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A Multi-state Molecular Epidemiological Survey of 16 Beef, Dairy, Poultry and Swine Farms to Facilitate Risk Assessment of Listeria monocytogenes

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To the Graduate Council:

I am submitting herewith a dissertation written by David Dean Rasmussen entitled "A Multi-state Molecular Epidemiological Survey of 16 Beef, Dairy, Poultry and Swine Farms to Facilitate Risk Assessment of Listeria monocytogenes." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Food Science and Technology.

F. Ann Draughon, Major Professor

We have read this dissertation and recommend its acceptance:

John New, John Mount, Svetlana Zivanovic

Accepted for the Council:

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Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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A MULTI-STATE MOLECULAR EPIDEMIOLOGICAL SURVEY OF 16 BEEF, DAIRY, POULTRY AND SWINE FARMS TO FACILITATE RISK ASSESSEMENT OF LISTERIA MONOCYTOGENES

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

David Dean Rasmussen
December 2004

DEDICATION

This dissertation is dedicated to Jesus Christ,

who along with my family and friends,

loved, strengthened and encouraged me to fulfill this dream

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I want to thank Jesus Christ, for all the blessings He has given me. It is only by His grace and love that I am here today.

To my family and close friends, especially Suzanne Mahan, thank you, for being so loving and supportive over the past three years. Thank you for believing in me and encouraging me. I Love You!

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ABSTRACT

Listeria monocytogenes is an intracellular pathogen that poses serious risks for immuno-compromised individuals. On the farm risk assessment is needed to reduce exposure of such individuals to this pathogen. Base line epidemiological surveys for this pathogen are needed to identify common ecological reservoirs and sources. A 21 month survey of 4 animal farm types for *L. monocytogenes* in five different states was conducted to determine the level of occurrence in various environments. An overall occurrence of 1.4% (20/1432 samples) for *L. monocytogenes* was observed in this 21 month survey. Each sample type (soil, bedding/litter, feed/grass and animal rectal swabs) produced an isolate of *L. monocytogenes*. The Winter and Spring of 2003 produced the greatest number of isolates (18 of 20 total isolates). Feed and animal rectal swabs exhibited the highest number of isolates, eight and 10, respectively. The Simpson's Index of Diversity for RiboPrinting™ of the isolates at 100% similarity was 0.9737, for PFGE with *Asc*I 0.9684 and PFGE with *Apa*I 0.9842, indicating a parity amongst the two characterization methods. Clonal (100% similar) isolates were seen amongst samples taken from non-contiguous states and multiple isolates from the same farm with differing genetic compositions were isolated, yet no clear regional relationships were evident. Enrichment types and enrichment incubation times were not significant for isolating *L. monocytogenes*, having equivalent odds for isolating *L. monocytogenes*. Selective plating media was nearly significant with a point estimate of 1.569 (p=0.0806), which when the highest season (third) was analyzed alone the significance level dropped (p=0.0584).

TABLE OF CONTENTS

PART		PAGE
I.	**LITERATURE REVIEW**……	1
	Listeria monocytogenes…….	2
	Prevalence of *Listeria monocytogenes*……..	5
	Molecular Identification and Characterization……....	16
	Risk Assessment and Management……	19
	Agricultural Surveillance……	21
	Purpose of Research……..……..	22
	References…..……..	23
II.	**A COMPARISON OF ENRICHMENT BROTHS AND SELECTIVE PLATING MEDIA FOR *LISTERIA MONOCYTOGENES* ISOLATED FROM FOUR ANIMAL FARM ENVIRONMENTS**……	30
	Abstract……	31
	Introduction…….	32
	Materials and Methods…….	35
	Experimental Design…….....	35
	Farm Sampling……..	36
	Methods Comparison…….....	36
	Biochemical Confirmation….. …….....	37
	Statistical Analysis…….....	37
	Results ……..	37
	Discussion…….…….	39
	References…….	41
	Appendix II: Tables and Figures…….…….	45
III.	**COMPARISON OF RIBOPRINTING™ AND PULSED-FIELD GEL ELECTROPHORESIS TO CHARACTERIZE LISTERIA MONOCYTOGENES TO EXAMINE GEOGRAPHICAL RELATIONSHIPS BETWEEN ISOLATES FROM VARIOUS ANIMAL FARM ENVIRONMENTS** …….....	48
	Abstract…….	49
	Introduction……..	50
	Materials and Methods……	53
	Comparison of PFGE and RiboPrinting™……	53
	Results …….	54
	Discussion……	57
	References…..……	60
	Appendix III: Tables and Figures……..	63

IV. **A MULTI-STATE SURVEY OF 16 BEEF, DAIRY, POULTRY AND SWINE FARMS FOR *LISTERIA MONOCYTOGENES***..... 68
Abstract..... 69
Introduction..... 70
Materials and Methods..... 72
Sample Collection..... 72
Isolation and Confirmation of *L. monocytogenes* 73
Molecular Characterization..... 73
Aerobic Plate Count..... 74
Total Coliforms/*Escherichia coli*..... 74
Fecal Streptococcus..... 75
Results 75
Discussion..... 76
References..... 83
Appendix IV: Tables and Figures..... 87

V. **APPENDIX: FARMER SAMPLING PROTOCOL**..... 99

VI. **Appendix: PFGE PROTOCOL**..... 104

VITA..... 111

LIST OF TABLES

TABLE		PAGE
PART II.		
1.	Media comparison for *L. monocytogenes* from farm environmental samples from September 2002 to May 2003 using logistic regression……..	46
2.	Media comparison for *L. monocytogenes* from farm environmental samples from March 2003 to May 2003 using logistic regression…….	47
PART III.		
1.	Type and frequency Dupont ID formed clusters amongst isolates of *L. monocytogenes* …….	64
PART IV.		
1	The occurrence of isolation of *L. monocytogenes* per animal and sample type in five states from September 2002 to May 2004……..……..	88
2	Seasonal distribution of *L. monocytogenes* by sample type for beef cattle farms over six seasons in four states…..	91
3	Seasonal distribution of *L. monocytogenes* by sample type for dairy cattle farms over six seasons in four states……..	92
4	Seasonal distribution of *L. monocytogenes* by sample type for chicken and turkey farms over six seasons in four states.	93
5	Seasonal distribution of *L. monocytogenes* by sample type for swine farms over six seasons in four states……	94
6	Comparison of isolation of *L. monocytogenes* and background microflora in beef cattle farm samples from September 2002 to May 2004……...	95
7	Comparison of isolation of *L. monocytogenes* and background microflora in dairy cattle farm samples from September 2002 to May 2004.....……	96
8	Comparison of isolation of *L. monocytogenes* and background microflora in chicken and turkey farm samples from September 2002 to May 2004……	97

9 Comparison of isolation of *L. monocytogenes* and background microflora in swine farm samples…….. … 98

LIST OF FIGURES

FIGURE **PAGE**

Part III.

1 Dendrogram of *Eco*RI digested and fingerprinted isolates of *L*. *monocytogenes* characterized by automated RiboPrinting™. Numbers identify each isolate. The dotted line represents average genetic similarity (90.5%) amongst all groups of isolates based upon the DuPont identification number. The block letters indicate the genetic cluster the isolates are grouped into based on the genetic similarity…….. 65

2 Dendrogram of *Asc*I digested and fingerprinted isolates of *Listeria monocytogenes* characterized by pulsed-field gel electrophoresis. Numbers identify each isolate. The dotted line represents average genetic similarity (90.5%) amongst all groups of isolates based upon the DuPont identification number. The block letters indicate the genetic cluster the isolates are grouped into based on the genetic similarity……..… 66

3 Dendrogram of *Apa*I digested and fingerprinted isolates of *Listeria monocytogenes* characterized by pulsed-field gel electrophoresis. Numbers identify each isolate. The dotted line represents average genetic similarity (90.5%) amongst all groups of isolates based upon the DuPont identification number. The block letters indicate the genetic cluster the isolates are grouped into based on the genetic similarity…….. 67

Part IV.

1 Distribution of *L. monocytogenes* across five states during 21 months…… …… 89

2 Number of *L. monocytogenes* isolates over 21 months in five states amongst four animal farm types……..…… 90

PART I: LITERATURE REVIEW

Listeria monocytogenes

The United States Department of Agriculture's (USDA) Food Safety and Inspection Service (FSIS) is committed to improving public health through food safety (FSIS 2004). One of the major activities of this objective is the use of risk assessment to evaluate risks in farm-to-table food safety strategies, as well as the evaluation of surveillance programs for the ability to provide accurate scientific knowledge for the establishment and improvement of public health programs.

The Centers for Disease Control and Prevention (CDC) reported in 2002 that *Listeria monocytogenes* had the highest hospitalization rate (86.7% of reported cases) and the highest mortality rate (18.0%) for all food-borne pathogens tested for (CDC 2002a). *L. monocytogenes* was responsible for less than 1% of total infections reported to FoodNet, a 10 state ongoing survey of foodborne illness, yet 19 of 51 human deaths reported were due to infection from *L. monocytogenes*. Human listeriosis incidence, new cases of human individuals who exhibit symptoms due to infection from *L. monocytogenes*, has declined over the past 14 years and in 2002 was 0.27 cases per 100,000 population, near the Healthy People 2010 objective of 0.25 cases per 100,000 population (CDC 2002a). Despite these encouraging figures, the mortality rate of this foodborne pathogen requires a comprehensive study for the prevention of this bacterium's propagation in the U.S. food chain.

Listeria monocytogenes is a facultative anaerobic, gram positive bacterial rod. Listeriosis, the disease resulting from infection from *L. monocytogenes*, is

an intracellular infection that makes individuals with weak immune systems most susceptible to this disease. *L. monocytogenes* is considered ubiquitous in the environment, that being the ability to grow in all environments, and able to grow at refrigeration temperatures. The prevalence of this microorganism in various food systems has lead to preventative action taken by regulators, such as the Food and Drug Administration's (FDA) zero-tolerance for the presence of *L. monocytogenes* on ready-to-eat meats. The introduction of *L. monocytogenes* into meat food systems is cause for the investigation of various animal farm environments for the prevalence of this pathogen (FDA 2001).

L. monocytogenes exhibits several physiological characteristics that provide for greater survival on the farm and in food processing plants. The ability of *L. monocytogenes* to grow at refrigeration temperatures (1˚C) and as high as 42˚C extends the possible habitats for growth and survival beyond most food borne pathogens (Junttila and others 1988). *L. monocytogenes* has shown to exhibit generation times of approximately 43h, 6.6h and 1.1h at 4˚C, 10˚C, 37˚C respectively (Barbosa and others 1994).

