* was encountered most frequently, and was isolated from a variety of grazing pastures and produce farms. *L. welshimeri* from this study grouped into six *sigB* allelic types, all from produce farms. Interestingly, a companion study also conducted in CO during this same time frame also reported an overrepresentation of *L. welshimeri* in samples obtained from wilderness areas, indicating this species to be common in CO (1). These results provide an important insight into the molecular ecology of *Listeria* spp. other than *L. monocytogenes* in agricultural environments.

There was significant genetic diversity among *Salmonella* isolates in the current study. A total of 11 unique *Xba*I PFGE patterns from 11 positive samples were identified (Figure 2.14). Despite previous studies reporting isolation of persistent *Salmonella* strains in the environment of produce production (30), we failed to identify persistent strains of *Salmonella* in any of the agricultural environments sampled, as no recurring PFGE patterns were identified. This could be due to differences in geography, production and management factors, or simply due to a low prevalence of *Salmonella* in farms enrolled in this study. Eleven serotypes in *Salmonella* isolates from this study were encountered, with *Salmonella* Oranienburg being the only serotype encountered that has been previously implicated in disease outbreaks. These results are consistent with previous studies that report widespread genetic diversity in *Salmonella* isolates from agricultural environmental samples, though each study reports a variety of serotypes not identified in the current study (30, 69).

We identified identical *Xba*I PFGE patterns in a soil and fecal sample that each tested positive for *E. coli* O157:H7 in this study (Figure 2.15). Interestingly, these samples were from two different pastures on the same day, where the ranches were less than a mile apart from one another. This suggests that transmission of the *E. coli* O157:H7 strain from one farm to another may have occurred. Cattle from both pastures had access to a mutual creek water source (North Poudre supply canal; Figures 2.7-8). Additional samplings of the creek source and the same locations that yielded positive fecal and soil samples were performed, with no repeat isolation of the strain. Evidence for transmission and subsequent long-term survival of *E. coli* O157:H7 into soil by fecal contamination has been demonstrated, suggesting this to be a likely route of transmission (55).

There was a high level of diversity among non-O157 STEC isolates obtained in this study. A total of 28 unique *Xba*I PFGE patterns were identified from 41 non-O157 STEC isolates from 20 non-O157 STEC positive samples (Figure 2.16), with many samples containing multiple strains of non-O157 STEC*,* a result consistent with previous studies of *E. coli* diversity in pasture environments (64, 65). Evidence for persistence of non-O157 STEC strains was not identified, a finding in contrast to several studies on *E. coli* O157:H7 in environmental samples from pastures (45, 65). On more than one occasion, multiple sample types during a single visit contained highly similar non-O157 STEC PFGE subtypes. For example, isolates CSU-M1-112 and CSU-M1-115 were recovered from soil and water samples, respectively, from pasture GP6 and PFGE typing revealed these isolates differed by a single band (Figure 2.16). Additionally, isolates CSU-M1-108 and CSU-M1-109 were recovered from a drag swab and fecal sample, respectively, from pasture GP5 and PFGE typing revealed these two isolates to differ by only a single band. These results suggest that non-O157 STEC strains may be disseminated into the environment of grazing pastures via contaminated feces or water.

O antigen serotype diversity of STEC was also assessed, with results indicating that non -O157 STEC isolates from this study can be grouped into 11 O-antigen types, (Table 2.6). Additionally, 10 isolates did not react with the standard antisera (Table 2.6), a finding consistent with several studies identifying a large number of presumptive non-O157 STEC isolates that were untypable by conventional serotyping (3, 34, 64). In this study, serotype O8 was most frequently encountered. A previous study on STEC in livestock production environments in

Nebraska also identified O8 as the most frequent serotype (64). Serotypes O3, O8, O14, O20, O113, and O168 were encountered in this study and have been previously implicated in human illness. For example, three clinical cases of HUS in Australia in 1998 were attributed to an *E. coli* O113:H21 strain (62). Upon screening with a multiplex PCR, the authors of this previous study reported these three strains to be stx_1 negative, stx_2 positive, *eae* negative, and EHEC *hlyA* positive (62), which matches the multiples PCR virulence profile of the O113 isolate identified in the current study.

While the set of virulence factors necessary to cause STEC related illness has not been strictly defined, several genes have been associated with the ability to cause severe disease in humans. STEC strains carrying stx_2 have been shown to have a higher likelihood of causing disease than strains carrying stx_1 alone or both stx_1 and stx_2 (12, 61). Of the non-O157 STEC isolates identified in this study, 53.3% carried stx_2 either alone or in combination with other virulence factors (*eae*, *hlyA*). Additionally, strains possessing *eae* and *hlyA* have been epidemiologically associated with severe disease, indicating that a combination of stx_2, *eae,* and *hlyA* may be a good indicator of the pathogenic potential of STEC strains (54). Out of the 30 STEC isolates identified in this study, 12 (40.0%) isolates carried a combination of *stx* (either stx_1, stx_2, *or both*), *eae,* and *hlyA*. These results indicate that a large percentage of STEC isolated from agricultural environments in Northern Colorado may have the ability to cause human illness.

Environmental sampling may provide insight into the molecular ecology and evolution of human foodborne pathogens. During the course of this study, we identified an atypical *Listeria* spp. from repeat samplings on pasture GP4. While these isolates displayed typical *Listeria* spp. morphology on selective and differential media such as MOX and LMPM, molecular analyses failed to consistently classify these isolates into a widely recognized species within the *Listeria* genus. A recent environmental sampling study, which employed similar methodologies to the current study, identified a proposed novel species (i.e., *Listeria marthii* sp. nov.) in a National forest in New York (31). The presumptive novel *Listeria* sp. isolates reported here display all of the basic phenotypic characteristics of the *Listeria* genus (i.e., Gram-positive short rod cell morphology, catalase positive and oxidase negative) with the exception of motility. Currently, all recognized members of the *Listeria* genus demonstrate motility when grown in nutrient rich broth at 25-30°C (71). However, motility at this temperate range in both semi-solid agar stabs and wet-mount slides was not observed. Additionally, sequence analysis indicates the genome to lack any motility factors. Interestingly, the non-motile novel *Listeria* sp. was isolated only after periods of heavy rainfall in the area. Rainwater leeching has been identified as a potential means of transport for non-motile bacteria (56).

Li and colleagues (2005) used 16S rDNA sequencing to identify typical bacteria that colonize the mid-gut of the fire ant (*Solenopsis invicta*). They identified a single *Listeria* sp. isolate, which showed 97% homology to the 16s rDNA sequence of the proposed novel *Listeria* spp. identified in the current study (50). The authors of this previous study deposited the sequence into the NCBI GenBank database with name *Listeria* sp. 102 and no efforts were made to characterize the isolate further. Whole genome sequencing of the presumptive novel *Listeria* spp. described in the current study revealed numerous metaloproteases, such as enhancin, as well as other putative adhesion factors. Enhancin has been identified in other Firmicutes such as *Bacillus anthracis*, and has been shown to enhance the infectivity of these organisms in insects through degradation of the mucin layer surrounding insect guts (49, 63). It is interesting that the first report of the novel *Listeria* spp. in the literature described the isolation of the microorganism from the gut of a fire ant (50). Together, these results indicate that the novel *Listeria* spp. presented here may have adapted to colonize an insect host.

Phylogenetic analysis was performed to determine the evolutionary position of these isolates within both the family Firmicutes and the genus *Listeria*. We used the methods of Wu and colleagues (2008) for our phylogenetic analysis, as this method involves analysis of 31 protein encoding gene sequences central to the lifecycle of the bacteria that are resistant to lateral gene transfer (98). Analysis reveals that the novel *Listeria* spp. isolate (designated as CSU M1-001) groups into the *Listeria* containing clade (Figure 2.18). Additionally, isolate CSU M1-001 seems to be more closely related on the nucleotide level to current *Listeria* than is *L. grayi*. These results indicate that M1-001 represents a new species of *Listeria,* and that the genus *Listeria* embraces three closely related yet distinct lines of descent: one containing *L. grayi*, one containing M1-001, and the third containing *L. monocytogenes*, *L. seeligeri, L. innocua, L. ivanovii,* and *L. welshimeri.*

Conclusions

We investigated the prevalence, distribution, and molecular epidemiology of four major foodborne pathogens in primary production agricultural environments (i.e., produce farms and grazing pastures) in Northern Colorado through a two-year longitudinal study. Our results indicate pathogens identified in these environments are rare and genetically diverse both within and between environments. Despite being isolated rarely, these pathogens were still present in primary production agriculture enviroments, and thus still pose a risk for transmission into the human food chain. Therefore, all food producers need to adhere to good agricultural practices in these environments. The results of this study provide important insights into the molecular epidemiology of these pathogens and will direct future research to reduce the incidence of foodborne pathogens entering the human food supply at the pre-harvest level.

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Chapter 2: Tables and Figures

Table 2.1: Description of produce farms in Northern Colorado chosen for this study.

Produce Farm ID	Size	Farming Practice	County
PF1	< 100 acres	Local Community Supported Agriculture Farm	Larimer
PF2	> 1,000 acres	Certified Organic	Larimer
PF3	< 100 acres	Certified Organic	Larimer
PF4	< 100 acres	Certified Organic	Adams
PF5	> 1,000 acres	Large conventional	Weld

Table 2.2: Description of pastures in Northern Colorado chosen for this study.

Ranch ID	**Size of Ranch**	**Herd Size (approximate)**	**Pasture Type**
GP1[a]	< 100 acres	20	Small Arid Pasture
GP2	> 1,000 acres	150	Arid Rocky Pasture
GP3	< 1,000 acres	40	Pond Pasture
GP4	> 1,000 acres	225	River Bottom Pasture
GP5	> 10,000 acres	315	Arid Rocky Pasture
GP6[b]	> 3,000 acres	180	River Bottom Pasture

[a] Removed from study in Fall 2009
[b] Introduced into study Summer 2010

Table 2.3: Description of oligonucleotide PCR primers used in this study.

Oligonucleotide	Sequence (5' to 3')	Purpose	Reference
sigB15-F	AAT ATA TTA ATG AAA AGC AGG TGG AG	For detection of *Listeria* spp. alternative sigma factor σ^B	Nightingale 2005
sigB16-R	ATA AAT TAT TTG ATT CAA CTG CCT T		
LM hly-β	TAG TTC TAC ATC ACC TGA GAC AGA	For detection of the listeriolysin O gene, *hlyA*, specific to *L. monocytogenes*	Norton 2001
LM hly-α	CCT AAG ACG CCA ATC GAA AAG AAA		
CAA1-invAF	GAA TCC TCA GTT TTT CAA CGT TTC	For detection of the invasion protein A gene *invA* in *Salmonella enterica*	Kim 2007
CAA2-invAR	TAG CCG TAA CAA CCA ATA CAA ATG		
EC hly-F	CCC TGG CAG ACC TTT GAT G	For detection of the hemoylsin gene, *hlyA*, in *E. coli*	This study
EC hly-R	CCG TGT CTT TTC TGA TAC TCA		
RfbF	GTG TCC ATT TAT ACG GAC ATC CAT G	For detection of the *rfbE* gene in *E. coli* O157:H7	Hu 1999
RfbR	CCT ATA ACG TCA TGC CAA TAT TGC C		
$FLIC_{h7}$-F	GCG CTG TCG AGT TCT ATC GAG C	For detection of a sequence specific for the H7 antigen in *E. coli* O157:H7	Gannon 1997
$FLIC_{h7}$-R	CAA CGG TGA CTT ATC GCC ATT CC		
IntF	GAC TGT CGA TGC ATC AGG CAA AG	For detection of intimin gene, *eaeA*, in *E. coli* O157:H7	Hu 1999
IntR	TTG GAG TAT TAA CAT TAA CCC CAG G		
SLT-IF	TGT AAC TGG AAA GGT GGA GTA TAC	For detection of *slt-I*, the gene encoding for shiga toxin I in pathogenic *E. coli*	Meng 1997
SLT-IR	GCT ATT CTG AGT CAA CGA AAA ATA AC		
SLT-IIF	GTT TTT CTT CGG TAT CCT ATT CCG	For detection of *slt-II*, the gene encoding for shiga toxin II in pathogenic *E. coli*	Meng 1997
SLT-IIR	GAT GCA TCT CTG GTC ATT GTA TTA C		
*eae*STECF	ATT ATG GAA CGG CAG AGG TTA AT	For detection of a conserved domain of intimin (encoded by *eae*) in pathogenic *E. coli*	Michigan State STEC Center, Online Protocol
*eae*STECR	ATC CCC ATC GTC ACC AGA GG		

Table 2.4: Percent and total number of samples positive for *L. monocytogenes*, *Salmonella*, *E. coli* O157:H7, and non-O157 STEC on produce farms and grazing pastures in Northern Colorado listed by sample type.

	Produce Farms				Grazing Pastures			
	L. monocytogenes	*Salmonella*	*E. coli* O157:H7	*Non-O157 STEC*	*L. monocytogenes*	*Salmonella*	*E. coli* O157:H7	*Non-O157 STEC*
Sample Type	**Percent Positive (Fraction)**				**Percent Positive (Fraction)**			
Composite Soil	1.7% (2/120)	0% (0/120)	0% (0/120)	0.8% (1/120)	0% (0/108)	0% (0/108)	0.9% (1/108)	2.8% (3/108)
Composite Fecal	Not found[a]	Not found	Not found	Not found	0% (0/105)	3.8% (4/105)	1.0% (1/105)	11.4% (12/105)
Composite Water	5.1% (2/39)	7.7% (3/39)	0% (0/39)	0% (0/39)	4.9% (2/41)	7.3% (3/41)	0% (0/41)	4.9% (2/41)
Individual Drag Swabs	1.7% (2/120)	0% (0/120)	0% (0/120)	0% (0/120)	0% (0/107)	0.9% (1/107)	0% (0/107)	1.9% (2/107)
Overall	**2.2% (6/279)**	**1.1% (3/279)**	**0% (0/279)**	**0.4% (1/279)**	**0.6% (2/361)**	**2.2% (8/361)**	**0.6% (2/361)**	**5.3% (19/361)**

[a] No fecal samples of suitable size for analysis were encountered from selected sampling sites on produce farms.

Table 2.5: Percent and total number of samples positive for *L. innocua, L. welshimeri, L. seeligeri, L. ivanovii,* and novel *Listeria* spp. on produce farms and grazing pastures in Northern Colorado listed by sample type.

	Produce Farms					**Grazing Pastures**				
	L. innocua	*L. welshimeri*	*L. seeligeri*	*L. ivanovii*	Novel *Listeria* spp.	*L. innocua*	*L. welshimeri*	*L. seeligeri*	*L. ivanovii*	Novel *Listeria* spp.
Sample Type	**Percent Positive (Fraction)**					**Percent Positive (Fraction)**				
Pooled Soil	0.8% (1/120)	1.7% (2/120)	0.8% (1/120)	0% (0/120)	0% (0/120)	3.7% (4/108)	0% (0/108)	0% (0/108)	0% (0/108)	4.6% (5/108)
Pooled Fecal	Not found[a]	Not found	Not found	Not found	Not found	0% (0/105)	1.0% (1/105)	0% (0/105)	0% (0/105)	5.7% (6/105)
Pooled Water	2.6% (1/39)	7.7% (3/39)	7.7% (3/39)	7.7% (3/39)	0% (0/39)	4.9% (2/41)	2.4% (1/41)	7.3% (3/41)	2.4% (1/41)	4.9% (2/41)
Drag Swabs	2.5% (3/120)	0.8% (1/120)	0.8% (1/120)	0% (0/120)	0% (0/120)	0% (0/107)	0% (0/107)	0% (0/107)	0% (0/107)	6.5% (7/107)
Overall	**1.8% (5/279)**	**2.2% (6/279)**	**1.8% (5/279)**	**1.1% (3/279)**	**0% (0/279)**	**1.7% (6/361)**	**0.6% (2/361)**	**0.8% (3/361)**	**0.3% (1/361)**	**5.5% (20/361)**

[a] Not Found

Table 2.6: Diversity among serotypes and virulence genes in shiga-toxin producing *E. coli* isolated in this study.