The ability of *L. monocytogenes* to tolerate higher osmotic conditions (halotrophic) enables it to survive in a greater diversity of environments. Normally most mesophilic pathogens require a water activity (a_w) of 0.93 or higher. *L. monocytogenes* has been shown to grow below 0.93 and even to 0.9 in BHI adjusted by glycerol when grown at 30˚C (Farber and others 1992). *L. monocytogenes* is classified as a facultative anaerobe, which indicates it is able to survive in both anaerobic and aerobic environments. The motility of *L.*

monocytogenes is typically, yet not always, present and is most active between 24˚C and 30˚C, yet still present at 10˚C and 37˚C (Galsworthy and others 1990).

Listeriosis is the disease resulting from infection with *L. monocytogenes*. Susceptible individuals are those with suppressed immune systems, such as cancer patients, AIDS patients, young children, elderly persons, and pre-born infants. Listeriosis is an intracellular pathogen, spreading directly from cell to cell. Büla and others (1995) reported that age was a significant favor ($p=0.01$) for onset of listeriosis, especially those over 65 years old. The Centers for Disease Control and Prevention (CDC) reported *L. monocytogenes* having an overall incidence rate of 0.26 cases per 100,000 people (CDC 2002a).

Listeriosis amongst healthy adults has been shown to result in a fever lasting on average 27 hours and diarrhea for 42 hours upon the initial sequelea (Dalton and others 1997). Vazquez-Boland and others (2001) suggest that up to 70% of human *L. monocytogenes* infections affect the central nervous system (CNS). The CNS infections are typically meningoencephalitic in origin, yet for most adult humans the encephalitic form is more common in infected animals, exhibiting mortality rates as high as 60%. Bacteremias and septicemias are another frequent pathway for infection in adult humans, with mortality rates as high as 70% for immuno-compromised adult humans (i.e., cancer patients, AIDS patients, etc.). Pre-born babies, neonates and their mothers, as well as the elderly, are humans hosts that are normally susceptible to listeriosis (Vazquez-Boland and others 2001). Clinical abnormality in humans begins after a reported median incubation period of 20 hours after ingestion (Dalton and others 1997).

Prevalence of *Listeria monocytogenes*

The FDA has a zero tolerance for the presence of *L. monocytogenes* on fully cooked or ready-to-eat products. *L. monocytogenes* has been implicated in several outbreaks, including the landmark 1985 Mexican soft-cheese case that resulted in several deaths. In 2002 the CDC reported an outbreak of listeriosis from precooked sliceable turkey deli meat mostly affecting the northeastern part of the United States of America (USA). In this outbreak 46 people were affected with listeriosis, with ten fatalities (including 3 miscarried or stillborn children). Twenty seven million pounds of fresh and frozen ready-to-eat turkey and chicken products were recalled after a May 1st production date until October 11th (CDC 2002b).

Mexican-style soft cheese was implicated as the vehicle for *L. monocytogenes* which caused 142 cases of listeriosis from January 1 until August 15 of 1985, leading to 48 deaths. The 48 deaths included 20 pre-born babies, 10 neonates and 18 non-pregnant adults. Serotype 4b was responsible for 82% of the cases of listeriosis. Unpasteurized milk was implicated as the source for the *L. monocytogenes* isolates. Only one of the 27 dairy farms that provide milk for the cheese produced at the factory which produced the contaminated cheese reported feeding silage during the three months preceding the outbreak, which is often implicated as the source for *L. monocytogenes* on the farm. *L. monocytogenes* was not found from any of the dairy herds providing milk when tested during June of 1985 (two weeks after the cheese factory was closed) (Linnan and others 1988).

Beckers and others (1987) reported that 10 out of 69 samples of imported soft cheese were positive for *L. monocytogenes*. Each of the soft cheeses was made from raw milk and some of the cheese samples contained 10^6 CFU of *L. monocytogenes* per gram of soft cheese. Raw milk taken from the Netherlands was tested for *L. monocytogenes*, resulting in 6 out of 137 samples proven to be positive for *L. monocytogenes*. Milk provides an adequate environment for survival and growth even at refrigeration temperatures. Fleming and others (1985) traced an outbreak of listeriosis among 49 people to pasteurized milk. The pasteurized milk was from a dairy plant that had received raw milk from a group of farms that had an outbreak of listeriosis amongst their dairy cattle at a time approximate to the time of the outbreak amongst the 49 infected people. Upon inspection of the dairy plant, no evidence of improper pasteurization was found, which indicates that large inoculums of *L. monocytogenes* in milk may survive pasteurization.

Other foods have also been implicated as a source for *L. monocytogenes*, including coleslaw. Schlech and others (1983) reported that manure from sheep infected with *L. monocytogenes* was used to fertilize soil used to grow cabbage, which was eventually used to produce coleslaw. In the summer of 1981 numerous listeriosis infections, both perinatal and adult cases, leading to an increased attack rate in two different regions of Canada. Coleslaw from the refrigerator of an adult with listeriosis was contaminated with *L. monocytogenes* serotype 4b, the same serotype that they were infected with. Schleck and others (1983), having determined by interviews that each infected person may have

eaten contaminated coleslaw, concluded that the increased incidence of listeriosis was due to the consumption of coleslaw infected with *L. monocytogenes*. Vegetables from “biologic agriculture”, those not using artificial fertilizers, has been implicated as a potential source for listeriosis (Allerberger and Guggenbichler 1989).

L. monocytogenes has been isolated from various farm environments. *L. monocytogenes* has been isolated from dairy feed (silage), soil and dairy cattle. The etiology of *L. monocytogenes* on the farm is unknown, yet a further understanding of other sources or reservoirs of *L. monocytogenes* may provide probable sources for continued contamination of the farm.

L. monocytogenes is thought to be ubiquitous in the environment, being able to grow in all environments. Weis and Seeliger (1975) reported that of 194 *L. monocytogenes* strains, 20.3% were found in plant and soil environments, especially those from uncultivated fields. They reported that 27.2% of the *L. monocytogenes* isolates derived from moldy fodder and wildlife feeding grounds, with another 33% being isolated from deer and birds. They noted that faded and decayed grass was a direct indicator of the presence of *Listeria.* Although Van Renterghem and others (1991) had noted problems of *L. monocytogenes* being isolated from inoculated soil after 8 weeks, Weis and Seeliger (1975) found in uncultivated fields (with overgrowth) half of all surface soil and one-third of soil at 10cm depth positive for *L. monocytogenes*, noting that results were repeatable for up to a 6 months. Weis and Seeliger (1975) did note that the lowest prevalence for *L. monocytogenes* was in land used for current agricultural use.

Welshimer (1960) showed *L. monocytogenes* surviving for greater than 295 days at no less than log 5 CFU/ml in fertile soil, having only decreases approximately 2.5 log CFU/ml from inoculation. Van Renterghem and others (1991) reported that 9 out of 10 *L. monocytogenes* isolates taken from 82 environmental samples were from fecal sources (4 from swine and 5 from cattle feces). They also found that *L. monocytogenes* does not survive more than 8 weeks, yet was detectable before 6 weeks in inoculated manure or soil samples. Despite this finding, 3 of 6 unwashed radishes grown for 3 months in *L. monocytogenes* inoculated soil were positive for *L. monocytogenes*, where no *L. monocytogenes* isolates were detected form 6 carrots grown in the inoculated soil. One of fifteen ground water samples was positive for *L. monocytogenes* (each 100ml filtered).

Arvanitidou and others (1997) reported that five *L. monocytogenes* isolates from 250 surface water samples were isolated. The isolates were found only in rivers (in Northern Greece) (none in the one lake sample) and seemed to be only present with less than 2 log CFU/100 ml. These samples were taken during the months of May and June, which may have had an affect upon the occurrence of *L. monocytogenes* in the sample environments. Six of fifteen samples (40%) taken from two rivers in Italy were positive for *L. monocytogenes* during the Spring and Summer of 1993 (Bernagozzi and others 1994). Thirty seven percent of 180 samples taken from lake and canal samples taken in September and December of 1981 from a northern province of the Netherlands were positive for *L. monocytogenes* (Dijkstra 1982).

Watkins and Sleath (1981) reported isolating *L. monocytogenes* from river water samples with counts ranging from 3 to over 180 CFU/litter. They also reported the enumeration of *L. monocytogenes* ranging from 700 to over 18000 CFU/litter of primary tank effluent of sewage treatment works. Knowing this sludge eventually is used to fertilize farms, the authors sampled soil from a farm receiving the sludge and reported *L. monocytogenes* being able to be isolated up to 49 days with a heavy treatment of sludge and up to 41 days with a light treatment of sludge, each starting with a count of greater than 180 CFU/50g soil. Watkins and Sleath (1981) also reported that *L. monocytogenes* was able to survive at least eight weeks in sewage sludge applied to farm land, maintaining a 2.26 log CFU/100g soil count. The relationship between sewage sludge contamination of surface water and soil is significant for understanding the distribution of *L. monocytogenes* in the environment.

Dijkstra (1982) reported that 67% of 33 water-effluent samples taken from a sewage treatment plant in the Netherlands was positive for *L. monocytogenes*, indicating sewage is a probable source for contamination of *L. monocytogenes* into the environment. This idea was reinforced by when Dijkstra (1982) also reported that 12% of 52 canal water samples which received the treated effluent contained *L. monocytogenes*. *L. monocytogenes* was found in 8.3% of the dewatered sewage sludge samples (28% solids) from Italy (29 MPN/g) (De Luca and others 1998). Although this is a decrease from the crude primary sludge (35.7% positive for *L. monocytogenes*, with 117 MPN/g) this example provides

evidence that “biosolids” (dewatered sewage sludge) distributed into farming soil may provide a source of *L. monocytogenes* to farm animals and crops.

River water containing contaminated sewage treatment plant effluent used to water farms could be a likely source for contamination, as Combarro and others (1997) reported that 44.3% of river water samples (from Spain) were positive for *L. monocytogenes*, with 58.8% of influent and 58.1% of effluent sewage samples also being positive for *L. monocytogenes* (sampled between September 1992 and February 1993 for the influent and effluence, with the river water being sampled between March 1992 and February 1993). Less than one kilometer before (upstream) a sewage treatment plant there was almost no *Listeria* counts, yet at the sewage treatment plant the *Listeria* counts increased to nearly 1 log MPN/100ml river water and is maintained at 0.5 log MPN/100ml river water for 23 kilometers (14.3 miles) (Combarro and others 1997).