		Virulence Genes present[b]			
O serogroup	**No. of Isolates[a]**	***hlyA***	***eae***	***stx-I***	***stx-II***
Not typable[c]	10	9	2	8	3
O8	6	5	0	0	6
O157	2	2	2	2	0
OX25	2	2	2	0	2
O84	2	2	0	2	2
O5	2	2	2	2	0
O113	1	1	0	0	1
O20	1	1	1	1	0
O174	1	1	0	1	1
O3, O15, O168[d]	1 each	0	0	0	1
Total Isolates	30	25	9	16	16

[a] Based on representative STEC isolates from each sample (based on PFGE patterns) in order to avoid analysis of genetically identical strains from the same sample.
[b] Numbers indicate how many isolates were positive for a specific gene based on combination of results of the multiplex PCR and the follow up conserved intimin domain PCR as detailed in the materials and methods.
[c] Not typable: Did not react with standard antisera
[d] Serogroups in the same row contain identical virulence patterns.

Table 2.7: Source description of *Listeria* spp. isolates representing unique *sigB* allelic types.

CSU FSL ID	***Listeria* species**	**Location of Isolation**	**Source**	**Month**	**Year**	***sigB* allelic type**
A1-046	*L. innocua*	GP1	Fecal	May	2009	23
A1-055	*L. welshimeri*	PF5	Water	May	2009	20
A1-058	*L. innocua*	PF4	Water	May	2009	21
A1-059	*L. monocytogenes*	PF4	Water	May	2009	6
A1-076	*L. innocua*	GP4	Water	June	2009	19
A1-101	*L. innocua*	PF3	Soil	July	2009	14
A1-106	*L. innocua*	GP1	Soil	July	2009	13
A1-137	*L. welshimeri*	PF4	Water	August	2009	25
A1-139	*L. welshimeri*	PF5	Water	August	2009	2
A1-140	*L. welshimeri*	PF5	Water	August	2009	17
A1-156	*L. welshimeri*	PF1	Soil	September	2009	18
M1-015	*L. monocytogenes*	PF4	Drag Swab	October	2009	12
M1-019	*L. innocua*	PF4	Drag swab	October	2009	24
M1-025	*L. monocytogenes*	PF1	Soil	June	2010	5
M1-029	*L. ivanovii*	PF1	Water	June	2010	8
M1-035	*L. welshimeri*	PF5	Soil	June	2010	7
M1-037	*L. monocytogenes*	PF4	Soil	June	2010	4
M1-052	*L. seeligeri*	GP3	Water	July	2010	11
M1-053	*L. seeligeri*	GP3	Water	July	2010	22
M1-072	*L. seeligeri*	PF2	Drag Swab	August	2010	15
M1-074	*L. innocua*	GP4	Soil	August	2010	16
M1-085	*L. innocua*	GP2	Soil	September	2010	10
M1-120	*L. ivanovii*	GP6	Water	November	2010	3
M1-122	*L. seeligeri*	GP6	Water	November	2010	1

Figure 2.1: Geographic Distribution of positive samples on Produce Farm 1.

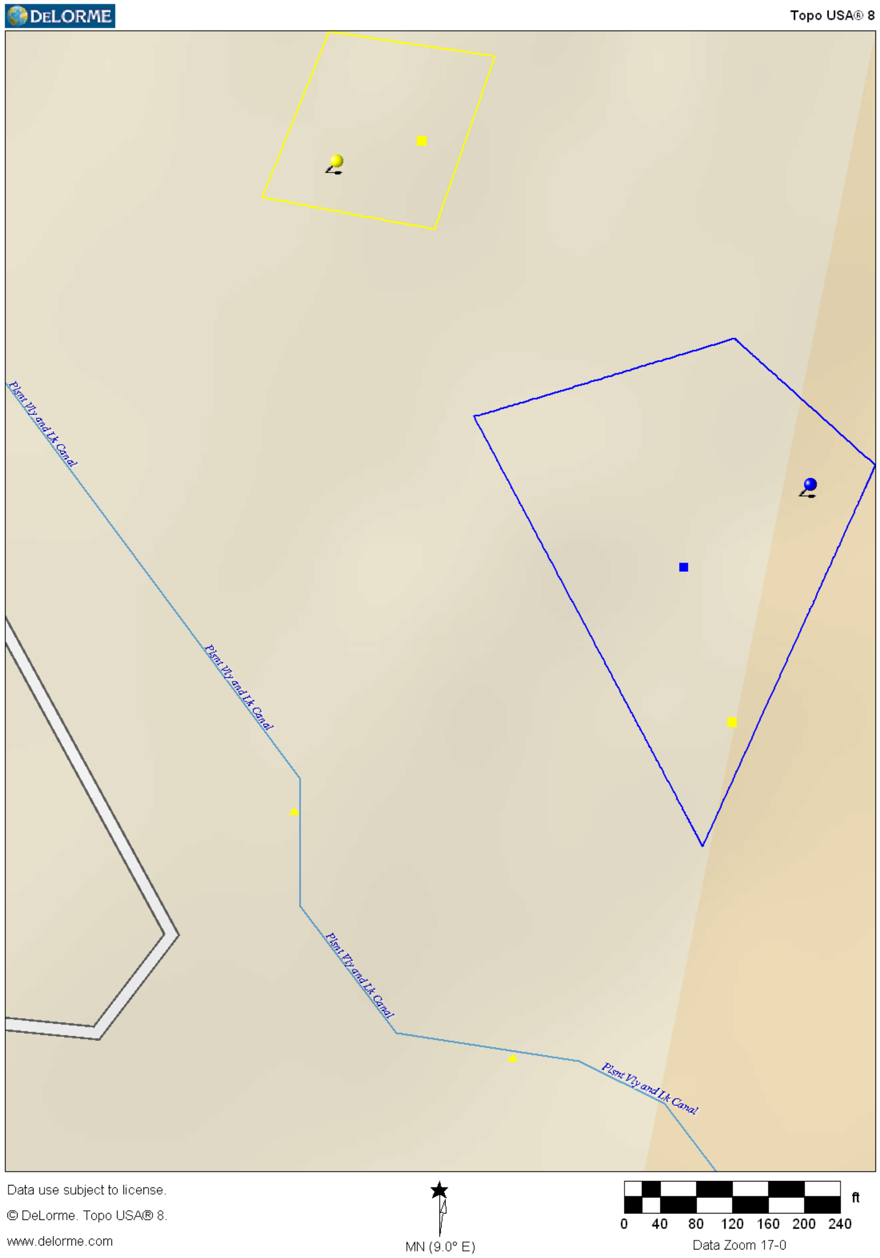


Figure 2.1: Map of Produce Farm 1 detailing the geographic distribution of positive samples for *L. monocytogenes* (blue) and other *Listeria* spp. (yellow). Sample types include soil (square), water (triangle), and drag swabs (pin). Colored box around drag swabs indicates approximate area sampled. Distance scale (ft) as indicated.

Figure 2.2: Geographic Distribution of positive samples on Produce Farm 2.

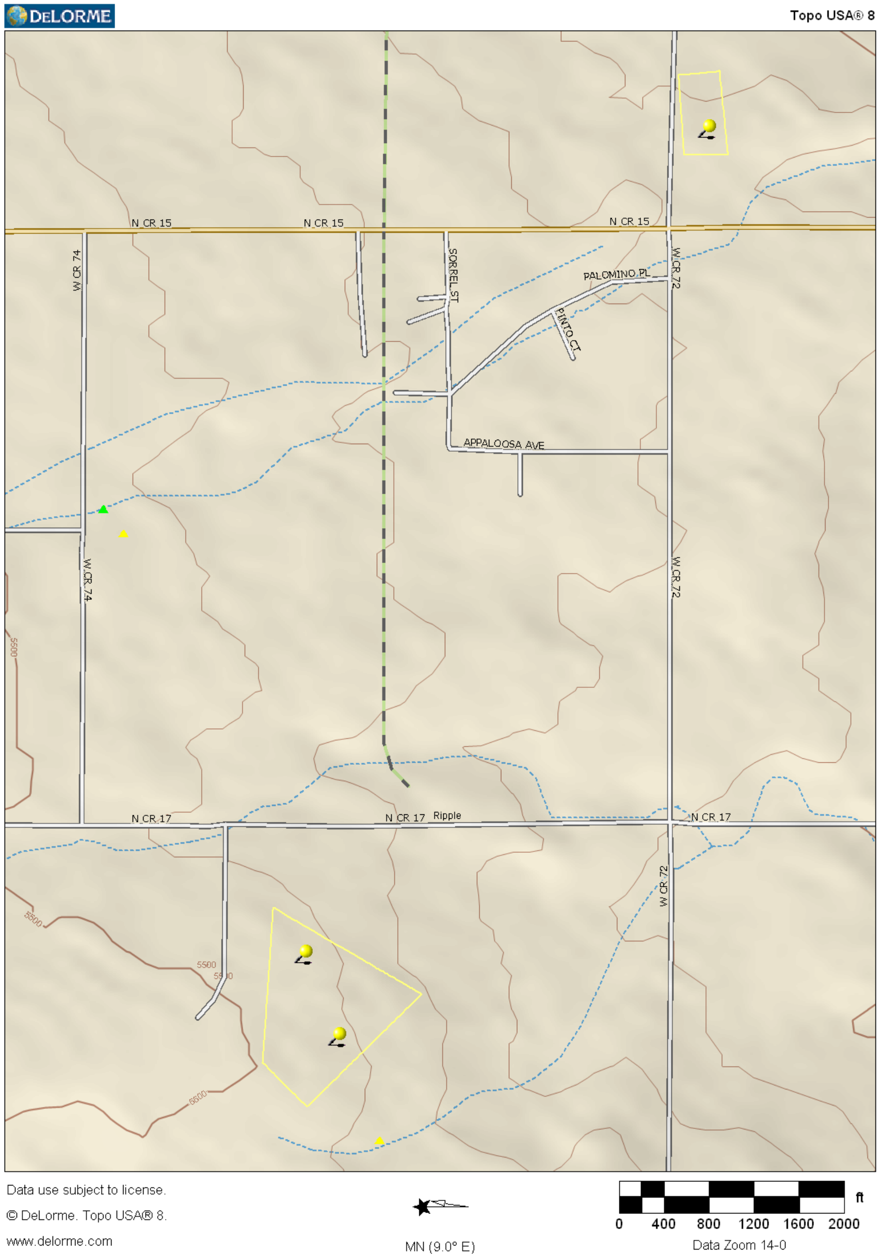


Figure 2.2: Map of Produce Farm 2 detailing the geographic distribution of positive samples for *Listeria* spp. (yellow) and *Salmonella* (green). Sample types include soil (square), water (triangle), and drag swabs (pin). Colored box around drag swabs indicates approximate area sampled. Distance scale (ft) as indicated.

Figure 2.3: Geographic Distribution of positive samples on Produce Farm 3.

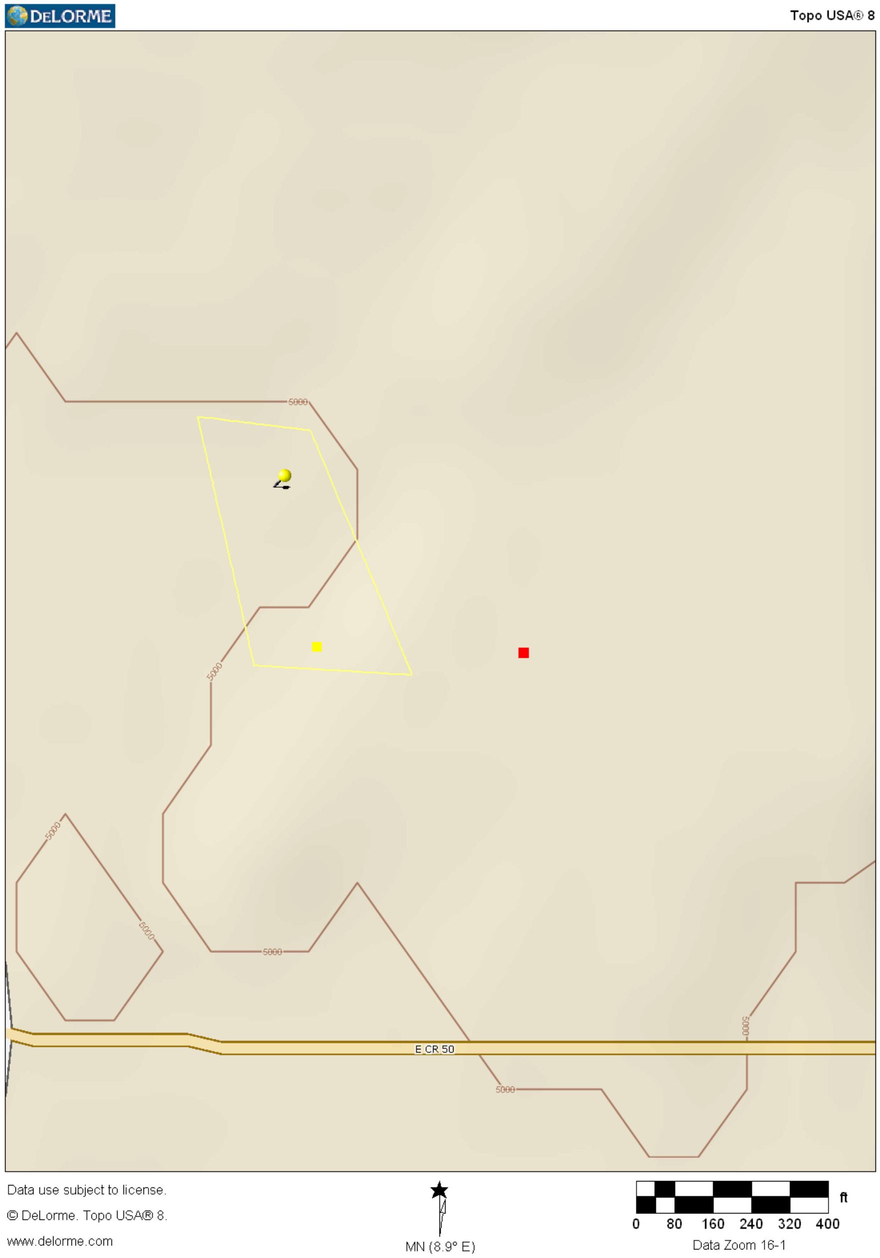


Figure 2.3: Map of Produce Farm 3 detailing the geographic distribution of positive samples for *Listeria* spp. (yellow) and STEC (red). Sample types include soil (square) and drag swabs (pin). Colored box around drag swabs indicates approximate area sampled. Distance scale (ft) as indicated.

Figure 2.4: Geographic Distribution of positive samples on Produce Farm 4.

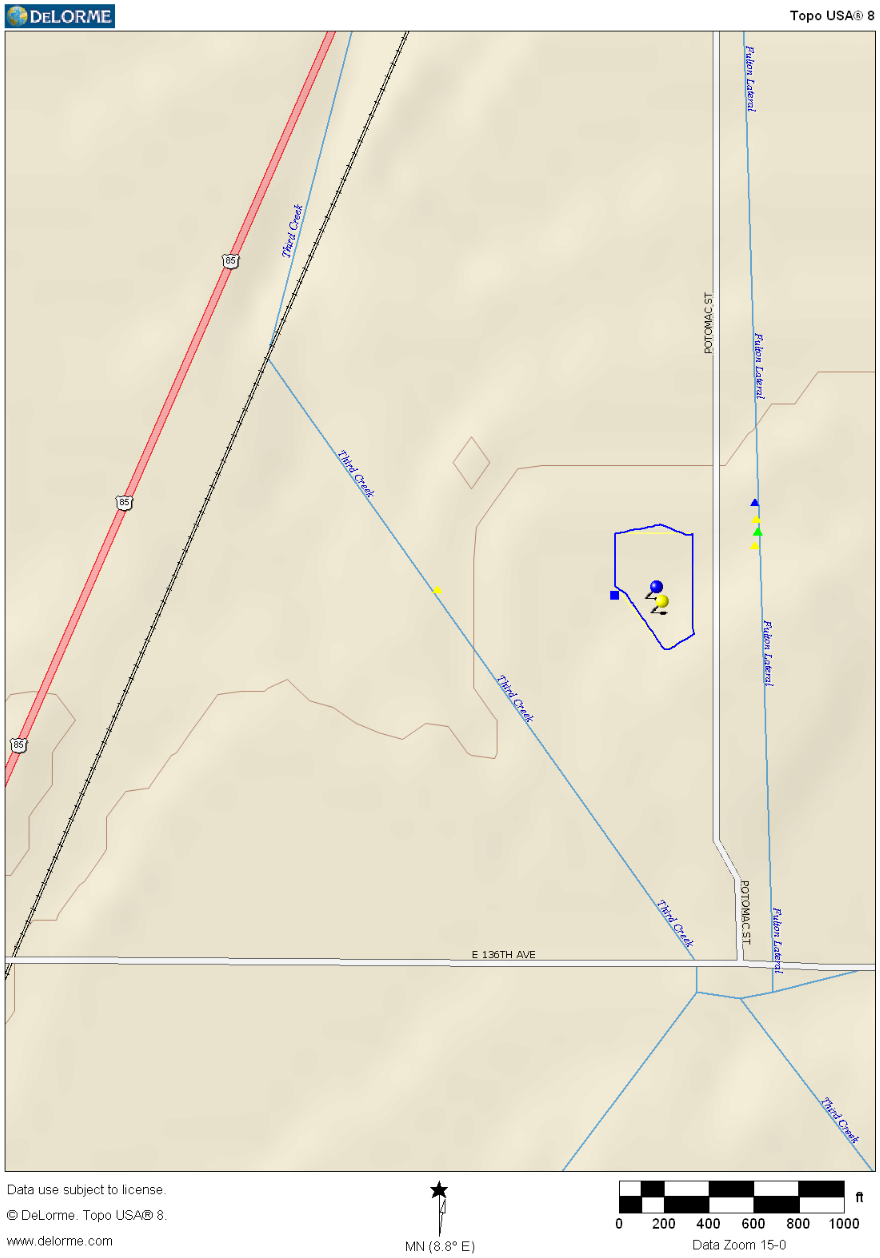


Figure 2.4: Map of Produce Farm 4 detailing the geographic distribution of positive samples for *L. monocytogenes* (blue), other *Listeria* spp. (yellow), and *Salmonella* (green). Sample types include soil (square), water (triangle), and drag swabs (pin). Colored box around drag swabs indicates approximate area sampled. Distance scale (ft) as indicated.

Figure 2.5: Geographic Distribution of positive samples on Produce Farm 5.

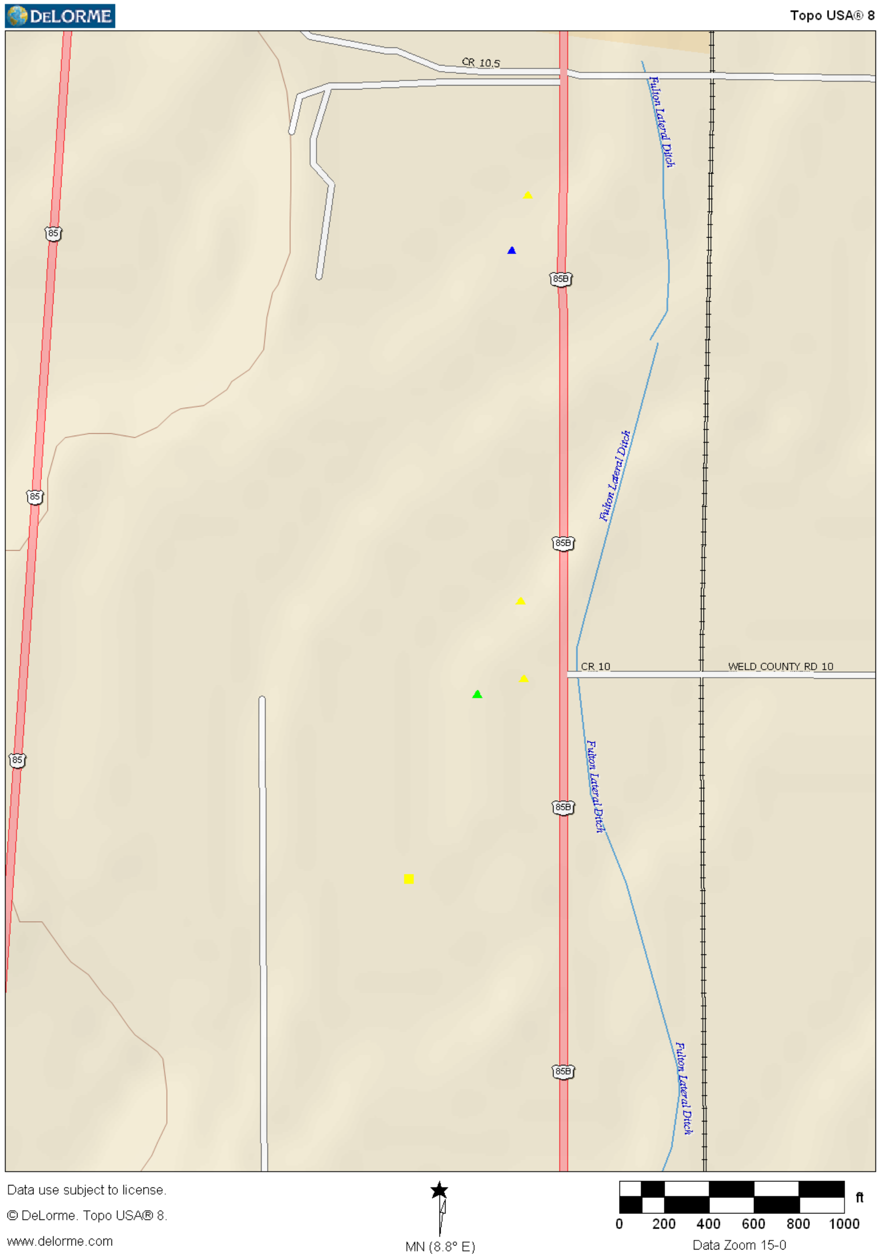


Figure 2.5: Map of Produce Farm 5 detailing the geographic distribution of positive samples for *L. monocytogenes* (blue), other *Listeria* spp. (yellow), and *Salmonella* (green). Sample types include soil (square), water (triangle), and drag swabs (pin). Colored box around drag swabs indicates approximate area sampled. Distance scale (ft) as indicated.

Figure 2.6: Geographic distribution of positive samples on Grazing Pasture 1.

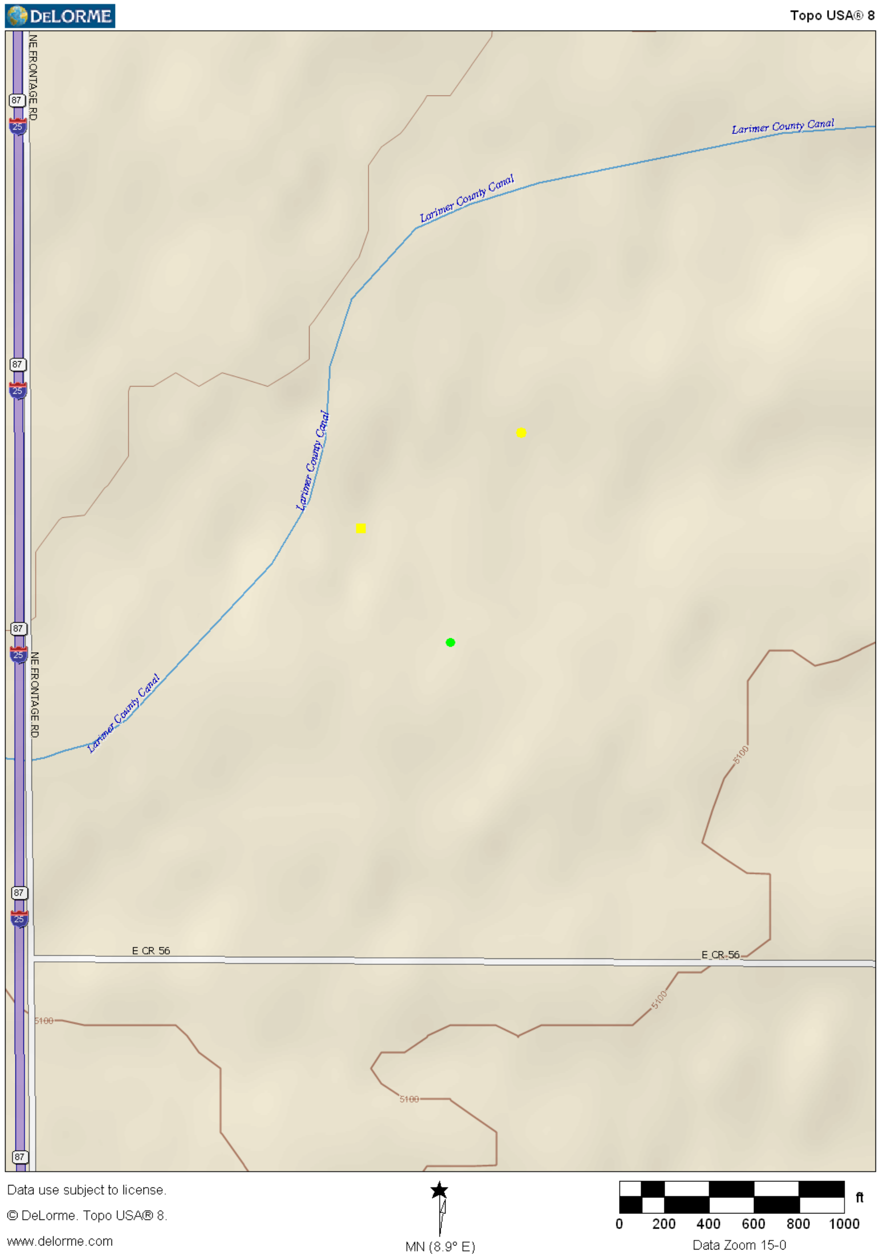


Figure 2.6: Map of Grazing Pasture 1 detailing the geographic distribution of positive samples for *Listeria* spp. (yellow) and *Salmonella* (green). Sample types include soil (square) and fecal (circle). Distance scale (ft) as indicated.

Figure 2.7: Geographic distribution of positive samples on Grazing Pasture 2.

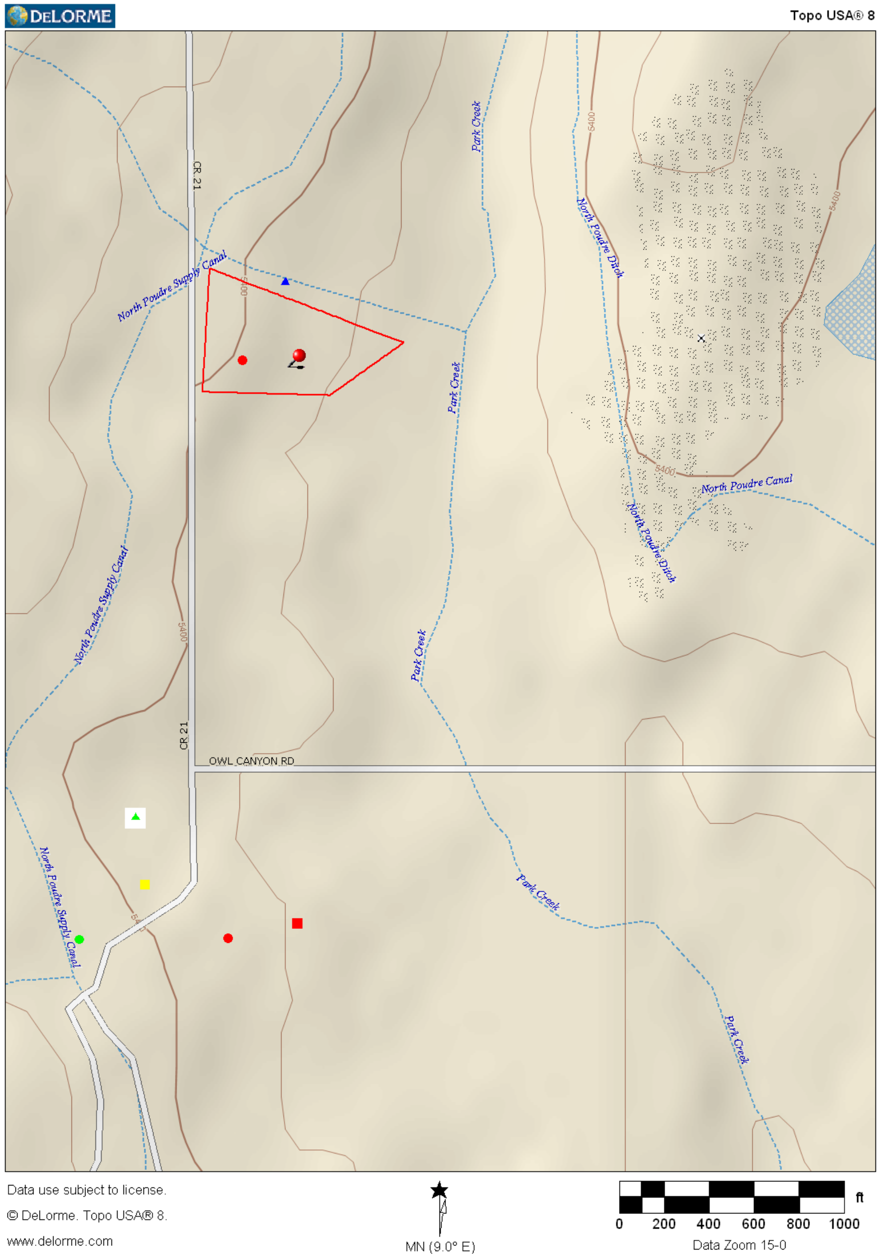


Figure 2.7: Map of Grazing Pasture 2 detailing the geographic distribution of positive samples for *L. monocytogenes* (blue), other *Listeria* spp. (yellow), *Salmonella* (green), and STEC (red). Sample types include soil (square), water (triangle), fecal (circle), and drag swabs (pin). Colored box around drag swabs indicates approximate area sampled. Distance scale (ft) as indicated.

Figure 2.8: Geographic distribution of positive samples on Grazing Pasture 3.

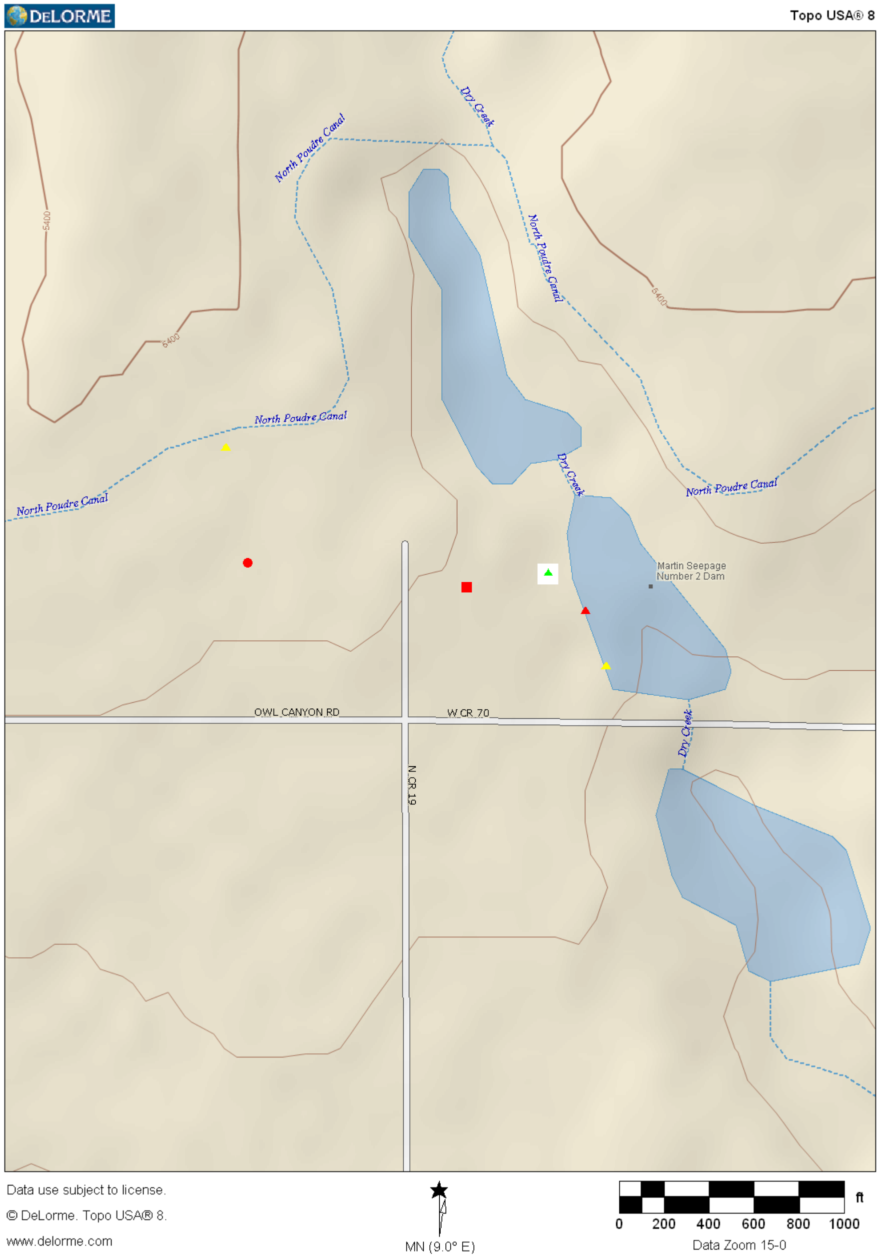


Figure 2.8: Map of Grazing Pasture 3 detailing the geographic distribution of positive samples for *Listeria* spp. (yellow), *Salmonella* (green), and STEC (red). Sample types include soil (square), water (triangle), and fecal (circle). Distance scale (ft) as indicated.