Decaying vegetative matter has also been shown to be an ideal source for *Listeria* species to survive and even grow. Silage, being fermented vegetative matter is therefore an ideal source for harboring *Listeria* species, including *L. monocytogenes*. Gray (1960) reported that mice fed “poor” silage were infected with *L. monocytogenes*, which was found in the liver of non-pregnant mice and fetuses of pregnant mice. Arimi and others (1997) reported isolating 6 *L. monocytogenes* isolates from silage, with each exhibiting a distinct ribotype.

Perry and Donnelly (1990) reported that 10% of corn silage, 28% hay silage and 60% grass silage samples from Vermont (USA) contained *Listeria* species. *L. monocytogenes* isolates were found in 3 of 13 isolates of corn silage

(n=129), 2 of 21 hay silage isolates (n=76), yet was not isolated from 5 grass silage samples. Ryser and others (1997), utilizing the same data as Perry and Donnelly, noted that *L. monocytogenes* was isolated from corn silage with a pH of less than 4.00 (ribogroups 19092 and 19193) and between 4.00 and 4.99 (ribogroup 54183) and from hay silage with a pH between 5.00 and 5.99 (ribogroups 19071 and 19075). The ability of *L. monocytogenes* isolates to survive at a pH less than 4.00 is troubling given acidity is a common hurdle for growth of foodborne pathogens. Skovgaard and Morgen (1988) also reported *L. monocytogenes* being isolated from silage at a pH less than 4.5. They also reported *L. monocytogenes* being isolated from hay, ammonia treated straw and debris from beet rinsing.

Ultimately, the prevalence of *L. monocytogenes* on farm environments directly impacts animals that are raised for meat production or to produce milk for human consumption. Between 1972 and 1994 0.04% of milk samples taken from dairy cattle were positive for *L. monocytogenes*, with 79% of bovine and 48% of human isolates being of the same ribogroups (using *Eco*RI) (Jensen and others 1996). Carlos and others (2001) reported that 13% of 1300 raw dairy cattle milk samples taken from southeast of Mexico City, Mexico were positive for *L. monocytogenes*, along with 6% positive for *L. ivanovii* and 4% positive for *L. seeligeri*. Jensen and others (1996) reported that milk from dairy cattle that was sampled for 23 years exhibited 448 *L. monocytogenes* positive sample from 1,132,958 dairy cow's milk. This may indicate that infected dairy cattle are contaminating the milk via a *L. monocytogenes* mastitis infection. Given that milk

is contaminated it is logical to consider the cattle as a potential contaminating source for the milk.

Yoshida and others (2000) provided evidence in Japan of various carriers of *L. monocytogenes* amongst mammalian (i.e., Red fox and Sika deer) and avian species (i.e., Crow and Green-winged teal). Sediment samples from a California coast exhibited a higher amount of *Listeria* isolates (27.3%) when domesticated animals (ie., cows and horses) were nearby as compared to sediment samples that did not have domesticated animals (no isolates and 16.7%) (Colburn and others 1990).

Eighteen percent of beef cattle from Rio de Janeiro were shown to be infected with *L. monocytogenes*, as determined by positive isolation from stool samples. The beef cattle were infected with differing numbers of serotypes ranging from one to seven different serotypes (Hofer 1983). Hofer (1983) also noted that 9 of the 11 infected beef cattle were in the greater than 5 years of age category, which might indicate the significance of age to harboring *L. monocytogenes*. None of the beef cattle were exhibiting sequellae from a *L. monocytogenes* infection and were considered “normal” and not sick.

The feces from 6 out of 73 herds of swine in Denmark during slaughter (March and April of 1988) exhibited *Listeria* species isolates (n=172), three of seven being *L. monocytogenes*. In contrast to this low percentage minced pork (11 of 51 from the same slaughter house) exhibited a higher prevalence, by having 6 *L. monocytogenes* isolates from 51 samples and 26 of the 51 samples being positive for *L. innocua*. No *L. monocytogenes* isolates form SPF (specific

pathogen free) herds (n=14) were present, showing that the controlled indoor environments were effective in preventing environmental contamination by *L. monocytogenes* (Skovgaard and Nørrung, 1989).

Animal feed may also provide an additional opportunity for exposure to *L. monocytogenes*. Skovgaard and Morgen (1988) reported that *L. monocytogenes* was isolated from 6 of 7 dairy farms in fecal and feed samples. The percentage of feed samples tested containing *L. monocytogenes* isolates ranged between 20 and 100%.

Chicken carcasses have been known to harbor *L. monocytogenes*, including on skin of necks. Materials (feces) from transport cages for chickens have also been found to be positive for *L. monocytogenes* (Skovgaard and Morgen, 1988). Schuchat and others (1992) noted that eating undercooked chicken also increased the risk of listeriosis amongst immunosuppressed patients (p=0.02). Vijayakrishna and others (2000) reported that *Listeria* species (likely, *L. monocytogenes*) caused a mortality rate of 40% for a broiler chicken flock of 700, which also resulted in a 80% morbidity rate and 50% case fatality rate.

The largest recall of meat products involved turkey products (CDC, 2002b). Although turkeys have not been the primary target of environmental studies, the possible link of turkey's introducing *L. monocytogenes* into a processing plant presents a real risk that should be investigated.

L. monocytogenes was isolated from 10.75% of 400 minced meat samples taken from butcheries in Switzerland, with serotypes 1/2a (n=19), 1/2c (n=12), 4b

(n=10) and 1/2b (n=2) (Fantelli and Stephan, 2001). Skovgaard and Morgen (1988) noted that the prevalence of *L. monocytogenes* in minced beef received from three different municipal food inspection laboratories in Denmark was 28% (19 of 67 samples).

The prevalence of *L. monocytogenes* in domestic households was investigated by Beumer and others (1996), who reported finding *L. monocytogenes* isolates in 10.2% of 206 bathroom samples, 5.0% of 522 kitchen related samples (dish clothes, kitchen sink and refrigerator). They also reported an *L. monocytogenes* isolate from a toothbrush (n=47). Other Listeria species besides the 54 total *L. monocytogenes* isolates were isolated from the 871 domestic samples (70 *L. innocua*, 3 *L. seeligeri*, 2 *L. grayi*, 2 *L. welshimeri*, and 1 *L. ivanovii*). The most frequent range quantified from dish-clothes and dish washing-up brushes was 3.5 to 4.0 log CFU/object, lending to a great probability for contamination of food contact surfaces (Beumer and others, 1996). Pinner and others (1992) found that 64% of refrigerators of listeriosis patients contained at least one food specimen with *L. monocytogenes*. The three most predominant foods exhibiting *L. monocytogenes* were beef (36%), poultry (31%) and pork (27%).

Seasonal affects were seen with the isolating of *L. monocytogenes* from dairy cattle milk, with 96 of 151 *L. monocytogenes* isolates found during the Spring (57) and Summer (39), where 55 of 151 *L. monocytogenes* isolates found during the Fall (30) and Winter (25), with this difference being shown to be statistically significant ($p<0.05$) (Carlos and others 2001). Carlos and others

(2001) reasoned that the mild winters in Mexico allow dairy cattle to graze all year long, thereby not using contaminated silage. The Winter exhibiting the lowest number of isolates contradicts previous studies (Fernandez-Garayzabal and others 1987), which have shown the Winter season being one for increased *L. monocytogenes* isolations.

Fernandez-Garayzabal and others (1987) reported that raw milk from a dairy processing plant in Madrid, Spain exhibited more *L. monocytogenes* isolates from November to March (78.3%) than the warmer months of April until October (30.2%). In support of these findings Rea and others (1992) report a rise in the isolation of *L. monocytogenes* from raw milk samples from 0 to 5% during the Spring and Summer to a baseline range of 35 to 37% during the Winter. They also found that higher total aerobic plate counts for the milk resulted during the Winter months. Factors that go beyond simple weather patterns must explain these contradicting, yet distinct seasonal patterns, such as feed type, housing, history of isolation of *L. monocytogenes* and climate (i.e., the Winter in California is not the same as the Winter in North Carolina). Colder temperatures have also been shown to increase the virulence of *L. monocytogenes*, which may be support for the influence of temperature upon increased frequency of listeriosis cases in the Winter (Picard-Bonnaud and Carbonnelle, 1989).

Human fecal samples taken from 1991-2 in the Bristol, England region resulted in 7 *Listeria* isolates with 4 of the 7 being *L. monocytogenes*, all of which were isolated in the months of June, July, August and September. In this same

time period in Bristol, July to September 1991, 10 of 17 *L. monocytogenes* isolates were found from human feces samples (n=115). Urban garden soil samples in Bristol produced one *L. monocytogenes* isolate (n=136), which was found between December 1991 and January 1992 (MacGowan and others, 1994). The identification of various risk factors for contamination and growth of *L. monocytogenes* is needed to help explain seasonal affects.

Molecular Identification and Characterization

There are numerous ways to characterize microorganisms, including biochemical, serological and molecular methodologies. Molecular characterization of bacteria has provided not only possible means for identification, yet differentiation between strains. To utilize molecular tools in an epidemiological function requires the ability to identify changes to the genome of a given microbe. Two different molecular techniques that provide such capabilities are pulse-field gel electrophoresis (PFGE) and RiboPrinting™. PFGE of genomic deoxyribonucleic acid (DNA) uses a restriction endonuclease to cleave the genome into segments of DNA of various sizes, which are then separated based on size in agarose exposed to a polarized charge. RiboPrinting™ is slightly different as it focuses only on ribosomal DNA and not the entire genome. Ribosomal DNA is thought to be highly conserved and provides a more stable avenue for identification. RiboPrinting™ may utilize various restriction endonucleases (i.e, EcoRI) like PFGE, yet an automated procedure created by Dupont's Qualicon, Inc. enables detection and recognition of the electrophoresed ribosomal DNA band pattern for identification of the

microbe. The band pattern is also then available for analysis by the creation of a dendrogram, which provides a schematic of the degree of relationship shared between various microbial strains.

L. monocytogenes strains implicated in clinical listeriosis for three different ruminant animals (goats, sheep and cattle) were found to have the same ribotypes as those found in silage fed to these animals (with one exception). Additional *L. monocytogenes* isolates with different ribogroups were found in the silage, not isolated from the clinical cases, indicating that certain strains were potentially more pathogenic for these ruminant animals (Wiedmann and others, 1996).