Figure 2.9: Geographic distribution of positive samples on Grazing Pasture 4.

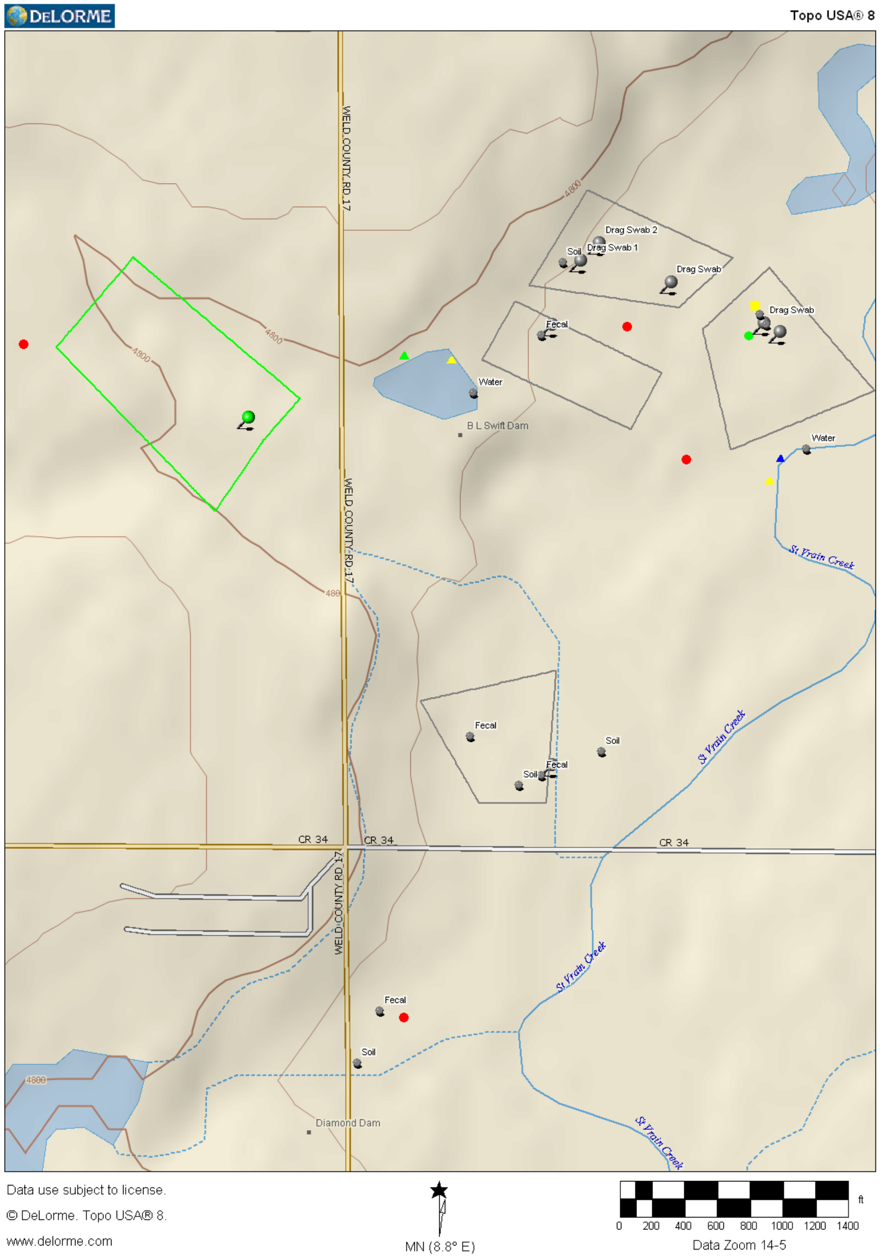


Figure 2.9: Map of Grazing Pasture 4 detailing the geographic distribution of positive samples for *L. monocytogenes* (blue), other *Listeria* spp. (yellow), *Salmonella* (green), and STEC (red). Sample types include soil (square), water (triangle), fecal (circle), and drag swabs (pin). Colored box around drag swabs indicates approximate area sampled. Additionally, novel *Listeria* spp. positive samples and sample types are indicated by grey dots. Distance scale (ft) as indicated.

Figure 2.10: Geographic distribution of positive samples on Grazing Pasture 5.

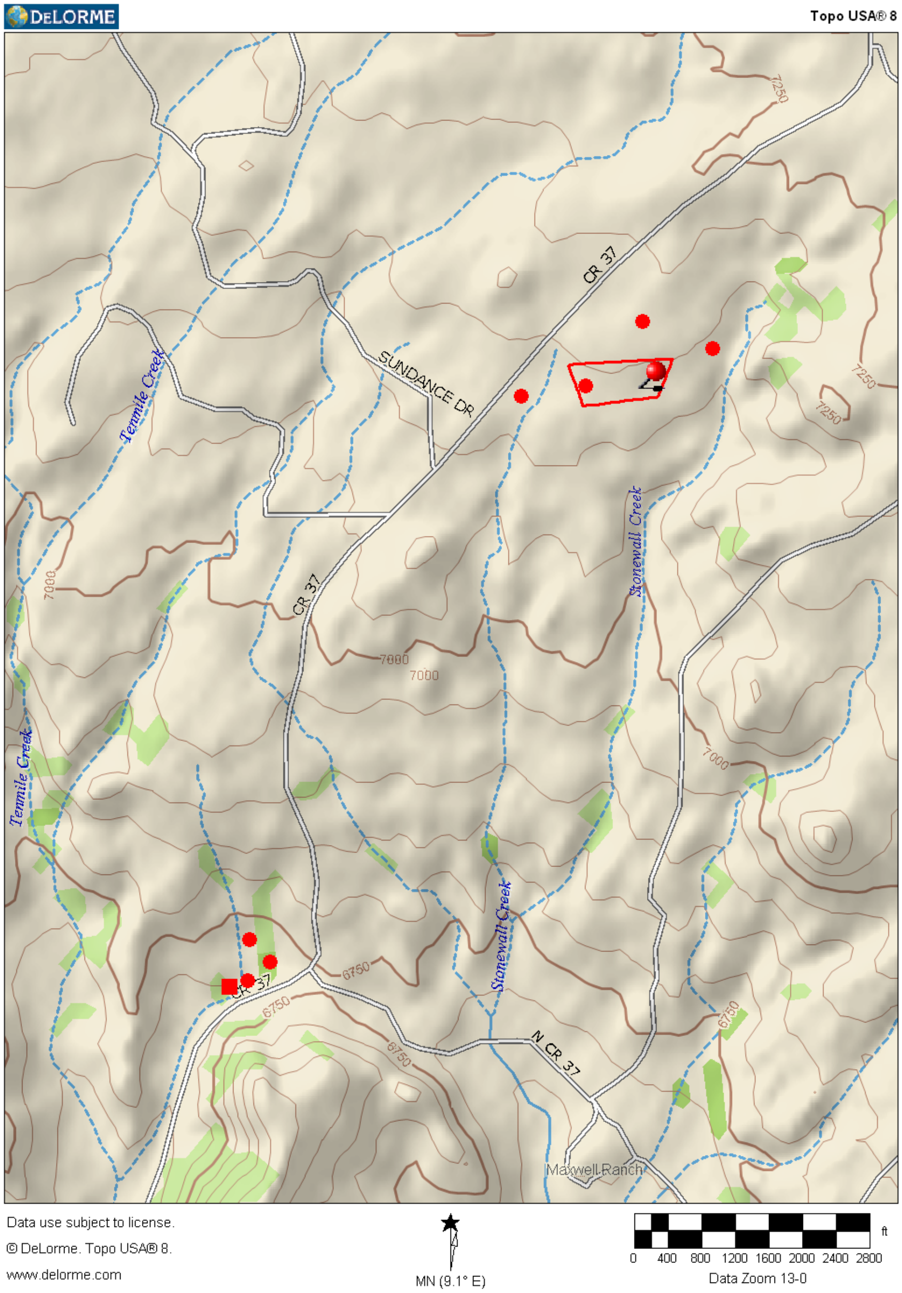


Figure 2.10: Map of Grazing Pasture 5 detailing the geographic distribution of positive samples for STEC (red). Sample types include soil (square), fecal (circle), and drag swabs (pin). Colored box around drag swabs indicates approximate area sampled. Distance scale (ft) as indicated.

Figure 2.11: Geographic distribution of positive samples on Grazing Pasture 6.

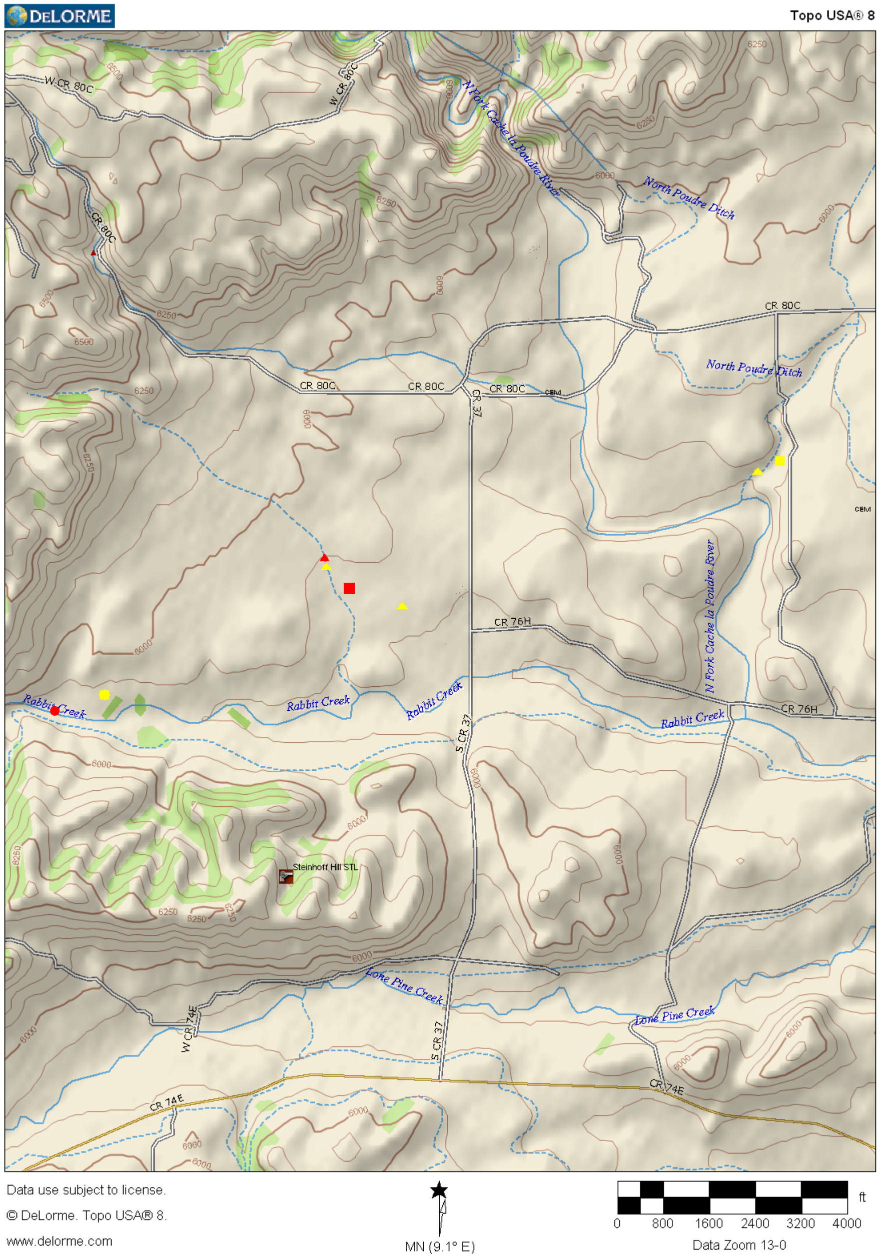


Figure 2.11: Map of Grazing Pasture 6 detailing the geographic distribution of positive samples for *Listeria* spp. (yellow) and STEC (red). Sample types include soil (square), water (triangle), and fecal (circle). Distance scale (ft) as indicated.

Figure 2.12: Prevalence of *Salmonella*, *L. monocytogenes,* and pathogenic *E. coli* (STEC) among month of isolation.

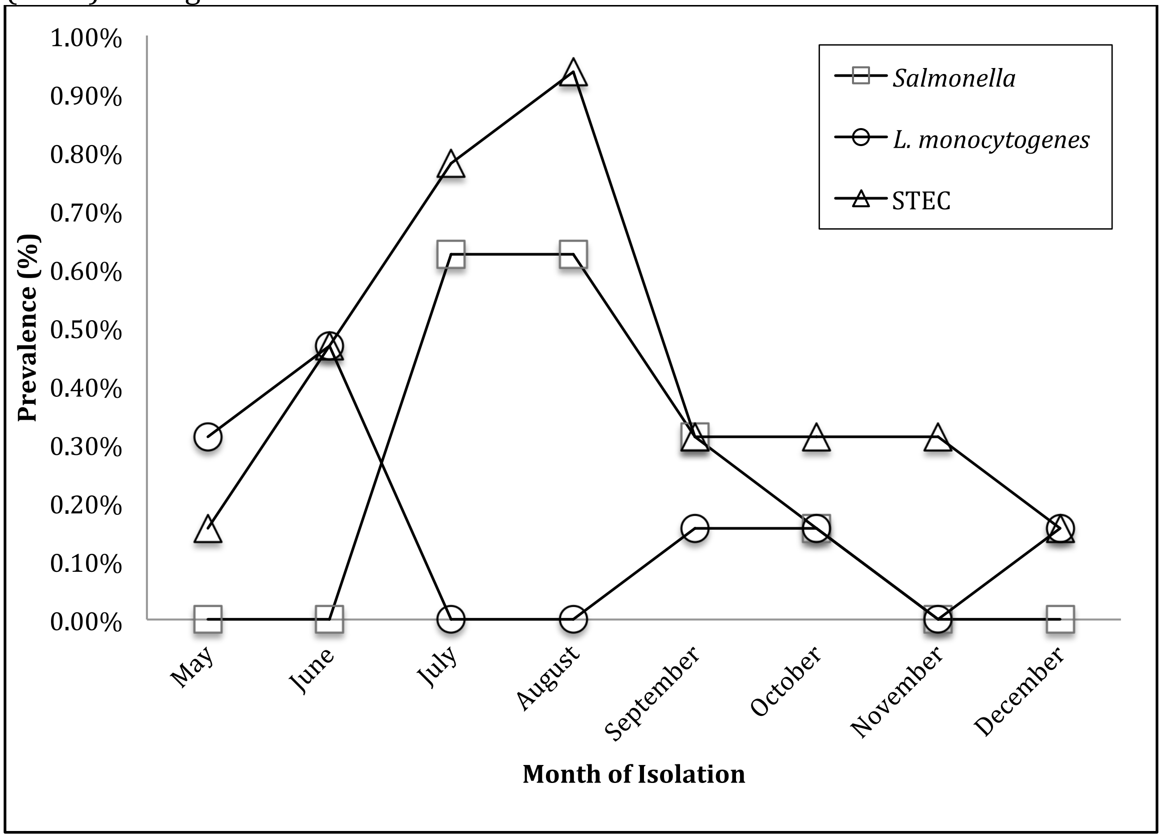
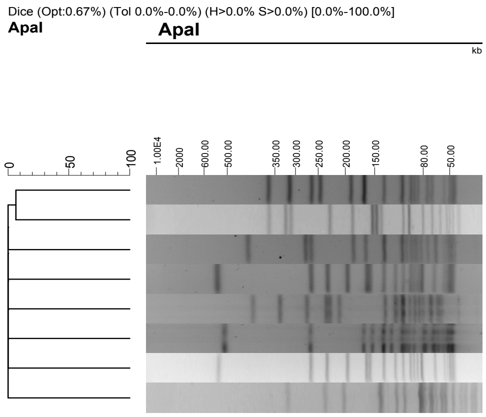


Figure 3: Graphical representation of the prevalence (defined as number of samples that tested positive divided by total number of samples analyzed) of *Salmonella*, *L. monocytogenes,* and pathogenic *E. coli* (STEC; represents both *E. coli* O157:H7 and non-O157 STEC) over time. Months listed represent two years of sampling (i.e., June represents June 2009 and June 2010). Winter months were not sampled in this project.

Figure 2.13: Dendrogram of *ApaI* and *AscI* PFGE patterns of *L. monocytogenes* isolates.

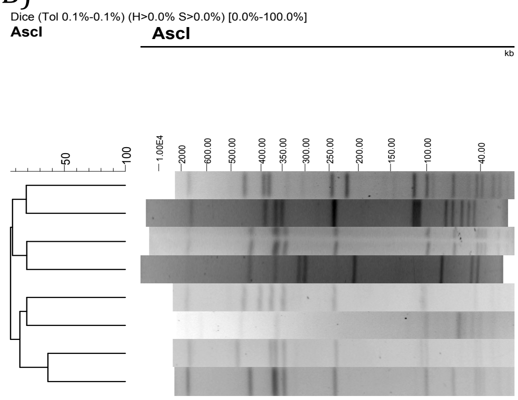
A)



Key	Source	Source (Specific)	Isolation Month	Isolation Year
CSU FSL A1-060	PF4	Irrigation Water	June	2009
CSU FSL M1-037	PF4	Pooled Soil	July	2010
CSU FSL A1-061	PF5	Irrigation Water	June	2009
CSU FSL A1-083	GP4	Pond Water	July	2009
CSU FSL M1-014	PF4	Drag Swab	November	2009
CSU FSL M1-020	GP2	Creek Water	November	2009
CSU FSL M1-025	PF1	Pooled Soil	June	2010
CSU FSL M1-090	PF1	Drag Swab	September	2010

2

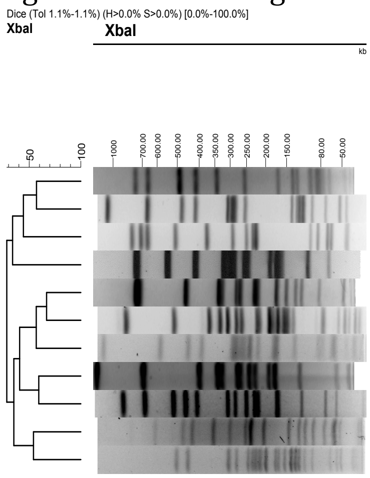
B)



Key	Source	Source (Specific)	Isolation Month	Isolation Year
CSU FSL M1-020	GP2	Creek Water	November	2009
CSU FSL M1-037	PF4	Pooled Soil	July	2010
CSU FSL M1-025	PF1	Pooled Soil	June	2010
CSU FSL M1-090	PF1	Drag Swab	September	2010
CSU FSL A1-060	PF4	Irrigation Water	June	2009
CSU FSL M1-014	PF4	Drag Swab	November	2009
CSU FSL A1-061	PF5	Irrigation Water	June	2009
CSU FSL A1-083	GP4	Pond Water	July	2009

Figure 2.13: Dendrogram created using the unweighted pair group matching algorithm and the Dice correlation coefficient using Bionumerics software to visualize similarity of *L. monocytogenes ApaI* (A) and *AscI* (B) patterns.

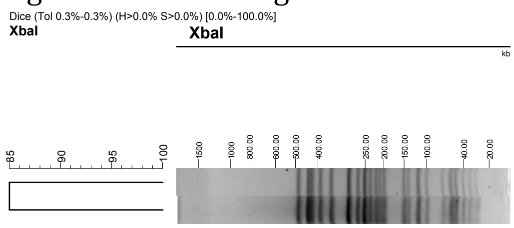
Figure 2.14: Dendrogram of *XbaI* PFGE patterns of *Salmonella* isolates.



Key	Source	Source - Specific	Isolation (Month)	Isolation (Year)	Serovar
CSU A1-115	PF2	Irrigation Water from.	July	2009	III 41:z4,z23:-
CSU M1-054	GP4	Pond Water	July	2010	Oranienburg
CSU M1-066	GP4	Drag Swab	August	2010	4,12:i:-
CSU A1-152	GP5	Bovine Fecal	September	2009	8:c:e,n,z15
CSU A1-125	GP2	Creek on Pasture	August	2009	IV 50:z52:z
CSU M1-064	GP4	Bovine Fecal	August	2010	III 38:(k):z35
CSU M1-095	PF4	Irrigation Water	October	2010	Rough O:d:1,7
CSU A1-117	GP1	Bovine Fecal	July	2009	III 61:-:1,5,7
CSU M1-058	PF5	Irrigation Ditch Water	July	2010	III 50:R:Z
CSU M1-070	GP4	Pond Water	August	2010	Unspecified
CSU M1-089	GP2	Bovine fecal	September	2010	Did not react with antisera

Figure 14: Dendrogram created using the unweighted pair group matching algorithm and the Dice correlation coefficient using Bionumerics software to visualize similarity of *Salmonella XbaI* PFGE patterns.

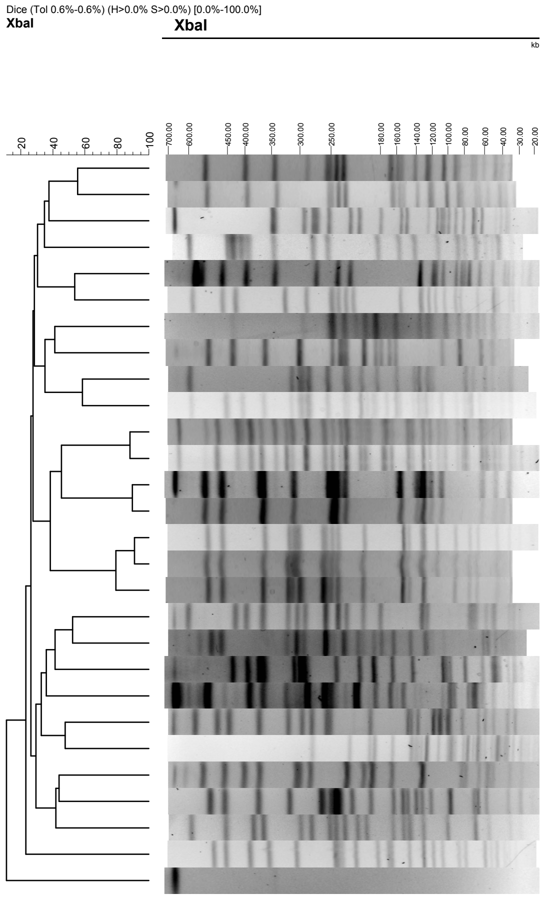
Figure 2.15: Dendrogram of *XbaI* PFGE patterns of *E. coli* O157:H7 isolates.



Key	Source	Source (Specific)	Isolation Month	Isolation Year
CSU FSL A1-128 GP2		Soil	August	2009
CSU FSL A1-136 GP3		Fecal	August	2009

Figure 15: Dendrogram created using the unweighted pair group matching algorithm and the Dice correlation coefficient using Bionumerics software to visualize similarity of *E. coli* O157:H7 *XbaI* PFGE patterns.

Figure 2.16: Dendrogram of *XbaI* PFGE patterns non-O157 STEC isolates.



Key	Serotype	Source	Source (Specific)	Isolation Year	Isolation Mc
CSU M1-112	O84	GP6	Soil	2010	November
CSU M1-115	O84	GP6	Creek Water	2010	November
CSU M1-045	Untypable	GP5	Bovine Fecal	2010	July
CSU M1-023	Untypable	GP2	Bovine Fecal	2009	December
CSU A1-065	Untypable	GP3	Pond Water	2009	June
CSU M1-088	OX25	GP2	Soil	2010	September
CSU A1-064	Untypable	GP3	Pond Water	2009	June
CSU M1-114	O8	GP6	Creek Water	2010	November
CSU M1-080	O5	GP5	Bovine Fecal	2010	August
CSU M1-083	O5	GP5	Bovine Fecal	2010	August
CSU M1-107	O113	GP5	Drag Swab	2010	October
CSU M1-124	Untypable	GP6	Bovine Fecal	2010	November
CSU A1-089	O8	GP5	Bovine Fecal	2009	June
CSU M1-110	O8	GP2	Bovine Fecal	2010	October
CSU M1-106	Untypable	GP5	Drag Swab	2010	October
CSU M1-108	O8	GP5	Drag Swab	2010	October
CSU M1-109	O8	GP5	Bovine Fecal	2010	October
CSU A1-090	O168	GP5	Bovine Fecal	2009	June
CSU M1-048	O20	GP4	Bovine Fecal	2010	July
CSU A1-082	O3	GP4	Bovine Fecal	2009	June
CSU A1-087	O174	GP5	Soil	2009	June
CSU A1-133	O15	GP2	Bovine Fecal	2009	August
CSU M1-049	Untypable	GP4	Bovine Fecal	2010	July
CSU A1-100	Untypable	PF3	Soil	2009	July
CSU M1-041	Untypable	GP5	Bovine Fecal	2010	July
CSU M1-022	Untypable	GP2	Bovine Fecal	2009	December
CSU M1-087	OX25	GP2	Soil	2010	September
CSU A1-088	O8	GP5	Bovine Fecal	2009	June

Figure 16: Dendrogram created using the unweighted pair group matching algorithm and the Dice correlation coefficient using Bionumerics software to visualize similarity of non-O157 STEC *XbaI* PFGE patterns.

Figure 2.17: Phylogenetic tree of unique *Listeria sigB* allelic types encountered in this study.

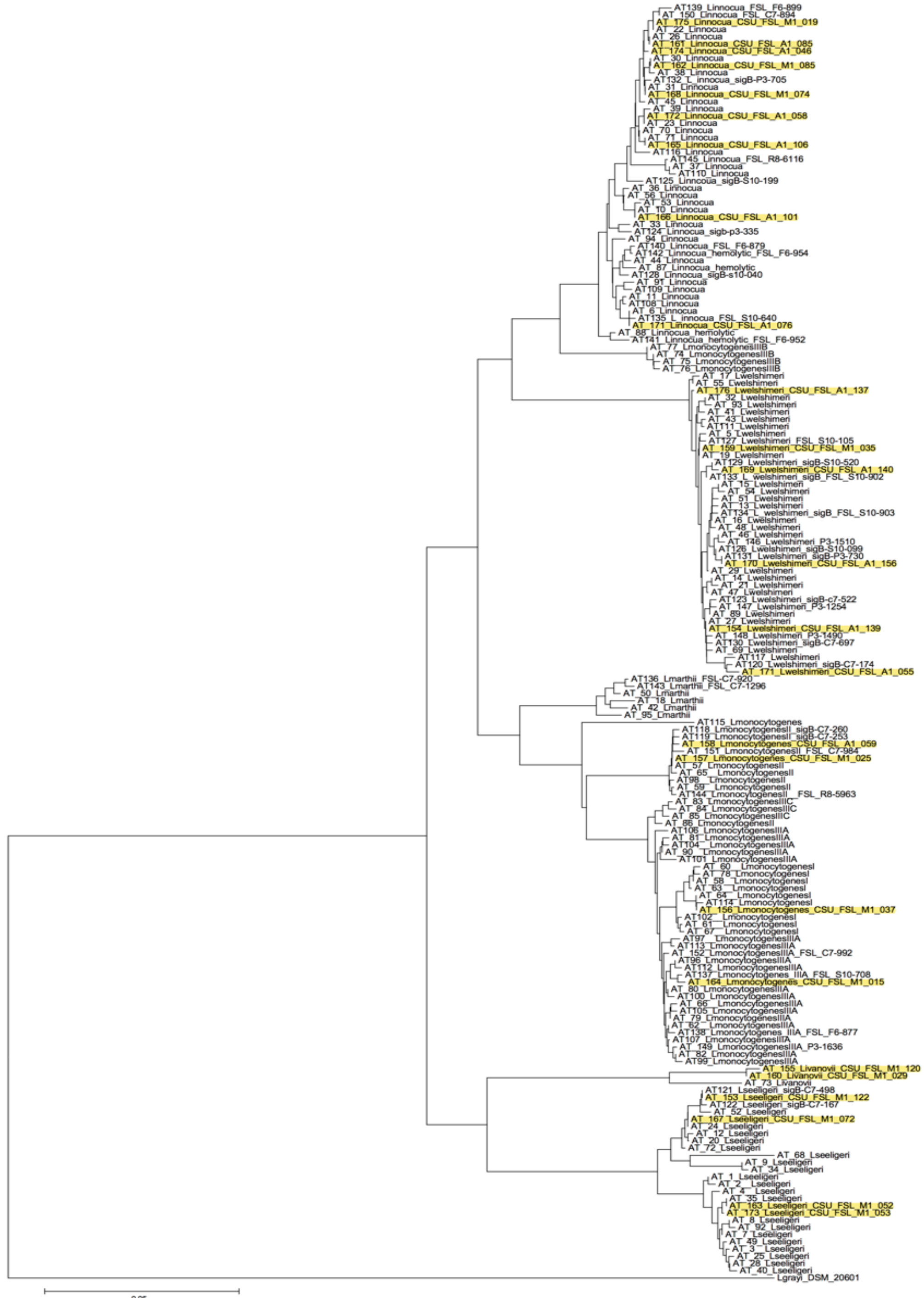


Figure 2.17: Phylogenetic tree of unique *Listeria sigB* allelic types inferred using the neighbor-joining method. Analysis involved 177 total *sigB* allelic types, including 25 *sigB* allelic types encountered during this study (highlighted in yellow). Each *sigB* sequence was trimmed to 660 nucleotides.

Figure 2.18**:** Phylogenetic tree displaying the location of the novel *Listeria* spp. within the family Firmicutes.

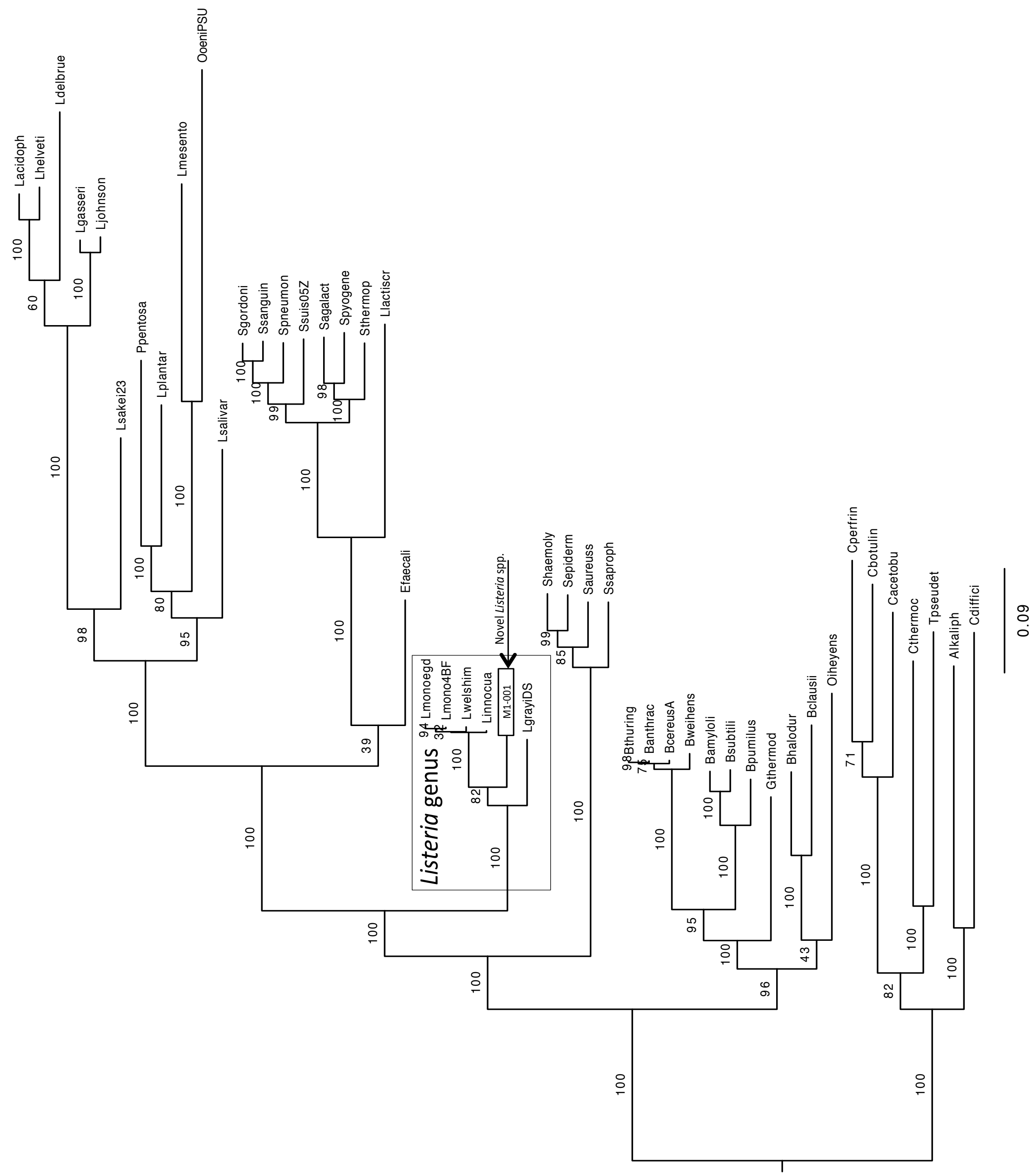


Figure 2.18: Phylogenetic tree inferred using the Maximum likelihood method (100 bootstrap replicates). The methods of Wu 2008 were used for phylogenetic analysis and were performed on concatenated amino acid sequences (totaling 7,149 aa) of 31 housekeeping genes from 50 taxa from the family Firmicutes. The novel *Listeria* sp. (M1-001) is marked with an arrow.