Jaradat and others (2002) noted that *L. monocytogenes* strains with 96.5 to 99% genetic similarity, as determined by RiboPrinting™, were found in two geographically distinct locations. In this same study all the *L. monocytogenes* strains were grouped into 4 clusters, with each cluster showing a 92 to 99% genetic homogeneity, each containing isolates from human clinical cases and food or food manufacturing samples. Beyond these situation clusters, three genetic lineages have been established for *L. monocytogenes* (Rasmussen and others, 1995). Different types of *L. monocytogenes* isolates have been seen in each lineage group, with less frequent *L. monocytogenes* isolates from humans in lineage group III (1 of 119 isolates) than for animal *L. monocytogenes* isolates (8 of 76 isolates; $p=0.003$). More *L. monocytogenes* isolates from humans have been found in lineage I than among animal cases ($p<0.001$) (Jeffers and others, 2001). *L. monocytogenes* isolated from animals was reported to be found in all

three genetic lineages, where human isolates were not found to be represented in the third lineage (serovar 4a) (Vazquez-Boland and others 2001).

Kerouanton and others (1998) reported that PFGE provided the greater discriminatory power than RiboPrinting™, and was the greatest of five typing methods for distinguishing between *L. monocytogenes* strains. They found that PFGE discerned 12 patterns with a high discriminatory power (discriminatory index (D.I.) = 0.886) and RiboPrinting™ 10 patterns with D.I. of 0.849. Kerouanton and others (1998) noted that the combined discrimination power of PFGE and RiboPrinting™ increased (D.I.=0.978), which was the highest of all paired techniques tested (included serotyping, zymotyping, random amplication of polymorphic DNA (RAPD) , PFGE and RiboPrinting™). RiboPrinting™ was unable to distinguish between strains of *L. monocytogenes* that were of differing origin, yet does provide a reliable and automated procedure for the identification of microbes, including *L. monocytogenes*. Serotyping provided the least differentiation between strains of *L. monocytogenes*.

The superiority of PFGE over RiboPrinting™ for distinguishing strains of *L. monocytogenes* was reinforced by Aarnisalo and others (2003) where PFGE exhibited a D.I. of 0.966 and ribotyping a D.I. of 0.906. They also compared restriction endonucleases for RiboPrinting™ (*Eco*RI and *Pvu*II), finding that *Eco*RI exhibited a higher D.I. (0.878) compared to *Pvu*II (D.I.=0.867), even though *Pvu*II produced three more ribotypes than *Eco*RI. This same pattern was observed by Louie and others (1996), with *Eco*RI producing 16 ribotypes and *Pvu*II 23 ribotypes. Louie and others (1996) also noted that RiboPrinting™ again

failed to discriminate between outbreak strains, yet PFGE was able to discern the differences. The disadvantage of PFGE is the time required to get results, lack of automation and labor intensiveness of the procedure, where ribotyping is highly reproducible, standardized across laboratories and requires minimal labor.

Risk Assessment and Management

Risk assessment seeks to answer three major questions: (1) What is the possible “risk” scenario?, (2) How likely is the risk scenario?, (3) What is the consequence if the risk scenario occurs? (Morales and McDowell, 1998). The product of the quantification of each of these questions, referred to as the “risk triplets”, quantifies the risk. Integrating the ability to quantify risk within hazard analysis critical control points (HACCP) programs enables risk managers the ability to connect food safety programs to public health impact (Buchanan and Whiting, 1998). Expanding HACCP to the farm complicates this integration, given the great diversity of farm environments and management practices. Measurable goals may be based upon the use of quantitative microbial risk assessment (QMRA). Dose-response relationships and pathogen baseline data are needed to assist risk managers to make quality decisions about how much risk is present concerning a given commodity or food product.

HACCP management system applied to the farm provides farms the ability to systematically prevent and manage disease on the farm. As with food processing plants, conducting a hazard analysis is needed to determine what risks are present for a particular farm environment, which essentially is risk assessment. Knowledge of animal health and physiology is required to

determine what potential risks are possible, yet epidemiological studies are needed to understand the link between possible hazards (disease) and critical control points (disease prevention).

Identification of risk factors, such as nutrition, climate, hygiene, housing conditions and animal management practices, must be integrated with data of baseline pathogen levels and infective dose levels for common animal and foodborne pathogens. Risk management entails applying the 2nd through the 6th steps of HACCP (identifying critical control points (CCP), setting critical limits, establishing monitoring of limits, establishing corrective actions when CCP are not controlled and verification procedures) (Noordhuizen and Welpelo, 1996).

Risk assessment for listeriosis on the farm or for animals introducing *L. monocytogenes* into the processing plants is needed given the pathogenicity and virulence of *L. monocytogenes* for both humans and farm animals. Quantification of the risk for listeriosis in humans has been initiated by the CDC's FoodNet program (CDC, 2002a). FoodNet quantifies the number of confirmed listeriosis cases in humans in selected states, noting hospitalization rates and death rates. The CDC's PulseNet provides the ability for epidemiological investigation of human listeriosis. PulseNet catalogues the genetic profile of major food bacterial pathogens responsible for human listeriosis via PFGE. Graves and Swaminathan (2001) have established a standardized protocol for subtyping *L. monocytogenes* by macrorestriction and PFGE for the PulseNet program.

The national distribution of foods lends to the possible spread of *L. monocytogenes* to different geographical regions. Animals and biological

fertilizer are probably distributed regionally, increasing the risk for finding related strains (genetically) of *L. monocytogenes* on the farm. To understand the exposure assessment of *L. monocytogenes* Rocourt and others (2003) noted that prevalence studies of *L. monocytogenes* in agriculture environments are needed.

Agricultural Surveillance

Van Renterghem and others (1991) noted that cold enrichment for *L. monocytogenes* from environmental samples was unacceptable given a recommended one to two month cold enrichment time (4˚C). They also showed that direct inoculation into selective media provided a competitive advantage against broths with high contamination of *Streptococcus* species. In contrast to this Van Renterghem and others (1991) reported that a pre-enrichment provided for more sensitive results than direct selective enrichment (without inoculation of the *Streptococcus* species). It is likely that samples taken from farm environments would be contain higher levels of *Streptococcus* species when contrasted with food manufacturing plant samples.

The FDA recommends the use of PALCAM (*polymixin B, acriflavin, lithium chloride, ceftazidime, aesculin, D-mannitol*) and modified oxford media (MOX) agars (FDA, 2003), where the USDA recommends only MOX as a selective agar (USDA, 2002). Capita and others (2001) reported that PALCAM recovered more *Listeria* species and *L. monocytogenes* isolates than MOX.

The FDA also recommends the use of buffered Listeria enrichment broth (BLEB) to enrich samples for the recovery of *L. monocytogenes* (FDA, 2003), with sampling of the enrichment at 24h and 48h. The USDA recommends a two

stage enrichment, UVM I for the primary enrichment and Fraser broth for the secondary enrichment (USDA, 2002). The UVM enrichment should be compared with BLEB for an one stage enrichment, with sampling at 24h and 48h.

Purpose of Research

The overall objective was to determine the occurrence of *Listeria monocytogenes* in farm environmental samples using classical microbiological and to understand the relationship between isolates using molecular tools. The study extends to five state-regions (California, Washington, Tennessee, Alabama, North Carolina), four farm animal types (beef cattle, dairy cattle, swine, chicken/turkey) and four environmental sample types (soil, feed/grass, bedding/feces, animal rectal swabs) being sampled during six consecutive seasons (Fall 2002 through Winter 2004). The first objective for this project is to compare the effectiveness of isolation methods, including enrichment and plating media and incubation times. The second objective for this project is to compare the effectiveness of molecular tools (Pulsed Field Gel Electrophoresis (PFGE) and Ribotyping®) in differentiating bacterial strains isolated on the farms. The third objective is to develop baseline data on occurrence of *L. monocytogenes* on 16 farms containing dairy cows, beef cattle, swine and poultry.

References

Aarnisalo, K., Autio, T., Sjöberg, A., Lundén, J., Korkeala, H., and Suihko, M.. 2003. Typing of *Listeria monocytogenes* isolates originating from the food processing industry with automated ribotyping and pulsed-field gel electrophoresis. J. Food Prot. 66(2):249-255.

Allerberger, F. and Guggenbichler, J.P.. 1989. Listeriosis in Austria: Report of an outbreak in 1986. Acta Microbiol. Hung. 36(2-3):149-152.

Arimi, S.M., Ryser, E.T., Pritchard, T.J., and Donnelly, C.W.. 1997. Diversity of *Listeria* ribotypes recovered from dairy cattle, silage, and dairy processing environments. J. Food Protect. 60(7): 811-816.

Arvanitidou, M., Papa, A., Constantinidis, T.C., Danielides, V., and Katsouyannopoulos, V.. 1997. The occurrence of Listeria spp. and Salmonella spp. in surface waters. Microbiol. Res. 152:395-397.

Barbosa, W.B., Cabedo, L., Wederquist, H.J., Sofos, J.N., and Schmidt, G.R.. 1994. Growth varation among species and strains of *Listeria* in culture broth. J. Food Prot. 57(9):765-769.

Beckers, H.J., Soentoro, P.S.S. and Delfgou-van Asch, E.H.M.. 1987. The occurrence of *L. monocytogenes* in soft cheeses and raw milk and its heat resistance to heat. Int. J. Food Microbiol. 4:249-256.

Bernagozzi, M., Bianucci, F., Sacchetti, R., and Bisbini, P.. 1994. Study of the prevalence of Listeria spp. in surface water. Zbl. Bakteriol. Mikrobiol. Hyg. 196:237-244.

Beumer, R.R., te Giffel, M.C., Spoorenberg, R., and Rombouts, F.M.. 1996. Listeria species in domestic environments. Epidemiol. Infect. 117:437-442.

Buchanan, R.L. and Whiting, R.C.. 1998. Risk assessment: a means for linking HACCP plans and public health. J. Food Prot. 61(11):1531-1534.

Büla, C.J., Bille, J., Glauser, M.P.. 1995. An epidemic of food-borne listeriosis in western Switzerland: Description of 57 cases involving adults. Clin. Infect. Dis. 20(1):66-72

Capita, R., Alonso-Calleja, C., Prieto, M., Garcia-Fernández, M.D.C., Moreno, B.. 2001. Comparison of PALCAM and modified oxford plating media for isolation of *Listeria* species in poultry meat following UVM II or frazier secondary enrichment broths. Food Microbiol. 18:555-563.