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CHAPTER III

Frequency and Distribution of Various Allelic Types of a Hypermutable 5' Region in *inlA* and Associations with Premature Stop Codons in *Listeria monocytogenes* Ribotype DUP-1039C Isolates

Chapter III Summary

Listeria monocytogenes uses the key virulence factor internalin A (InlA; encoded by *inlA*) to traverse host cell barriers during establishment of a systemic infection. Multiple naturally occurring mutations leading to a premature stop codon (PMSC) in *inlA* have been reported worldwide, and these mutations have been shown to be virulence attenuating in guinea pig infection models. Ribotype DUP-1039C is a broadly distributed molecular subtype within *L. monocytogenes* that has been commonly isolated along the food continuum. Ribotyope DUP-1039C isolates have been shown to carry three distinct PMSC mutations in *inlA,* including a frameshift mutation in a 5' homopolymeric tract of adenine restudies (PMSC4), a C to T substitution leading to a PMSC at aa 562 (PMSC 7), and a G to A substitution leading to a PMSC at aa 684 (PMSC 12). Additionally, DUP-1039C isolates carry one of three allelic types of a 5' hypermutable region of *inlA*, which may have a role in phase variation. A selection of DUP-1039C isolates available from the PathogenTracker database (n=368) were screened for known *inlA* PMSCs and the allelic type of the 5’ hypermutable region by two multiplex single nucleotide polymorphism (SNP) genotyping assays. SNP genotyping assays included (i) a previously described single nucleotide extension assay and (ii) a newly developed probe-based assay, which uses differentially labeled probes to differentiate between wild type (WT; AAAAAAA), frameshift (FS; AAAAAA), and interrupted HT (IHT; AAGAAAA) 5’ allelic types of *inlA*. DUP-1039C isolates carrying a PMSC in *inlA* were not uniformly distributed among isolation sources. *inlA* PMSC type 4 (n=19) and PMSC type 7 (n=25) were associated ($P < 0.05$) with isolation from food and food environments, while WT isolates encoding a full-length InlA (n=184) were associated ($P < 0.05$) with non-food environments and mammalian hosts. Interestingly, all DUP-1039C isolates carrying *inlA* PMSC7 also had the IHT *inlA* allele in the hypermutable 5’ region, and these isolates persisted within a single NY state seafood processing plant. The 5’ IHT allelic type was frequently encountered among isolates from diverse sources, though at lower frequencies as compared to the WT allele. Together, the results may help to explain the underlying mechanisms behind the ability of lineage II *L. monocytogenes* isolates to adapt to both human and environmental niches.

Introduction

Listeria monocytogenes is a foodborne pathogen and the etiological agent of listeriosis, a potentially life-threating invasive disease that can affect both humans and animals (30). Over 99% of human listeriosis cases are estimated to be foodborne, and the disease carries exceptionally high hospitalization (94.0%) and mortality rates (15.9%) for a bacterial foodborne pathogen (24). Despite having been isolated from a variety of food products, the majority of human listeriosis infections (i.e., sporadic and epidemic cases) appear to be related to consumption of contaminated ready-to-eat (RTE) foods (25). Cross contamination of RTE foods in the food processing environment after the lethality step represents a major route in which RTE foods become contaminated with *L. monocytogenes* (26).

Numerous studies indicated that not all *L. monocytogenes* strains are equally associated with invasive disease. McLauchlin and colleagues (1990) determined that four (1/2a, 1/2b, 1/2c, and 4b) of the 13 serotypes of *L. monocytogenes* were responsible for 93% of 1,363 listeriosis cases examined from the U.K. (10). Additionally, multiple studies using both DNA band-based and sequence based subtyping methods showed that *L. monocytogenes* isolates form a structured population composed of at least 4 divergent lineages (i.e., lineages I, II, III, IV) (3, 15, 21, 33, 34). The majority of *L. monocytogenes* isolates belong to lineages I and II, and while both lineages contain serotypes associated with human clinical cases, a number of studies indicate that lineage I strains are overrepresented among human clinical cases as compared to lineage II strains, which are common in natural and farm environments, as well as animal listeriosis cases and sporadic human clinical cases (3, 6). Lineage III and IV strains are rare in general and are most commonly isolated from ruminants (21, 31).

The virulence factor internalin A (InlA; encoded by *inlA*) facilitates the crossing of the host intestinal barrier during establishment of a systemic infection by *L. monocytogenes* (9). Recent studies indicate that a significant proportion (around 45%) of *L. monocytogenes* isolates from RTE foods carry unique mutations leading to a premature stop codon (PMSC) in *inlA* (12, 29, 32). *L. monocytogenes* isolates carrying a PMSC in *inlA* produce a truncated form of InlA that is secreted rather than anchored to the bacterial cell wall, and have been shown to be virulence-attenuated in a guinea pig model (1, 12, 14, 28). Numerous studies suggest lineage II strains carry an *inlA* PMSC more frequently than lineage I strains, which may help to explain why lineage I strains are overrepresented in human clinical cases (5, 14, 19). To date, a total of 18 unique mutations leading a PMSC in *inlA* have been identified worldwide, and some mutations (i.e., PMSC types 1, 3, and 4) are more common than others (29). Additionally, certain PMSC mutations in *inlA* are associated with specific ribotypes. For example, ribotyope DUP-1039C isolates have been shown to carry three distinct PMSC mutations in *inlA,* including a frameshift mutation in a 5' homopolymeric tract of adenine restudies (PMSC 4), a C to T substitution leading to a PMSC at aa 562 (PMSC 7), and a G to A substitution leading to a PMSC at aa 684 (PMSC 12). (19, 22, 29).

Homopolymeric (HT) tracts of DNA are hypermutable regions frequently associated with insertion and deletion mutations due to slipped-strand mispairing (4). The hypermutability of HTs have led them to be implicated as a mechanism for phase variation (the rapid switch between two different states of protein expression) in a number of bacteria (7, 20). Previous research by our group identified a hypermutable 5' *inlA* poly(A) HT in *L. monocytogenes,* with isolates carrying either a "AAAAAAA" (WT; full frame *inlA*), or a "AAAAAA" (FS; frameshift mutation leading to truncated *inlA*) sequence beginning at nt 6 in the *inlA* open reading frame (Table 3.1). Additionally, an interrupted HT having the sequence

“AAGAAAA” (IHT; full frame *inlA*) has also been identified in all lineage I and III isolates, as well as a subset of lineage II isolates (Table 3.1) (18).

L. monocytogenes DUP-1039C isolates represent a diverse lineage II ribotype and are commonly isolated from a wide variety of sources, including food, food environments (i.e., processing plants), non-food environments (i.e., wilderness areas), and in some instances, human clinical and animal clinical cases (3, 13, 17, 23, 27). Additionally, DUP-1039C *L. monocytogenes* isolates are known to have several unique PMSC mutations in *inlA,* including PMSC types 4, 7, and 12 (29). Given the diversity and broad distribution of these isolates along the food continuum, we assembled a diverse collection (n=368) of *L. monocytogenes* DUP-1039C isolates and screened them using both a newly developed multiplex oligonucleotide probe-based genotyping assay and a previously described sequenced-based multiplex single nucleotide extension genotyping assay (27). The probe-based assay allowed us to determine the frequency and distribution of the three known alleles of a hypermutable region of *inlA*, while the sequence-based assay allowed us to determine the frequency and distribution of known *inlA* PMSC mutations. Collectively, the two assays allowed us to explore the associations between a hypermutable region in *inlA*, *inlA* PMSC mutations, and isolate source in a collection of DUP-1039C *L. monocytogenes* strains.

Materials and Methods

Bacterial isolates for genotyping experiments. Using the publicly available database PathogenTracker (www.pathogentracker.net), we assembled a representative set (n=368) of DUP-1039C *L. monocytogenes* isolates from a variety of sources. A total of 51 food (3), 82 animal (both clinical and non-clinical) (11), 19 food environment (8), 10 non-food environment (i.e., natural and urban areas) (23) , 51 farm environment (13), and 15 human clinical isolates were selected (3). Additional information on isolates used in this study can be found in supplemental table S3.1 in the appendix.

Detection of *inlA* PMSC mutations using a multiplex single nucleotide extension genotyping assay. We used a previously described multiplex single nucleotide extension genotyping assay to screen DUP-1039C *L. monocytogenes* isolates for known *inlA* PMSC mutations (27). This sequence-based assay detects known *inlA* PMSC mutations by the extension of unlabeled primers with fluorescently labeled dideoxynucleoside triphosphates (27). In the current study, this genotyping assay was used to screen 228 of the 368 total DUP-1039C *L. monocytogenes* isolates, as results for 140 of these isolates have been previously described by our group (27, 29).

Screening of the 5' *inlA* HT genotype using a newly developed multiplex oligonucleotide probe-based genotyping assay. We developed a real time PCR assay based on competitive hybridization of fluorescently labeled oligonucleotide probes (TaqMan probes, Applied Biosystems, Carlsbad, CA) to determine the allelic type of a hypermutable sequence of a 5' region of *inlA* in all 368 isolates. A summary of primers and probes designed for this assay can be found in Table 3.2. Primers RHO1*inlA*TqMnF and RHO2*inlA*TqMnR were used to amplify a 134 bp fragment of the 5' region of *inlA.* Oligonucleotide probes with MGB quencher dyes were designed, including RHO8-*inlA*-TqMn-WT, RHO9-*inlA*-TqMn-FS, and CSM1-*inlA*-

TqMn-IHT, which are specific for the wild type HT the frameshift HT, and the guanine interrupted HT, respectively. Each reaction contained 12.5 μl of the TaqMan Universal PCR Master Mix reagent (Applied Biosystems, Carlsbad, CA), 5 μM of each primer, 25 μM of each probe, and dH_2O to a volume of 25 μl. Cycling conditions were performed according to the TaqMan Universal PCR Master Mix kit manufacturer's instructions. Positive controls, for which DNA sequencing confirmed genotypes were reported by Orsi et. al (19), were used in each run for each of the tested genotypes. A reaction containing water instead of lysate or colony was used as a negative control. The genotype of each isolate was assigned depending on which probe had the highest final signal above the 1.0 ΔRn threshold after 40 cycles. If inconclusive results (i.e., no probe signal or multiple probe signals) were encountered, samples were re-ran up to three times. Upon failure of the third run to clear the ambiguity, the isolate was sequenced using *inlA* sequencing primers (Table 3.2). DNA sequencing was performed at the CSU Proteomics and Metabolomics facility (Fort Collins, CO), with samples prepared using ABI BigDye Terminator v3.1 sequencing chemistry and processed on an ABI 3130xL genetic analyzer. Sequences were assembled and proofread in DNAStar Lasergene software (DNAStar 8, Madison, WI).

Statistical analysis. All statistical analyses were performed using Statistical Analysis Systems (SAS) software (version 9.2; SAS Institute, Cary, NC), and P values of <0.05 were considered to be statistically significant in all cases. Chi-square tests of independence or Fisher's exact tests (as appropriate) were implemented using the SAS frequency procedure to describe (i) the distribution of *inlA* PMSC mutations among DUP-1039C *L. monocytogenes* isolates across source (i.e., animal clinical, animal non-clinical, food, food environment, non food environment, and human clinical), and (ii) the distribution of hypermutable 5' region in *inlA* among DUP-1039C *L. monocytogenes* isolates across source.

Results and Discussion

In the current study, we screened a collection of *L. monocytogenes* DUP-1039C isolates using both a newly developed probe-based assay and an existing sequence-based assay to gain a better understanding of both the frequency and distribution of *inlA* PMSC mutations and the nature of a hypermutable 5' region of *inlA*. Our results support increasing evidence that *L. monocytogenes* isolates from food and food environments frequently contain PMSC mutations in *inlA* leading to a truncated InlA protein as compared to isolates from human and animal cases. Additionally, the newly developed probe-based assay described here represents a high throughput means of interrogating the allelic type of a hypermutable 5' region of *inlA* in *L. monocytogenes* isolates. Results from this assay indicate the WT allele to be common in human and animal isolates, the FS allele to be common in food isolates, and the IHT allele to be common in isolates from both food and food environments.

Multiplex genotyping of *L. monocytogenes* DUP-1039C isolates indicates isolates carrying a PMSC in *inlA* are more common in foods or food associated environments than mammalian hosts. We used an existing sequence-based assay developed by our group to screen 228 *L. monocytogenes* DUP-1039C isolates to support an ongoing effort to investigate the distribution of *inlA* PMSC mutations among *L. monocytogenes* isolates from various sources (27, 29). These 228 isolates were chosen for screening because they represent DUP-1039C *L. monocytogenes* isolates not previously screened by our group from studies which were focused on a much larger and diverse set of *L. monocytogenes* ribotypes (27, 29). Categorical data analyses were performed to describe the distribution of *inlA* PMSC mutations among different isolate sources (i.e., human clinical, food, food environment, non-food environment, farm environment, animal clinical, and animal non-clinical) for isolates screened by the SNP genotyping assay. Chi-square tests of independence indicated that *L. monocytogenes* isolates containing *inlA* PMSC mutations were not uniformly distributed among these isolation sources ($P < 0.05$). More specifically, among DUP-1039C *L. monocytogenes* strains screened in this study, isolates containing a PMSC mutation in *inlA* were frequently sourced from food and food-associated environments; whereas, isolates encoding a full-length InlA were overrepresented among non-food environments, farm environments, and mammalian hosts (Table 3.3). These results are consistent with previous studies that indicate *inlA* PMSC mutations are frequently encountered in *L. monocytogenes* lineage II isolates sourced from food and food related environments, which may help explain the apparent underrepresentation of lineage II *L. monocytogenes* isolates from human and animal cases (12, 14, 27, 29, 32).

In this study, PMSC type 7 represented the most frequently encountered (n=25) mutation event leading to a truncated InlA previously described among DUP-1039C isolates (Figure 3.1). PMSC type 4 was the only other mutation type encountered (n=19; Figure 3.1), as PMSC type 12 was not identified. Source information available in PathogenTracker revealed PMSC type 7 mutations encountered in this study (n=25) to be exclusive to *L. monocytogenes* DUP-1039C isolates collected from an individual seafood processing plant in New York during 1998 through 2000 (16). In a screen of numerous *L. monocytogenes* isolates from both human clinical cases and from foods across several ribotypes, Van Stelten and colleagues (2008) identified *inlA* PMSC type 7 only among ribotypes DUP-1039C and DUP-1048A and only among isolates from this same seafood plant (27). These results indicate *L. monocytogenes* carrying *inlA* PMSC type 7 representing two distinct *EcoRI* ribotypes (i.e., DUP-1039C and DUP-1048A), which were isolated from multiple sites (i.e., drains, food contact surfaces, finished product, raw materials) across a time period of nearly two years within an individual seafood plant.

inlA PMSC type 4 was encountered in 19 isolates screened in this study (Figure 3.1). PathogenTracker source information revealed isolates with *inlA* PMSC type 4 in this study to be from diverse sample types, including human sporadic cases, food (i.e., raw ingredients, finished product), food environments, and non-food environments, with these sources often being from unique geographical locations. Previous studies have reported identifying *inlA* PMSC type 4 in *L. monocytogenes* isolates in multiple sample types from various locations (2, 19). Together, these results indicate that this mutation has likely accumulated at the population level, perhaps because it occurs within a hypermutable 5' poly(A) HT in *L. monocytogenes* isolates (29).

A high-throughput probe-based genotyping assay shows the WT allele of a hypermutable region of *inlA* to be predominant among a set of DUP-1039C *L. monocytogenes* isolates from diverse sources.

We developed a multiplex probe-based real time PCR assay to investigate the frequency and distribution of alleles of a hypermutable 5' region of *inlA* in lineage II *L. monocytogenes* isolates (19). Unlike the sequence-based assay which can only identify the WT and HT alleles within this region, the probe-based assay is capable of identifying the three known (i.e., WT, FS, and IHT) 5' *inlA* allelic types based on competitive hybridization of oligonucleotide probes. More specifically, the probe complimentary to the given *inlA* allele will preferentially bind to the template DNA in the initial cycles of the reaction, and thus will be amplified to greater levels than that of the other probes by the end of the reaction. Since this assay and the previously described sequence-based assay are both capable of detecting *inlA* PMSC type 4, direct comparison of results were made to ensure results from both assays were in agreement. Of the 368 isolates screened, only two isolates displayed ambiguous results, and targeted *inlA* sequencing was used to resolve these ambiguities. The sequence-based assay failed to detect PMSC 4 in one isolate, whereas the probe-based assay failed to detect *inlA* PMSC type 4 in a different isolate, indicating that both assays are capable of detecting *inlA* PMSC type 4 with highly accurate and reproducible results.

Results of the probe-based assay showed the majority of DUP-1039C *L. monocytogenes* isolates to carry the WT *inlA* allele, though the FS *inlA* allele was overrepresented in food isolates and the IHT allele was overrepresented in food and food-environment isolates (Figure 3.2). The overrepresentation of the FS allele (which leads to a PMSC in *inlA*) in food isolates has been previously reported in several studies (12, 19, 29, 32). *L. monocytogenes* strains containing the FS allele may represent generalist strains that have evolved a phase variation mechanism for *inlA*, allowing for selective silencing of *inlA* in environments (i.e., food and food processing environments) where infectivity is not needed (19).

Previous studies by our group have shown the IHT allele in *inlA* allows for stable expression of this key virulence gene (18). Additionally, the IHT allele is exclusive among all lineage I and III isolates which are associated with human and animal clinical cases, respectively (18). This information has led to the hypothesis that the presence of the IHT allele in *inlA* among some lineage II isolates resulted from horizontal gene transfer and allows for adaptation to colonization/infection of mammalian hosts (19). We thus expected to see overrepresentation of the IHT allele in isolates sourced from human and animal clinical cases; however, the IHT allele was overrepresented among isolates from food and food environment sources (Figure 3.2). Further examination of isolate information in PathogenTracker revealed the majority of these isolates from food and food environments containing the IHT *inlA* allele (n=79) to also have *inlA* PMSC type 7, and were all obtained from the same seafood processing plant in New York during 1998 through 2000, as noted above (16). Additionally, a smaller portion (n=13) of *L. monocytogenes* isolates from food samples obtained in this particular seafood plant were characterized by the A_2GA_4 5' *inlA* HT without a downstream *inlA* PMSC type 7 (i.e., encoding a full length InlA). Therefore, the results of this probe-based assay suggest that a DUP-1039C *L.*

monocytogenes strain displaying the lineage I ancestral IHT allele was introduced into the environment of this particular NY seafood plant, and a subsequent point mutation yielding *inlA* PMSC type 7 allowed this strain to flourish in the environment of this plant, where it regularly outcompeted strains encoding a full length *inlA*.

Conclusions

Studies have shown *L. monocytogenes* lineages to have varying pathogenic potential, and lineage II isolates, while most commonly encountered in food and food settings, are sometimes implicated in sporadic human clinical and animal clinical listeriosis cases. In an attempt to better understand this dual role of lineage II *L. monocytogenes* strains, we used a sequence-based genotyping assay to screen a diverse set of DUP-1039C *L. monocytogenes* isolates to determine the frequency and distribution of *inlA* PMSC mutations among these strains. Additionally, we developed and implemented a high throughput multiplex probe-based assay to identify the various alleles of a hypermutable region of *inlA* among these isolates. Results from this study contribute to a large body of evidence that *L. monocytogenes* isolates from food and food environments frequently contain PMSC mutations in *inlA*. Additionally, the IHT allele of *inlA*, which is thought to be associated with disease adapted lineage II isolates, is frequently encountered among isolates from diverse sources, though at lower frequencies as compared to the wild type A_7 allele.

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their assistance with freezing down isolates and with preparation of bacterial lysates.

Chapter 3: Tables and Figures

Table 3.1: List of allelic types associated with a hypermutable region in the 5’ end of *inlA*.

Allelic types of a hypermutable 5' region of *inlA*

Wild Type *inlA*

5'-gtg ag<u>a aaa aaa</u> cga tat gta tgg ttg aaa agt ata-3'

inlA with PMSC4 — PMSC4

5'-gtg ag<u>a aaa aa</u>c gat atg tat ggt tga aaa gta t-3'

inlA with HT interruption

5'-gtg ag<u>a aga aaa</u> cga tat gta tgg ttg aaa agt ata-3'

Table 3.2: List of primers and probes used in this study.

Primer or Probe	Sequence (5' to 3')	Purpose	Reference
RHO1*inlA*TqMnF	CGG ATG CAG GAG AAA ATC CTA TAC	Amplification of a 134 bp fragment of the 5' region of *inlA*	This study
RHO2*inlA*TqMnR	CGC TGC CAA ATA CTA ATA TTG CTA CTA G		
inlA proF	TTT TAA AAG GTG GAA TGA CA	Sequencing primer for entire *inlA* ORF	Orsi 2007
inlA proR	GAA GCG TTG TAA CTT GGT CTA		
inlA F1	CAG GCA GCT ACA ATT ACA CA	Sequencing primer for entire *inlA* ORF	Orsi 2007
inlA S1R	GGA CTG ATG TTA CTT ATT TGG Tb		
inlA F2	AAG ATA TAG GCA CAT TGG CGA GTT	Sequencing primer for entire *inlA* ORF	Orsi 2007
inlA S2R	CGT ACT GAA ATY CCA KTT AGT TCCb		
inlA seq	GTG GAC GGC AAA GAA ACA AC	Sequencing primer for entire *inlA* ORF	Orsi 2007
F *inlA* R	ATA TAG TCC GAA AAC CAC ATC T		
RHO8-*inlA*-TqMn-WT	TAGTGAGAAAAAAACGATATG; Reporter: FAM; Quencher: MGB	TaqMan probe for detection of WT *inlA* allele	This study
RHO9-*inlA*-TqMn-FS	AGTGAGAAAAAACGATATG; Reporter: VIC; Quencher: MGB	TaqMan probe for detection of FS *inlA* allele	This study
CSM1-*inlA*-TqMn-IHT	ATAGTGAGAAGAAAACGATATG; Reporter: NED Quencher: MGB	TaqMan probe for detection of IHT *inlA* allele	This study

Table 3.3: Frequency and distribution of *inlA* genotypes across isolate source.

Isolate Source	No. of isolates with indicated *inlA* genotype (no. of isolates screened)[a,b]	
	Truncated *inlA*[c]	Full length *inlA*
Human Clinical	2 (15)	13 (15)
Food	34 (51)	17 (51)
Food Environment	7 (19)	12 (19)
Non-Food Environment[d]	1 (10)	9 (10)
Farm Environment	0 (51)	51 (51)
Animal Clinical	0 (22)	22 (22)
Animal non-Clinical	0 (60)	60 (60)

[a] Chi-square analysis of a 2x7 (*inlA* genotype by isolate source) contingency table determined unequal distribution of PMSC strains across all sources ($P < 0.001$).
[b] A total of 228 isolates were screened by the multiplex SNP genotyping assay, as results for 140 of the DUP-1039C *L. monocytogenes* isolates in this study have been described previously by Van Stelten et al. 2008 and Van Stelten et al. 2010.
[c] Number of isolates containing any PMSC type.
[d] Represent isolates from urban and natural (i.e., wilderness areas) environments

Figure 3.1: Frequency and distribution of *inlA* PMSC types among source of isolates.

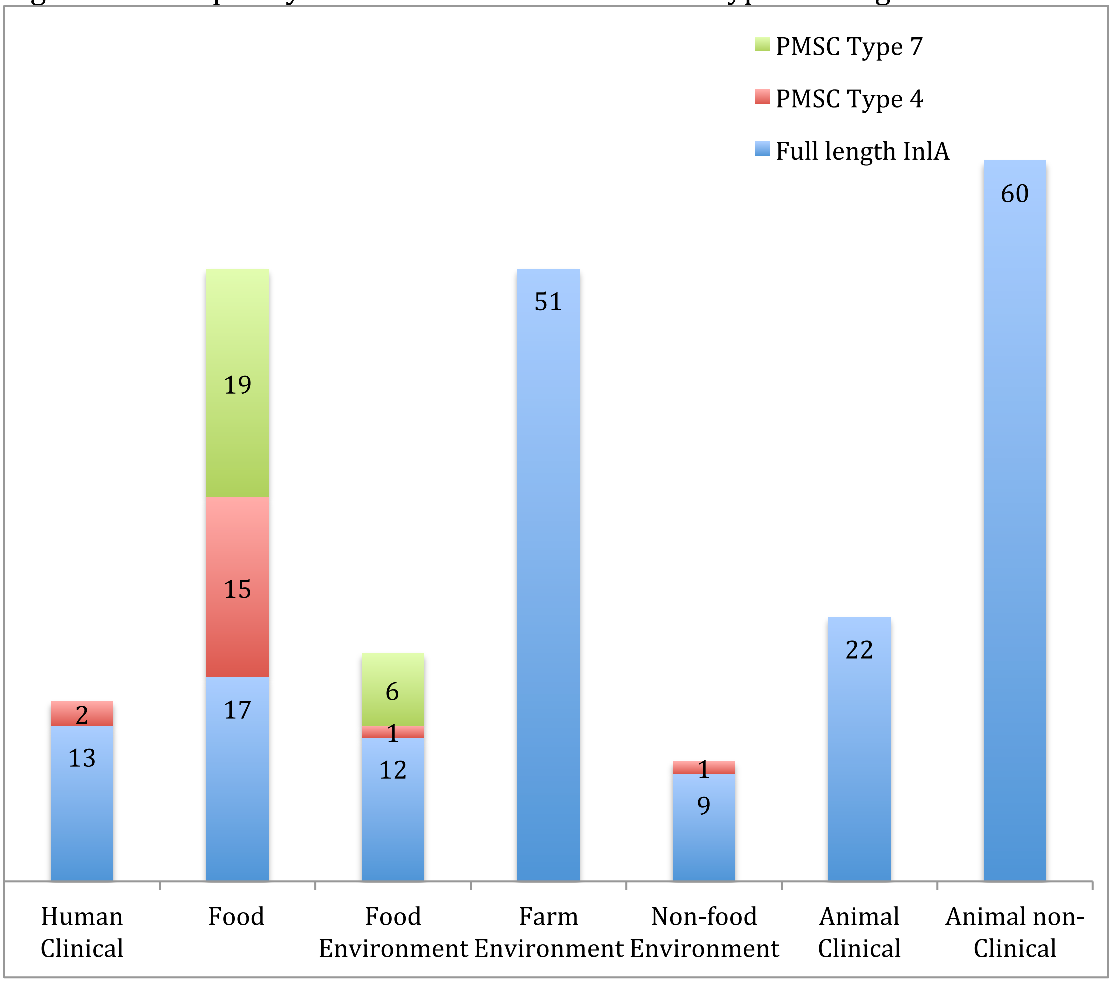
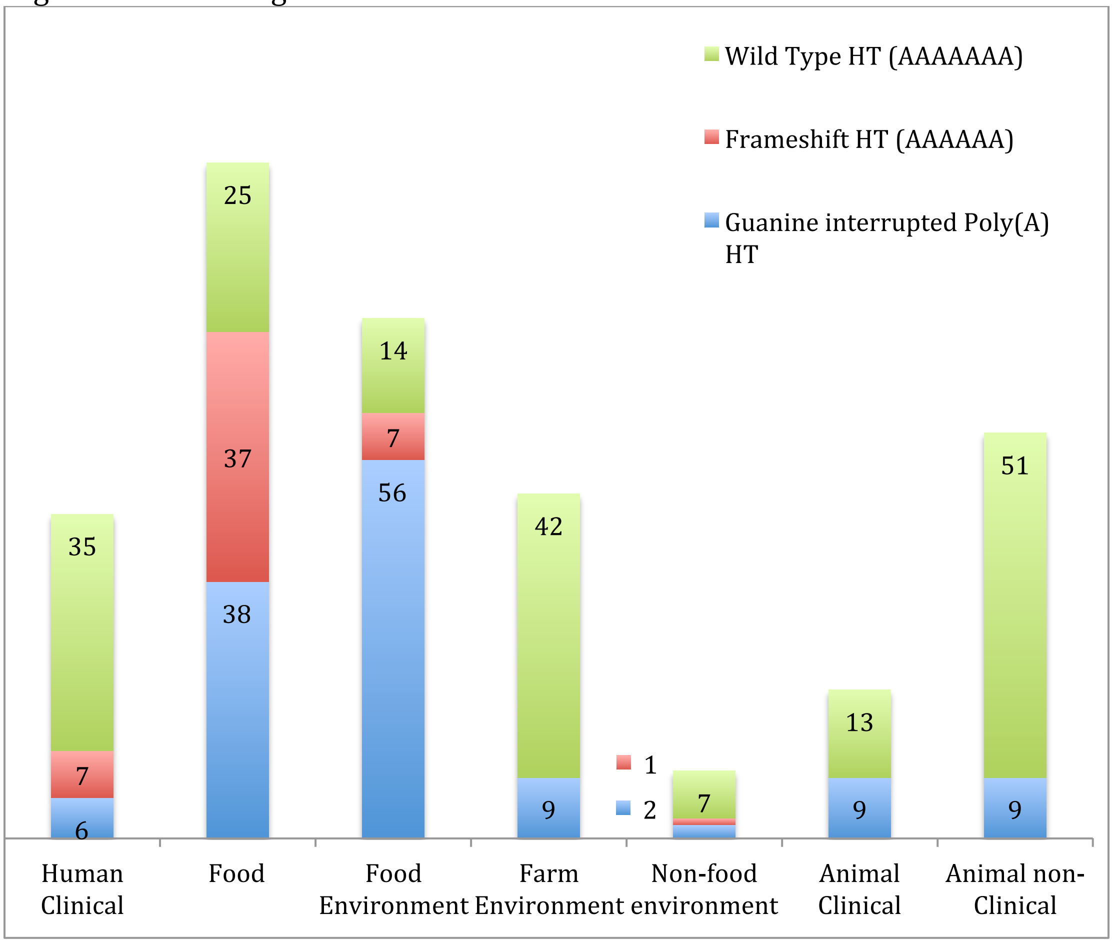


Figure 3.2: Frequency and distribution of known allelic types of a hypermutable 5' region of *inlA* among source of isolates.



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APPENDIX

Detailed information on the isolates used in this study.