Carlos, V.S., Oscar, R.S. and Irma, Q.R.E. 2001. Occurrence of Listeria species in raw milk in farms on the outskirts of Mexico City. Food Micobiol. 18:177-181.

CDC. 2002a. Centers for Disease Control and Prevention. FoodNet 2002 annual report. http://www.cdc.gov/foodnet/annual/2002/2002executive_summary.pdf

CDC. 2002b. Centers for Disease Control and Prevention. Public health dispatch: Outbreak of Listeriosis - northeastern United States, 2002. Morbidity and Mortality Weekly Report. 51(42):950-951.

Colburn, K.G., Kaysner, C.A., Abeyta, C., Wekell, M.M.. 1990. *Listeria* species in a California coast estuarine environment. Appl. Environ. Microbiol. 56(7):2007-2011.

Colby, M.M. and Johnson, Y.J.. 2002. Potential uses for geographic information system-based planning and decision support technology in intensive food animal production. Anim. Health. Res. Rev. 3(1):31-42.

Combarro, M.P., Gonzalez, M., Araujo, M., Amezaga, A.C., Sueiro, A.R., and Garrido, M.J. 1997. Listeria species incidence and characterization in a river receiving town sewage from a sewage treatment plant. Water Sci. Technol. 35:201-204.

Dalton, C.B., Austin, C.C., Sobel, J., Hayes, P.S., Bibb, W.F., Graves, L.M., Swaminathan, B., Proctor, M.E., and Griffin, P.M.. 1997. An outbreak of gastroenteritis and fever due to *Listeria monocytogenes* in milk. New. Engl. J. Med. 336(2):100-106.

De Luca, G., Zanetti, F., Fateh-Moghadm, P., and Stampi, S.. 1998. Occurrence of Listeria monocytogenes in sewage sludge. Zbl. Hyg. Umweltmed. 201:269-277.

Dijkstra, R.G.. 1982. The occurrence of Listeria monocytogenes in surface water of canals and lakes, in ditches of one big polder and in the effluents and canals of a sewage treatment plant. Zbl. Bakteriol. Mikrobiol. Hyg. 176:202-205.

Fantelli, K and Stephan, R.. 2001. Prevalence and characteristics of shigatoxin-producing *Escherichia coli* and *Listeria monocytogenes* strains isolated from minced meat in Switzerland. Intl. J. Food Micobiol. 70(1-2):63-69.

Farber, J.M., Coates, F., Daley, E.. 1992. Minimum water activity requirements for the growth of *Listeria monocytogenes*. Lett. Appl. Microbiol. 15:103-105.

Fernandez-Garayzabal, J.F., Dominguez, L., Vazquez, J.A., Gomez-Lucia, A., Rodriguez Ferri, E.R. and Suarez, G. 1987. Occurrence of Listeria monocytogenes in raw milk. Vet. Rec. 120:258-259.

Fleming, D.W., Cochi, S.L., MacDonald, K.L., Brondum, J., Hayes, P.S., Plikaytis, B.D., Holmes, M.B., Audurier, A., Broome, C.V., Reingold, A.L.. 1985. Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. N. Engl. J. Med. 312:404-407.

FDA. 2001. United States Food and Drug Administration. www.foodsafety.gov/~dms/lmrisk1.html

FDA. 2003. United States Food and Drug Administration. Bacteriological Analytical Manual. www.cfsan.fda.gov/~ebam/bam-10.html

FSIS. 2004. Food Safety Inspection Service. www.fsis.usda.gov/PDF/Fulfilling_the_Vision.pdf

Galsworthy, S.B., Girdler, S., and Koval, S.F.. 1990. Chemotaxis in *Listeria monocytogenes*. Acta Microbiol. Hung. 37:81-85.

Graves, L.M. and Swaminathan, B.. 2001. PulseNet standardization protocol for subtyping *Listeria monocytogenes* by macrorestriction and pulsed-field gel electrophoresis. Intl. J. Food Micro. 65:55-62.

Gray, M.L.. 1960. Silage feeding and listeriosis. J. Amer.Vet. Med. Assoc. 136:205-208.

Hofer, E.. 1983. Bacteriologic and epidemiologic studies on the occurrence of *Listeris monocytogenes* in healthy cattle. Zentral. fuer Bakteriol. Mikrobiol. und Hyg. Series A. 256(2):175-183.

Jaradat, Z.W., Schutze, G.E., Bhunia, A.K.. 2002. Genetic homogeneity among *Listeria monocytogenes* strains from infected patients and meat products from two geographic locations determined by phenotyping, ribotyping and PCR analysis of virulence genes. Intl. J. Food Microbiol. 76:1-10.

Jeffers, G.T., Bruce, J.L., McDonough, P.L., Scarlett, J., Boor, K.J., Wiedmann, M.. 2001. Comparative genetic characterization of *Listeria monocytogenes* isolates from human and animal listeriosis cases. Microbiol. 147:1095-1104.

Jensen, N.E., Aarestrup, F.M., Jensen, J., Wegener, H.C.. 1996. Listeria monocytogenes in bovine mastitis. Possible implication for human health. Intl. J. Food Microbiol. 32:209-216.

Junttila, J.R., Niemelä, S.U., and Hirn, J.. 1988. Minimum growth temperatures of *Listeria monocytogenes* and non-haemolytic *Listeria*. J. Appl. Bacteriol. 65:321-327.

Kerouanton, A., Brisabois, A., Denoyer, E., Dilasser, F., Grout, J., Salvat, G., Picard, B.. 1998. Comparison of five typing methods for the epidemiological study of *Listeria monocytogenes*. Intl. J. Food Microbiol. 43:61-71.

Linnan, M.J., Mascola, L., Lou, X.D., Goulet, V., May, S., Salinen, N.C., Hird, D.W., Yonekura, M.L., Hayes, P., Weaver, R., Audurier, A., Plikaytis, B.D., Fannin, S.L., Kleks, A., Broome, C.V.. 1988. Epidemic listeriosis associated with Mexican style cheese. N. Engl. J. Med. 319:823-828.

Louie, M., Jayaratne, P., Luchsinger, I., Devenish, J., Yao, J., Schlech, W., Simor, A.. 1996. Comparison of ribotyping, arbitrarily primed PCR, and pulsed-field gel electrophoresis for molecular typing of *Listeria monocytogenes*. J. Clin. Microbiol. 34(1):15-19.

MacGowan, A.P., Bowker, K., McLauchlin, J., Bennett, P.M., and Reeves, D.S.. 1994. The occurrence and seasonal changes in the isolation of Listeria spp. in shop bought foodstuffs, human faeces, sewage and soil from urban sources. Intl. J. Food Microbiol. 21:325-334.

Morales, R.A. and McDowell, R.M.. 1998. Risk assessment and economic analysis for managing risks to human health from pathogenic microorganisms in the food supply. J. Food Prot. 61(11):1567-1670.

Noordhuizen, J.P.T.M. and Welpelo, H.J.. 1996. Sustainable improvement of animal health care by systematic quality risk management according to the HACCP concept. Vet. Quart. 18(4):121-126.

Perry, C.M. and Donnelly, C.W.. 1990. Incidence of *Listeria monocytogenes* in silage and its subsequent control by specific and nonspecific antagonism. J. Food Prot. 53(8):642-647.

Picard-Bonnaud, F., Cottin, J., Carbonnelle, B.. 1989. Preservation of the virulence of *Listeria monocytogenes* in different sorts of soil. Acta Microbiol. Hung. 36(2-3):269-272.

Pinner, R.W., Schuchat, A., Swaminathan, B., Hayes, P.S., Deaver, K.A., Weaver, R.E., Plikaytis, B.D., Reeves, M., Broome, C.V., Wenger, J.D., and the Listeria Study Group. 1992. Role of foods in sporadic listeriosis: II. Microbiologic and epidemiologic investigation. J. Amer. Med. Assoc. 267(15):2046-2050.

Rasmussen, O.F., Skouboe, P., Dons, L., Rossen, L., and Olsen, J.E.. 1995. *Listeria monocytogenes* exists in at least three evolutionary lines: evidence from flagellin, invasive associated protein and listeriolysin O genes. Microbiol. 141:2053-2061.

Rea, M.C., Cogan, T.M., and Tobin, S.. 1992. Incidence of pathogenic bacteria in raw milk in Ireland. J. Appl. Bacteriol. 73:331-336.

Rocourt, J., Ben Embarek, P., Toyofuku, H., Schlundt, J.. 2003. Quantitative risk assessement of *Listeria monocytogenes* in ready-to-eat foods: the FAO/WHO approach. Immun. Med Microbiol. 35:263-267.

Ryser, E.T., Arimi, S.M., and Donnelly, C.M.. 1997. Effects of pH on distribution of Listeria ribotypes in corn, hay, and grass silage. Appl. Environ. Microbiol. 63:3695-3697.

Schlech, W.F.III, Lavigne, P.M., Bortolussi, R.A., Allen, A.C., Haldane, E.V., Wort, A.J., Hightower, A.W., Johnson, S.E., King, S.H., Nicholls, E.S., Broome, C.V.. 1983. Epidemic listeriosis-evidence for transmission by food. N. Engl. J. Med. 308:203-206.

Schuchat, A., Deaver, K.A., Wenger, J.D., Plikaytis, B.D., Mascola, L., Pinner, R.W., Reingold, A.L., Broome, C.V., and the Listeria Study Group. 1992. Role of foods in sporadic listeriosis: I. Case-control study of dietary risk factors. J. Amer. Med. Assoc. 267(15):2041-2045.

Skovgaard, N. and Morgen, C.. 1988. Detection of Listeria spp. in faeces from animals, in feeds, and in raw foods of animal origin. Intl. J. Food Microbiol. 6(3):229-242.

Skovgaard, N. and Norrung, B.. 1989. The incidence of Listeria spp. in faeces of Danish pigs and in minced pork meat. Int. J. Food Microbiol. 8:59-63.

USDA. 2002. United States Department of Agriculture. Food Safety and Inspection Service. Microbiology Laboratory Guidebook. www.fsis.usda.gov/ophs/microlab/mlg8.0 3.pdf

Van Renterghem, B., Huysman, F., Rygole, R., and Verstraete, W.. 1991. Detection and prevalence of Listeria monocytogenes in the agricultural ecosystem. J. Appl. Bacteriol. 71:211-217.

Vazquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Dominguez-Bernal, G., Goebel, W., Gonzalez-Zorn, B., Wehland, J., and Kreft, J.. 2001. *Listeria* Pathogenesis and molecular virulence determinants. Clin. Micobiol. Rev. 14(3):584-640.