Cornell FSL ID	Isolate Source	year	state	country
FSL C1-098	human	1998	NY	
FSL C1-115	human	1998		
FSL C1-117	human	1998		
FSL E1-001	animal, clinical	1997	NY	
FSL E1-010	animal, clinical	1998	NY	
FSL E1-015	animal, clinical	1997	NY	USA
FSL E1-017	animal, clinical	1998	NY	USA
FSL E1-019	animal, clinical	1998	NY	USA
FSL E1-023	animal, clinical	1996	NY	USA
FSL E1-043	animal, clinical	2000	NY	USA
FSL E1-046	animal, clinical	1999	NY	USA
FSL E1-056	animal, clinical	1999	NY	USA
FSL E1-122	animal, clinical	2000	PA	USA
FSL e1-123	animal, clinical	2001	NY	USA
FSL E1-126	animal, non-clinical	2001	NY	USA
FSL E1-153	environment, farm	2001	NY	USA
FSL E1-202	environment, farm	2001		USA
FSL F2-034	human	1999		USA
FSL F2-035	human	1999		USA
FSL F2-115	human	1999	MI	USA
FSL F2-170	human	1999	NY	USA
FSL F2-215	human	1999	OH	USA
FSL F2-240	food	2000	NY	USA
FSL F2-250	food	2000	NY	USA
FSL F2-255	environment, food	2000		USA
FSL F2-256	environment, food	2000		USA
FSL F2-257	environment, food	2000		USA
FSL F2-258	environment, food	2000		USA
FSL F2-259	environment, food	2000		USA
FSL F2-260	environment, food	2000		USA
FSL F2-261	environment, food	2000		USA
FSL F2-262	environment, food	2000		USA
FSL F2-263	environment, food	2000		USA
FSL F2-264	environment, food	2000		USA
FSL F2-265	environment, food	2000		USA
FSL F2-308	human	2000	OH	USA
FSL F2-362	human	2000	NY	USA
FSL F2-364	human	2000		USA
FSL F2-365	human	2000		USA
FSL F2-373	food	2000	NY	USA
FSL F2-374	food	2000	NY	USA
FSL F2-388	food	2000	CA	USA

FSL F2-396	food	2000	NY	USA
FSL F2-512	human	2000	NY	USA
FSL F2-546	human	2000	NY	USA
FSL F2-553	human	2000	NY	USA
FSL f2-560	human	2000	NY	USA
FSL F2-562	human	2000		USA
FSL F2-640	food	2001	NY	USA
FSL F2-664	human	2001	NY	USA
FSL F2-683	human	2001	OH	USA
FSL F2-723	food	2001	NY	USA
FSL F2-724	food	2001	NY	USA
FSL F2-725	environment, food	2001	NY	USA
FSL F2-727	animal, non-clinical	2002	NY	USA
FSL F2-728	animal, non-clinical	2002	NY	USA
FSL F2-729	animal, non-clinical	2002	NY	USA
FSL F2-732	environment, farm	2002	NY	USA
FSL F2-733	environment, farm	2002	NY	USA
FSL F2-741	environment, farm	2002	NY	USA
FSL F2-923	food	2002	NY	
FSL f2-924	food	2002	NY	USA
FSL F2-935	food	2002	NY	USA
FSL F2-936	food	2002	NY	
FSL F2-937	food	2002	NY	
FSL F2-939	food	2002	NY	USA
FSL F2-940	environment, food	2002	NY	USA
FSL F2-941	environment, food	2002	NY	USA
FSL F2-943	environment, food	2002	NY	USA
FSL F2-944	environment, food	2002	NY	
FSL F2-947	environment, food	2002	NY	
FSL F2-950	food	2002	NY	USA
FSL F2-957	food	2002	NY	
FSL F2-958	food	2002	NY	USA
FSL F2-978	human	2002	NY	USA
FSL F2-980	human	2002	NY	USA
FSL F3-007	human	2002	NY	USA
FSL F3-043	animal, non-clinical	2002	VT	USA
FSL F3-056	animal, non-clinical	2002	NY	USA
FSL F3-142	animal, non-clinical	2002	NY	USA
FSL F3-230	animal, non-clinical	2003		
FSL F3-290	environment, farm	2003		USA
FSL F3-303	environment, farm			USA
FSL F3-305	environment, farm			USA
FSL F3-311	environment, farm			USA
FSL F3-323	environment, farm			USA
FSL F3-532	human	2003	NY	USA
FSL F3-544	human	2003	NY	USA
FSL F3-548	human	2003	NY	USA
FSL F3-549	human	2003	NY	USA
FSL F3-550	animal, clinical	2003	NY	
FSL F3-581	human	2004	NY	USA
FSL F3-582	animal, clinical	2004	NY	USA
FSL F3-583	animal, clinical	2004	NY	

FSL F3-776	human	2003	MI	
FSL F3-784	human	2003	MI	
FSL F3-791	human	2003	MI	USA
FSL F3-797	human	2004	MI	USA
FSL F3-817	human	2004	NY	USA
FSL F3-824	human	2004	NY	
FSL F3-865	human	2004	NY	USA
FSL H1-003	environment, food	2000		USA
FSL H1-018	environment, food	2000		USA
FSL H1-024	environment, food	2000		USA
FSL H1-028	environment, food	2000		
FSL H1-034	environment, food	2000		USA
FSL H1-051	environment, food	2000		USA
FSL H1-052	environment, food	2000		
FSL H1-055	environment, food	2000		
FSL H1-059	environment, food	2000		
FSL H1-061	environment, food	2000		
FSL H1-067	environment, food	2000		
FSL H1-077	environment, food	2000		
FSL H1-085	food	2000		
FSL H1-088	food	2000		
FSL H1-091	food	2000		
FSL H1-094	environment, food	2000		
FSL H1-097	environment, food	2000		
FSL H1-103	environment, food	2000		USA
FSL H1-129	environment, food	2000		USA
FSL H1-134	environment, food	2000		USA
FSL H1-182	environment, food	2000		USA
FSL H1-239	environment, food	2000		USA
FSL H1-240	environment, food	2000		USA
FSL H1-266	food	2000		
FSL H1-268	food	2000		
FSL H1-276	environment, food	2000		
FSL H1-294	food	2000		
FSL H1-298	food	2000		
FSL H1-310	environment, food	2000		
FSL H1-314	food	2000		
FSL H1-347	food	2000		
FSL H1-353	environment, food	2000		
FSL H1-367	environment, food	2000		
FSL H1-371	food	2000		
FSL H1-375	environment, food	2000		
FSL H1-379	environment, food	2000		
FSL H1-399	environment, food	2000		
FSL H1-402	environment, food	2000		
FSL H1-430	environment, food	2000		
FSL H1-434	environment, food	2000		
FSL H1-451	environment, food	2000		
FSL H1-463	environment, food	2000		
FSL H1-474	environment, food	2000		
FSL H1-478	environment, food	2000		
FSL H1-486	environment, food	2000		

FSL H1-493	food	2000		
FSL H1-496	food	2000		
FSL H1-497	food	2000		
FSL H1-501	food	2000		
FSL H1-502	food	2000		
FSL H1-514	food	2000		
FSL H1-518	food	2000		
FSL H1-522	food	2000		
FSL H1-525	food	2000		
FSL H1-529	food	2000		
FSL H1-533	food	2000		
FSL H1-542	food	2000		
FSL H4-104	environment, farm	2003	NY	
FSL H4-107	environment, farm	2003	NY	
FSL H4-119	environment, farm	2003	NY	USA
FSL H4-129	environment, farm	2003	NY	USA
FSL H4-148	environment, farm	2003	NY	USA
FSL H4-158	animal, non-clinical	2003	NY	USA
FSL H4-396	environment, farm	2003	NY	USA
FSL H4-449	environment, farm	2003	NY	
FSL H4-654	animal, non-clinical	2004	NY	
FSL H4-850	animal, non-clinical	2004	NY	
FSL H4-861	animal, non-clinical	2004	NY	
FSL H4-869	animal, non-clinical	2004	NY	
FSL H4-871	animal, non-clinical	2004	NY	
FSL H4-981	animal, non-clinical	2004	NY	
FSL H5-011	animal, non-clinical	2004	NY	
FSL H5-012	animal, non-clinical	2004	NY	
FSL H5-093	animal, non-clinical	2004	NY	
FSL H5-116	animal, non-clinical	2004	NY	
FSL H5-169	animal, non-clinical	2004	NY	USA
FSL H5-184	animal, non-clinical	2004	NY	
FSL H5-194	animal, non-clinical	2004	NY	
FSL H5-249	environment, farm	2004	NY	
FSL H5-251	animal, non-clinical	2004	NY	
FSL H5-260	animal, non-clinical	2004	NY	
FSL H5-277	animal, non-clinical	2004	NY	
FSL H5-295	animal, non-clinical	2004	NY	
FSL H5-297	animal, non-clinical	2004	NY	
FSL H5-338	environment, farm	2004	NY	USA
FSL H5-346	animal, non-clinical	2004	NY	USA
FSL H5-416	animal, non-clinical	2004	NY	USA
FSL H5-425	animal, non-clinical	2004	NY	USA
FSL H5-439	animal, non-clinical	2004	NY	USA
FSL H5-446	animal, non-clinical	2004	NY	USA
FSL H5-449	environment, farm	2004	NY	USA
FSL H5-458	animal, non-clinical	2004	NY	USA
FSL H5-459	animal, non-clinical	2004	NY	USA
FSL H5-461	animal, non-clinical	2004	NY	USA
FSL H5-476	animal, non-clinical	2004	NY	
FSL H5-483	animal, non-clinical	2004	NY	
FSL H5-484	animal, non-clinical	2004	NY	

FSL H5-487	animal, non-clinical	2004	NY	
FSL H5-493	animal, non-clinical	2004	NY	
FSL H5-494	animal, non-clinical	2004	NY	
FSL H5-497	animal, non-clinical	2004	NY	
FSL H5-498	animal, non-clinical	2004	NY	
FSL H5-504	animal, non-clinical	2004	NY	
FSL H5-508	animal, non-clinical	2004	NY	
FSL H5-513	animal, non-clinical	2004	NY	
FSL H5-571	environment, farm	2004	NY	
FSL H5-573	environment, farm	2004	NY	USA
FSL J1-022	human			USA
FSL J1-047	human			USA
FSL J1-196	human	1997	NY	USA
FSL J1-216	human	1997	NY	USA
FSL J2-002	animal, clinical	1992	NY	USA
FSL J2-003	animal, clinical	1993	NY	USA
FSL J2-005	animal, clinical	1987	NY	USA
FSL J2-011	animal, clinical	1991	NY	USA
FSL J2-018	animal, clinical	1995	NY	USA
FSL J2-020	animal, clinical	1986	NY	USA
FSL J2-032	animal, clinical	1996	NY	USA
FSL K2-237	environment, food	2000		USA
FSL L3-511	environment, food	2002		USA
FSL L3-802	food	2002		USA
FSL L4-160	food	2002		USA
FSL L4-161	food	2002		USA
FSL L4-182	environment, food	2002		USA
FSL L4-257	environment, food	2002		
FSL L4-332	environment, food	2002		USA
FSL L4-368	food	2002		USA
FSL L4-396	environment, food	2002		USA
FSL L4-467	food	2002		USA
FSL L4-469	food	2002		USA
FSL L4-471	food	2002		USA
FSL L4-473	food	2002		USA
FSL M2-073	Human			USA
FSL N1-037	environment, food	1998		USA
FSL N1-054	food	1998		USA
FSL N1-055	food	1998		USA
FSL N1-058	food	1998		USA
FSL N1-059	food	1998		USA
FSL N1-062	food	1998		
FSL N1-063	food	1998		
FSL N1-104	environment, food	1998		
FSL N1-109	food	1998		
FSL N1-174	environment, food	1998		
FSL N1-232	human	1998		
FSL N1-240	human	1998	NY	
FSL N1-253	food	1998		
FSL N1-263	human			
FSL N1-325	environment, food	1998		
FSL N1-328	environment, food	1998		

FSL N1-344	environment, food	1998		
FSL N1-345	environment, food	1998		
FSL N1-346	environment, food	1998		
FSL N1-348	food	1998		
FSL N1-395	environment, food	1998		
FSL N1-405	environment, food	1998		
FSL N1-449	environment, food	1998		
FSL N3-127	animal, non-clinical		NY	
FSL N3-130	animal, non-clinical		NY	
FSL N3-131	animal, non-clinical		NY	
FSL N3-160	environment, farm	2002	NY	
FSL N3-166	environment, farm	2002	NY	USA
FSL N3-185	environment, farm	2002	NY	USA
FSL N3-224	animal, non-clinical	2002	NY	USA
FSL N3-226	animal, non-clinical	2002	NY	USA
FSL N3-229	animal, non-clinical	2002	NY	USA
FSL N3-260	environment, farm	2002	NY	USA
FSL N3-273	environment, farm	2002	NY	
FSL N3-302	environment, farm	2002	NY	
FSL N3-303	animal, non-clinical	2002	NY	
FSL N3-310	environment, farm	2002	NY	
FSL N3-312	environment, farm	2002	NY	
FSL N3-316	animal, non-clinical	2002	NY	
FSL N3-336	environment, farm	2002	NY	
FSL N3-687	environment, farm	2002	NY	
FSL N3-786	environment, farm	2002	NY	
FSL N3-804	environment, farm	2002	NY	
FSL N3-814	environment, farm	2002	NY	
FSL N3-815	environment, farm	2002	NY	
FSL N3-816	environment, farm	2002	NY	
FSL n3-967	animal, non-clinical	2002	NY	
FSL N4-008	environment, farm	2002	NY	
FSL N4-030	environment, farm	2002	NY	
FSL N4-041	environment, farm	2002	NY	
FSL N4-043	environment, farm	2002	NY	
FSL N4-198	environment, farm	2001	NY	
FSL N4-225	environment, farm	2002	NY	
FSL N4-227	environment, farm	2002	NY	
FSL N4-241	environment, farm	2002	NY	
FSL N4-290	animal, clinical	2001	NY	
FSL N4-335	animal, non-clinical	2002	NY	
FSL N4-373	environment, farm	2002	NY	
FSL N4-375	environment, farm	2002	NY	
FSL N4-379	environment, farm	2002	NY	
FSL N4-381	environment, farm	2002	NY	
FSL N4-383	environment, farm	2002	NY	
FSL N4-536	environment, farm	2002	NY	
FSL N4-661	animal, non-clinical	2002	NY	
FSL N4-663	animal, non-clinical	2002	NY	
FSL N4-694	animal, non-clinical	2002	NY	
FSL N4-695	animal, non-clinical	2002	NY	
FSL R2-046	food	2000	MD	

FSL R2-047	food	2000	MD	
FSL R2-093	food	2000	CA	
FSL R2-100	food	2000	CA	
FSL R2-118	food	2001	CA	
FSL R2-120	food	2001	CA	
FSL R2-129	food	2001	MD	
FSL R2-130	food	2001	MD	
FSL R2-169	food	2001	CA	
FSL R2-184	food	2001	CA	
FSL R2-189	food	2001	MD	China
FSL R2-230	food	2001	CA	China
FSL R2-241	food	2001	MD	England
FSL R2-242	food	2001	MD	
FSL R2-244	food	2001	MD	USA
FSL R2-272	food	2001	MD	USA
FSL R2-305	human	2001	MD	
FSL R2-306	human	2001	MD	USA
FSL R2-308	human	2001	MD	
FSL R2-328	food	2001	MD	
FSL R2-360	food	2001	CA	USA
FSL R2-365	food	2001	CA	USA
FSL R2-376	food	2001	MD	
FSL r2-382	food		CA	
FSL R2-383	food	2001	CA	
FSL R2-404	food	2001	CA	USA
FSL R2-419	food	2001	MD	USA
FSL R2-440	food	2001	CA	USA
FSL R2-441	food	2001	CA	USA
FSL R2-442	food	2001	CA	USA
FSL R2-443	food	2001	CA	
FSL R2-462	food	2001	MD	
FSL R2-466	food	2001	MD	USA
FSL R2-472	food	2001	MD	USA
FSL R2-483	food	2001	MD	USA
FSL R2-496	food	2001	CA	USA
FSL R2-546	food	2002	CA	
FSL R2-553	food	2002	CA	USA
FSL R2-561	human			USA
FSL R2-637	human			USA
FSL R2-641	human			USA
FSL R2-656	food			USA
FSL R2-657	food			USA
FSL R2-674	food			USA
FSL S4-380	environment, non-food	2002	NY	USA
FSL S4-497	environment, non-food	2002	NY	USA
FSL S4-640	environment, non-food	2002	NY	USA
FSL S4-742	environment, non-food	2002	NY	USA
FSL S4-821	environment, non-food	2002	NY	USA
FSL S4-835	environment, non-food	2002	NY	USA
FSL S4-880	environment, non-food	2002	NY	USA
FSL S4-914	environment, non-food	2002	NY	USA
FSL S6-033	environment, non-food	2002	NY	USA

FSL S6-147	environment, non-food	2002	NY	USA
FSL T1-023	environment, food	2001		
FSL T1-061	environment, food	2001		
FSL T1-119	environment, food			USA
FSL T1-181	food	2001		USA
FSL T1-189	environment, food	2001		
FSL T1-295	environment, food	2001		USA
FSL T1-301	food	2001	LA	
FSL T1-358	environment, food	2001		
FSL T1-362	food	2001		
FSL T1-580	food	2001		
FSL T1-584	environment, food	2001		England
FSL T1-946	environment, food	2001		
FSL W1-047	food			