Vijayakrishna, S., Venkata-Reddy, T., Varalakshmi, K., and Subramanyam, K.V.. 2000. Listeriosis in broiler chicken. Indian Vet. J. 77(4):285-286.

Watkins, J. and Sleath, K.P.. 1981. Isolation and enumeration of Listeria monocytogenes from sewage, sewage sludge and river water. J. Appl. Bacteriol. 50:1-9.

Wiedmann, M., Bruce, J.L., Knorr, R., Bodis, M., Cole, E.M., McDowell, C.I., McDonough, P.L., Batt, C.A.. 1996. Ribotyping diversity of *Listeria monocytogenes* strains associated with outbreaks of listeriosis in ruminants. J. Clin. Microbiol. 34(5):1086-1090.

Weis, J. and Seeliger, H.P.. 1975. Incidence of Listeria monocytogenes in nature. Appl. Microbiol. 30:29-32.

Welshimer, H.J.. 1960. Survival of Listeria monocytogenes in soil. J. Bacteriol. 80:316-320.

Yoshida, T. Sugimoto, T., Sato, M., and Hirai, K.. 2000. Incidence of Listeria monocytogenes in wild animals in Japan. J. Vet. Med. Sci. 62:673-675.

PART II: A COMPARISON OF ENRICHMENT BROTHS AND SELECTIVE PLATING MEDIA FOR *LISTERIA MONOCYTOGENES* ISOLATED FROM FOUR ANIMAL FARM ENVIRONMENTS

Abstract

Listeria monocytogenes is a dangerous intracellular food borne pathogen, especially for immuno-compromised individuals. Farm based risk assessment for this pathogen must be done to determine what factors are associated with isolation from the environment and animals. Optimization of isolation methods for *L. monocytogenes* from animal and farm environments is needed to best conduct risk assessment. Buffered Listeria Enrichment Broth (BLEB) and UVM-I should be compared at both 24h and 48h for a one stage enrichment. In addition to enrichment, PALCAM and MOX selective plating media recommended for isolating *L. monocytogenes* from food samples, yet should be compared for isolation from farm environments. Enrichment types and enrichment incubation times were not significant for isolating *L. monocytogenes*, having equivalent odds for isolating *L. monocytogenes*. Selective plating media was nearly significant with a point estimate of 1.569 (95% confidence interval (CI) of 0.9466 to 2.6006) (p=0.0806), which when the highest season (third) was analyzed alone the significance level dropped (p=0.0584). The point estimate of the 3rd seasons of 2.022 (95% CI of 0.9754 to 4.1916) indicated a two-to-one odds that PALCAM will isolate *L. monocytogenes* when compared to MOX.

Introduction

The Centers for Disease Control and Prevention (CDC) reported in 2002 that *Listeria monocytogenes* had the highest hospitalization rate (86.7% of reported cases) and the highest mortality rate (18.0%) for all food-borne pathogens tests (CDC 2002a). *L. monocytogenes* was responsible for less than 1% of total infections reported to FoodNet, yet 19 of 51 deaths due to foodborne illness were due to infection from *L. monocytogenes*. Human listeriosis incidence, the new cases amongst humans exhibiting symptoms due to infection with *L. monocytogenes*, has declined over the past 14 years and in 2002 was 0.26 cases per 100,000 population, near the Healthy People 2010 objective of 0.25 cases per 100,000 population (CDC 2002a). Despite these encouraging figures, the mortality rate of this foodborne pathogen requires a comprehensive study for the prevention of this bacterium's propagation in the U.S. food chain.

Listeria monocytogenes is a facultative anaerobic, gram positive bacterial rod. Listeriosis, the disease resulting from infection from *L. monocytogenes*, is an intracellular infection that makes individuals with weak immune systems most susceptible to this disease. *L. monocytogenes* is considered ubiquitous in the environment and able to grow at refrigeration temperatures. The prevalence and virulence of this microorganism in various food systems has lead to preventative action taken by regulators, such as the Food and Drug Administration's (FDA) zero-tolerance for the presence of *L. monocytogenes* on ready-to-eat meats (FDA). The introduction of *L. monocytogenes* into meat food systems is cause

for the investigation of various animal farm environments for the occurrence of this pathogen.

L. monocytogenes exhibits several physiological characteristics that provide for greater survival on the farm and in food processing plants. The ability of *L. monocytogenes* to grow at refrigeration temperatures (1˚C) and as high as 42˚C extends the possible habitats for growth and survival beyond most food borne pathogens (Junttila and others, 1988). *L. monocytogenes* has been shown to exhibit generation times of approximately 43h, 6.6h and 1.1h at 4˚C, 10˚C, 37˚C, respectively (Barbosa and others, 1994).

The ability of *L. monocytogenes* to tolerate higher osmotic conditions (halotrophic) enables it to survive in a greater diversity of environments. Normally most mesophilic pathogens require a water activity (a_w) ready of 0.93 or higher. *L. monocytogenes* has been shown to grow below 0.93 and even to 0.9 in BHI adjusted by glycerol when grown at 30˚C (Farber and others, 1992). The motility of *L. monocytogenes* via a flagella that is typically, yet not always, present and is most active between 24˚C and 30˚C, yet still present at 10˚C and 37˚C (Galsworthy and others, 1990).

Mexican-style soft cheese was implicated as the vehicle for *L. monocytogenes* which caused 142 cases of listeriosis from January 1 until August 15 of 1985, leading to 48 deaths (Linnan and others, 1988). Schlech and others (1983) reported that manure from sheep infected with *L. monocytogenes* was used to fertilize soil used to grow cabbage, which was eventually used to produce coleslaw. Weis and Seeliger (1975) reported that of 194 *L.*

monocytogenes strains, 20.3% were found in plant and soil environments, especially those from uncultivated fields, which concurs with the conclusion of Welshimer (1960), that *L. monocytogenes* has a saprophytic life.

Beef cattle have been shown to harbor *L. monocytogenes* (Hofer 1983). Swine have also been shown to carry *L. monocytogenes* (Skovgaard and Nørrung, 1989). Jensen and others (1996) provided evidence for the dairy cattle carrying *L. monocytogenes*. Chicken carcasses have been known to harbor *L. monocytogenes*, including on skin of necks. Materials (feces) from transport cages for chickens have also been found to be positive for *L. monocytogenes* (Skovgaard and Morgen, 1988). The largest recall of meat products involved turkey products. Although turkeys have not been the primary target of environmental studies, the possible link of turkey's introducing *L. monocytogenes* into a processing plant presents a risk that should be examined. Animal feed may also provide an additional opportunity for exposure to *L. monocytogenes* (Skovgaard and Morgen, 1988).

Van Renterghem and others (1991) noted that cold enrichment for *L. monocytogenes* from environmental samples was unacceptable given a recommended one to two month cold enrichment time (4˚C). They also showed that direct inoculation into selective media provided a competitive advantage against broths with high contamination of *Streptococcus* species. In contrast to this Van Renterghem and others (1991) reported that a pre-enrichment provided for more sensitive results than direct selective enrichment (without inoculation of the *Streptococcus* species). It is likely that samples taken from farm

environments would be contain higher levels of *Streptococcus* species when contrasted with food manufacturing plant samples.

The FDA recommends the use of Buffered Listeria Enrichment Broth (BLEB) to enrich samples for the recovery of *L. monocytogenes* (FDA 2003), with sampling of the enrichment at 24h and 48h. The USDA recommends a two stage enrichment, UVM-I for the primary enrichment and Fraser broth for the secondary enrichment (USDA 2002). The UVM enrichment should be compared with BLEB for an one stage enrichment, with sampling at 24h and 48h.

The FDA recommends the use of PALCAM (Polymixin B, Acriflavin, Lithium chloride, Ceftazidime, Aesculin, d-Mannitol) and Modified Oxford Media (MOX) agars (FDA 2003), where the USDA recommends only MOX as a selective agar (USDA 2002). Capita and others (2001) reported that PALCAM recovered more *Listeria* species and *L. monocytogenes* isolates than MOX.

The study extends to five state-regions, four farm animal types and four environmental sample types being sampled during 3 consecutive seasons. The objective for this project is to compare the effectiveness of isolation methods for *L. monocytogenes* from various farm environments, by comparing enrichment broths, enrichment incubation times and plating media.

Materials and Methods

Experimental Design

Environmental samples were collected from four different animal farm types located across five different states (Alabama, California, North Carolina, Tennessee, Washington). The farm types consisted of beef cattle, dairy cattle,

swine, avian farms (chicken and turkey). Four sample types were collected from each farm type, including two animal rectal swabs for the 1st season and 10 swabs for the 2nd and 3rd seasons, two soil samples, two bedding/litter/fecal samples, and two feed/grass/hay samples from each farm. Sampling covered three seasons, beginning the Fall of 2002 and continuing through Spring of 2004. Samples were collected by farmers, following a detailed sampling protocol (Appendix V), who then shipped samples overnight to the University of Tennessee's Food Safety Center of Excellence (Knoxville, TN.). Upon receiving, samples were refrigerated until analyzed.

Farm Sampling

Twenty five grams of each environmental farm sample was analyzed, being removed form a larger sample container and immediate placed within an enrichment broth. All environmental samples were manually stomached in sterile filter bags (Labplas, Inc., Québec, Canada) for one minute. Rectal swabs (Becton Dickson™ Culture Swabs™ with Cary-Blair medium (Sparks, MD.)) were placed into Universal Preenrichment Broth (Difco™, Beckton Dickson, Sparks, MD.), shaken and then refrigerated until sampling occurred, at which point 1 ml of broth was removed and transferred into 6 ml of enrichment broth.

Methods Comparison

The first goal for the evaluation of isolation methodologies involved the comparison of two enrichment media, BLEB (Oxoid Ltd., Basingstoke, Hampshire, England) and UVM-I broth (Difco™, Beckton Dickson, Sparks, MD.) for isolation of *L. monocytogenes* from farm environmental samples. Enrichment

incubation time was also tested, with the one-stage enrichments sampled at 24h and 48h of incubation at 30°C. Lastly, a comparison of selective media was done by plating both enrichments on duplicate plates of two different selective media, PALCAM (Difco™, Beckton Dickson, Sparks, MD.) and MOX (Difco™, Beckton Dickson, Sparks, MD.), which were incubated for 48h at 35°C.

Biochemical Confirmation

Suspect *L. monocytogenes* isolates were purified on Trypticase Soy Agar with 0.6% Yeast Extract (Difco™, Beckton Dickson, Sparks, MD.). Tumbling motility was checked under wet-mount microscopy, followed by a carbohydrate fermentation test utilizing 0.5% solutions of mannitol, rhamnose and xylose (Difco™, Beckton Dickson, Sparks, MD.). Upon confirmation of the carbohydrate tests, positive isolates were tested for hemolysis on a sheep's blood agar plates, including the CAMP (Christie, Atkins, and Munch-Peterson) test utilizing beta-lysin disks (Remel, Inc., Lenexa, KS.) (FDA 2003).

Statistical Analysis

Statistical analysis was accomplished via logistical regression (p=0.05) using SAS (Cary, NC), with enrichment media (BLEB, UVM), selective plating media (PALCAM, MOX) and enrichment time (24h, 48h) as the independent variables and the dependent variable being presence or absence of *L. monocytogenes*.

Results

The first three seasons produced 4,928 possible outcomes, out of which there were 64 *L. monocytogenes* isolates. The potential outcomes were a

product of animal farm type, season, sample type, enrichment type, plating media type, and enrichment time. Only 2 sets of animal rectal swabs were collected for the first season, this was increased to 10 sets of swabs for the second and third season.

None of the three independent variables were significant at the preset alpha value of 0.05, with enrichment media with a chi-square p value of 0.6149, enrichment time with a chi-square p value of 0.8013 and plating media with a chi-square p value of 0.0806. Plating media would have been a significant factor with an alpha at 0.1. Odds ratio estimates for PALCAM and MOX produced a point estimate of 1.569 and a 95% Wald confidence limits of 0.947 to 2.601. Given that this confidence interval contains the 1.0 value, this indicates that there is an equal possibility for a positive outcome (presence of *L. monocytogenes*) using either plating media (Table 1).

When the third season was analyzed alone, which exhibited the greatest number of *L. monocytogenes* isolates (33 of 64), the odds ratio estimates for plating media exhibited a point estimate of 2.022, with PALCAM as the reference variable and a 95% Wald CI of 0.9754 to 4.1916, while exhibiting a chi-squared p value of 0.0584. The CI contained the value 1.0, therefore the CI contains the probability that the odds are equal for getting the same outcome. For this third season (Spring), the point estimate of 2.022 indicates that there is twice the odds that a positive outcome (presence of *L. monocytogenes*) will occur when PALCAM agar is used. PALCAM is nearly statistically significant at a 5% alpha value and with a higher occurrence of *L. monocytogenes* might have been

statistically significant, as was the case with season three compared to the first two seasons. Enrichment type and time were both not statistically significant ($p=0.8606$ for both) (Table 2).

Discussion

Our results indicate that enrichment broth type was not statistically significant, which might explain why various studies (Patel and Beuchat 1995; Lund and others 1991) have shown conflicting data concerning the effectiveness of enrichment broths (UVM and BLEB) for isolating *L. monocytogenes*. Many of the comparisons were done using UVM and LEB, yet BLEB is now recommended by the FDA given its additional buffering capacity. For heat injured cells, UVM-I has been recommended over LEB for enrichment due to the presence of phosphate buffers in UVM-I. LEB lacked buffering which could lead to fatal decreases in pH (Bailey and others 1990b; Ferron and Michard 1993).

Patel and Beuchat (1995) provided contrary evidence, reported LEB to be superior to UVM and Fraser Broth for recovering heat treated *L. monocytogenes* cells. UVM had been shown to recover *L. monocytogenes* better than LEB from raw milk, yet level of occurrence might have influenced the outcome (Lund and others 1991). Noah and others (1991) showed LEB superior to both UVM and BLEB. LEB has been shown significantly ($p<0.05$) better for enumerating *L. monocytogenes* from ground beef compared to UVM-I and UVM-II (Yu and Fung 1991). Knabel and Theilen (1995) reported LEB superior to UVM under more anaerobic conditions (Nitrogen gas flushed tubes and addition of 0.5g/L cysteine). Given the parity of these two enrichment broths, choosing either broth

or both broths, as Ryser and others (1996) recommended for ensuring isolation of present *Listeria monocytogenes* from raw refrigerated meat and poultry products, is warranted.

PALCAM exhibited an odds ratio of two to one (95% CI of 0.9754 to 4.1916) in the 3rd season which means that PALCAM has been shown to be more effective than MOX for isolating Listeria species and *L. monocytogenes* when enriched by UVM-I for raw, refrigerated poultry meat (Capita and others 2001), yet Lund and others (1991) showed Oxford (non-modified) equally as effective as PALCAM for isolating *L. monocytogenes* from raw milk. The presence of mannitol as a differential agent provides an advantage for PALCAM over MOX. This additional advantage was significant for distinguishing *L. monocytogenes* from the diverse microflora found in the environmental samples not eliminated by the selective enrichments or plating media.

The lack of statistical significance for enrichment time for isolating *L. monocytogenes* agreed with Bailey and others (1990a), who had shown *L. monocytogenes* being equivalently recovered from two stage enrichments and 48h primary enrichments. Noah and others (1991) reported that two stage enrichments for both BLEB and UVM-I didn't recover *L. monocytogenes* in seafood as well as compared to a primary enrichment sampled at 48h.

References

Bailey, J.S., Fletcher, D.L., Cox., N.A.. 1990a. Effect of enrichment media and sampling protocol on recovery of *Listeria monocytogenes*. J. Food Prot. 53(6):505-507.

Bailey, J.S., Fletcher, D.L., Cox, N.A.. 1990b. Efficacy of enrichment media for recovery of heat-injured *Listeria monocytogenes*. J. Food Prot. 53(6):473-477.

Barbosa, W.B., Cabedo, L., Wederquist, H.J., Sofos, J.N., and Schmidt, G.R.. 1994. Growth varation among species and strains of *Listeria* in culture broth. J. Food Prot. 57(9):765-769.

Capita, R., Alonso-Calleja, C., Prieto, M., Garcia-Fernández, M.D.C., Moreno, B.. 2001. Comparison of PALCAM and modified oxford plating media for isolation of *Listeria* species in poultry meat following UVM II or frazier secondary enrichment broths. Food Microbiol. 18:555-563.

CDC. 2002a. Centers for Disease Control and Prevention. FoodNet 2002 annual report. www.cdc.gov/foodnet/annual/2002/2002executive_summary.pdf

CDC. 2002b. Centers for Disease Control and Prevention. Public health dispatch: Outbreak of Listeriosis - northeastern United States, 2002. Morbidity and Mortality Weekly Report. 51(42):950-951.

Farber, J.M., Coates, F., Daley, E.. 1992. Minimum water activity requirements for the growth of *Listeria monocytogenes*. Lett. Appl. Microbiol. 15:103-105.

FDA. 2003. United States Food and Drug Administration. Bacteriological Analytical Manual. www.cfsan.fda.gov/~ebam/bam-10.html

Ferron, P. and Michard, J.. 1993. Distribution of *Listeria* spp. In confectioners' pastries from western France: comparison of enrichment methods. Intl. J. Food Microbiol. 18:289-303.

Galsworthy, S.B., Girdler, S., and Koval, S.F.. 1990. Chemotaxis in *Listeria monocytogenes*. Acta Microbiol. Hung. 37:81-85.

Hofer, E.. 1983. Bacteriologic and epidemiologic studies on the occurrence of *Listeris monocytogenes* in healthy cattle. Zentral. fuer Bakteriol. Mikrobiol. und Hyg. Series A. 256(2):175-183.

Jensen, N.E., Aarestrup, F.M., Jensen, J., Wegener, H.C.. 1996. Listeria monocytogenes in bovine mastitis. Possible implication for human health. Intl. J. Food Microbiol. 32:209-216.

Junttila, J.R., Niemelä, S.U., and Hirn, J.. 1988. Minimum growth temperatures of *Listeria monocytogenes* and non-haemolytic *Listeria*. J. Appl. Bacteriol. 65:321-327.

Knabel, S.J. and Thielen, S.A.. 1995. Enhanced recovery of severely heat-injured, thermotolerant *Listeria monocytogenes* from USDA and FDA primary enrichment media using a novel, simple, strictly anaerobic method. J. Food Prot. 58(1):29-34.

Linnan, M.J., Mascola, L., Lou, X.D., Goulet, V., May, S., Salinen, N.C., Hird, D.W., Yonekura, M.L., Hayes, P., Weaver, R., Audurier, A., Plikaytis, B.D., Fannin, S.L., Kleks, A., Broome, C.V.. 1988. Epidemic listeriosis associated with Mexican style cheese. N. Engl. J. Med. 319:823-828

Lund, A.M., Zottola, E.A., Pusch, D.J. 1991. Comparison of methods for isolation of Listeria from raw milk. J. Food Prot. 54(8):602-606.

Noah, C.W., Perez, J.C., Ramos, N.C., McKee, C.R., Gipson, M.V. 1991. Detection of *Listeria* spp. In naturally contaminated seafoods using four enrichment procedures. J. Food Prot. 54(3):174-177.

Patel, J.R. and Beuchat, L.R.. 1995. Evaluation of enrichment broths for their ability to recover heat-injured *Listeria monocytogenes*. J. Appl. Bacteriol. 78:366-372.

Ryser, E.T., Arimi, S.M., Bunduki, M.M., Donnelly, C.W.. 1996. Recovery of different *Listeria* ribotypes from naturally contaminated, raw refrigerated meat and poultry products with two primary enrichment media. 62(5):1781-1787.

Schlech, W.F.III, Lavigne, P.M., Bortolussi, R.A., Allen, A.C., Haldane, E.V., Wort, A.J., Hightower, A.W., Johnson, S.E., King, S.H., Nicholls, E.S., Broome, C.V.. 1983. Epidemic listeriosis-evidence for transmission by food. N. Engl. J. Med. 308:203-206.

Skovgaard, N. and Morgen, C.. 1988. Detection of Listeria spp. in faeces from animals, in feeds, and in raw foods of animal origin. Intl. J. Food Microbiol. 6(3):229-242.

Skovgaard, N. and Norrung, B.. 1989. The incidence of Listeria spp. in faeces of Danish pigs and in minced pork meat. Int. J. Food Microbiol. 8:59-63.

USDA. 2002. United States Department of Agriculture. Food Safety and Inspection Service. Microbiology Laboratory Guidebook. www.fsis.usda.gov/Ophs/Microlab/ Mlg8.03.pdf

Van Renterghem, B., Huysman, F., Rygole, R., and Verstraete, W.. 1991. Detection and prevalence of Listeria monocytogenes in the agricultural ecosystem. J. Appl. Bacteriol. 71:211-217.

Weis, J. and Seeliger, H.P.. 1975. Incidence of Listeria monocytogenes in nature. Appl. Microbiol. 30:29-32.

Welshimer, H.J.. 1960. Survival of Listeria monocytogenes in soil. J. Bacteriol. 80:316-320.

Yu, L.S.L. and Fung, D.Y.C.. 1991. Evaluation of FDA and USDA procedure for enumerating *Listeria monocytogenes* in ground beef. Food Microbiol. 8:69-74.

Appendix II: Tables and Figures

Table 1. Media comparison for *L. monocytogenes* from farm environmental samples from September 2002 to May 2003 using logistic regression.

Hypothesis	***Point Estimate***	***95% Confidence Interval***	***Significance Level***
BLEB v. UVM-I	0.881	0.537 – 1.444	0.6149
24h v. 48h Enrichment	0.939	0.573 – 1.538	0.8013
PALCAM v. MOX	1.569	0.947 – 2.601	0.0806

Table 2. Media comparison for *L. monocytogenes* from farm environmental samples from March 2003 to May 2003 using logistic regression.

Hypothesis	***Point Estimate***	***95% Confidence Interval***	***Significance Level***
BLEB = UVM-I	0.940	0.472 – 1.872	0.8606
24h = 48h Enrichment	0.940	0.472 – 1.872	0.8606
PALCAM = MOX	2.022	0.975 – 4.192	0.0584

PART III: COMPARISON OF RIBOPRINTING™ AND PULSED-FIELD GEL ELECTROPHORESIS TO CHARACTERIZE LISTERIA MONOCYTOGENES TO EXAMINE GEOGRAPHICAL RELATIONSHIPS BETWEEN ISOLATES FROM VARIOUS ANIMAL FARM ENVIRONMENTS

Abstract

Listeria monocytogenes is a dangerous food borne pathogen, especially for immuno-compromised individuals. Contaminated fully cooked meat products present a significant problem for these weakened individuals and a challenge for processors of ready to eat meat products. Risk assessment for *L. monocytogenes* on farm environments is needed to reduce the probability for introduction into meat production environments. Molecular biological tools such as automated RiboPrinting™ and pulsed field gel electrophoresis (PFGE) may provide insight concerning the source of *L. monocytogenes* contaminated meat producing animals by identifying genetic relationships between contaminated animals and sources or carriers on the farm. Furthermore, identification of regional relationships may provide evidence for associated factors leading to contamination on the farm environment. The Simpson's Index of Diversity for RiboPrinting™ of the isolates at 100% similarity was 0.9737, for PFGE with *Asc*I 0.9684 and PFGE with *Apa*I 0.9842, indicating a parity amongst the two characterization methods. Clonal (100% similar) isolates were seen amongst samples taken from non-regional states and multiple isolates from the same farm with differing genetic compositions were isolated, yet no clear regional relationships were evident. Multiple animal contamination of one farm was observed, yet no environmental source was identified. Although both PFGE and RiboPrinting™ provide equivalent diversity profiles, significant differences in how isolates relate were observed.

Introduction

The Centers for Disease Control and Prevention (CDC) reported in 2001 that Listeria had the highest hospitalization rate (86.7% of reported cases) and the highest mortality rate (18.0%) for all food-borne pathogens tests (CDC, 2002a). This alarming trend placed *L. monocytogenes* as a high priority for research and prevention to limit it's exposure to the U.S. food supply.

Integrating the ability to quantify risk within hazard analysis critical control points (HACCP) programs enables risk managers the ability to connect food safety programs to public health impact (Buchanan and Whiting 1998). The CDC's PulseNet provides the ability for epidemiological investigation of human listeriosis. PulseNet catalogues the genetic profile of major food bacterial pathogens responsible for human listeriosis via PFGE. The application of this program to animal isolates of *L. monocytogenes* is needed to provide a greater comprehension of the prevalence of *L. monocytogenes* on the farm.

The national distribution of foods and commercial animal feeds lends to the possible spread of *L. monocytogenes* to different geographical regions. Given that animals and biological fertilizer are distributed less nationally and probably more regionally, the risk for finding identical strains (genetically) of *L. monocytogenes* on the farm is not high, yet if found would indicate a specific relationship.

L. monocytogenes has been isolated from a variety of farm environments, including dairy feed (silage), soil and dairy cattle (Arimi and others 1997). Since the etiology of *L. monocytogenes* on the farm is not well known, these data may

identify potential sources or reservoirs of *L. monocytogenes* and may provide probable sources for continued contamination of the farm.

Animal feed may also provide an additional opportunity for exposure to *L. monocytogenes*. Skovgaard and Morgen (1988) reported that *L. monocytogenes* was isolated from 6 of 7 dairy farms in fecal and feed samples. The percentage of feed samples tested containing *L. monocytogenes* isolates ranged between 20 and 100%.

Poultry have also been found to harbor *L. monocytogenes*. Chicken carcasses have been known to harbor *L. monocytogenes*, including on skin of necks (Skovgaard and Morgen 1988). The largest recall of meat products involved turkey products (CDC 2002b). Although turkeys have not been the primary target of environmental studies, the possible link of turkey's introducing *L. monocytogenes* into a processing plant presents a real risk that should be investigated.

Molecular characterization of *L. monocytogenes* is a powerful tool for identification and differentiation between strains. Two common molecular techniques that provide such capabilities are pulse-field gel electrophoresis (PFGE) and RiboPrinting™ (Qualicon, Inc., Wilmington, DE). An example of how these tools can be used is when *L. monocytogenes* strains were implicated in clinical listeriosis for three different ruminant animals (goats, sheep and cattle) and were found to have the same ribotypes as those found in silage fed to these animals (with one exception) (Wiedmann and others 1996). This indicates the power of molecular subtyping.

Jaradat and others (2002) noted that *L. monocytogenes* strains with 96.5 to 99% genetic similarity, as determined by RiboPrinting™, were found in two geographically distinct locations, indicating a wide spread dispersion of diverse strains. In this same study, the *L. monocytogenes* strains were grouped into 4 clusters, with each cluster showing a 92 to 99% genetic homogeneity. Each of these clusters contained isolates from human clinical cases and from food or food manufacturing samples, which again indicates a wide dispersion of similar isolates in the environment.

Ultimately, the prevalence of *L. monocytogenes* on farm environments directly impacts animals that are raised for meat production or to produce milk for human consumption. Between 1972 and 1994, 0.04% of milk samples taken from dairy cattle were positive for *L. monocytogenes*. In addition, 79% of bovine and 48% of human isolates were of the same ribogroups (using *Eco*RI) (Jensen and others 1996). The relationship between the occurrence of *L. monocytogenes* and its impact upon human population through food is of significant concern and mandates further investigation.

The objective of this research was to compare the effectiveness of two molecular tools of genetic characterization (Pulsed Field Gel Electrophoresis (PFGE) and Ribotyping™) for differentiating *L. monocytogenes* strains isolated from a variety of farm samples. Thus the hypothesis is that there is a high degree of diversity amongst isolates of *L. monocytogenes*. The study extends to five state-regions (California, Washington, Tennessee, Alabama, North Carolina), four farm animal types (beef cattle, dairy cattle, swine, chicken/turkey) and four

environmental sample types (soil, feed/grass, bedding/feces, animal rectal swabs) being sampled during six consecutive sampling periods (Fall 2002 through Winter 2004).

Materials and Methods

Comparison of PFGE and RiboPrinting™

Twenty *L. monocytogenes* isolates from 16 beef cattle, dairy cattle, poultry and swine farm environments collected over 21 months (September 2002 to May 2004). From each farm, various samples were collected, including diverse feed types, bedding or litter samples, soil samples and animal rectal swabs. The 16 farms were spread out over five states, Alabama, California, North Carolina, Tennessee and Washington to provide a diverse geographical representation of *L. monocytogenes* isolates.

Comparison of the effectiveness of two molecular tools, PFGE and Ribotyping® (Qualicon, Inc., Wilmington, DE.), was done to determine if Ribotyping® can provide the same specificity as PFGE for *Listeria monocytogenes*, which is considered the "gold-standard" for molecular characterization. *Eco*RI was the restrictive endonuclease used for Ribotyping® of *L. monocytogenes* (Qualicon, Inc. 1999). Ribotyping® was performed without modification according to Qualicon's published protocol (Qualicon, Inc. 1999). Isolates were also electrophoresed according to the PFGE protocol on a CHEF Mapper (BioRad Laboratories, Hercules, CA). Isolated DNA were digested using *Apa*I and *Asc*I (New England Biolabs, Beverly, MA.) as recommended by the

current PulseNet protocol from the CDC (Graves and Swaminathan 2001). The new PulseNet standard for PFGE is Salmonella braenderup (ATCC#: BAA-664), which was utilized per correspondence with Efrain Ribot, Ph.D., the Chief of the PulseNet Methods Development Laboratory.

Simpson's index of diversity was calculated to compare the degree of diversity measured by each analysis, calculated as follows: [1-D, where D equal to the sum of n(n-1) divided by N(N-1), where n is the total number of organisms of a particular species, and N is the total number of organisms of all species] (Simpson 1949). The average genetic similarity for each dendrogram was also measured to enable cluster analysis. Dendrograms were created using BioRad's Molecular Analyst Software version Fingerprinting plus vs. 1.6, with a 1.5% tolerance, 0.5% optimization, UPGMA clustering and dice coefficient.

Results

Twenty *L. monocytogenes* isolates were characterized from the 1420 samples collected over 21 months. Table 1 provides the Dupont ID (DID) numbers and the respective percent similarities, along with a group number for the groups of isolates with similar DID numbers. A cluster analysis of *Eco*RI, *Asc*I, and *Apa*I provided 3 clusters (A-C), five clusters (A-E), and four clusters (A-D), respectively, based upon average genetic similarity of DID groups (Figures 1-3).

Twenty two isolates were RiboPrinted™ with *Eco*RI, two of which were duplicates, producing nine Dupont ID numbers. Multiple isolates with the same DID occurred, with 14 of 20 isolates occurring in three DID groupings. Jaradat

and others (2002) noted that isolates with a DID percent similarity below 90% are probably a newly identified isolate. Figure 1 is the dendrogram for the *Eco*RI RiboPrinted™ isolates, where at 100% similarity 11 isolate groupings were identified for the 20 isolates and 6 isolate groupings at the 90.5% similarity level. The following isolates were 100% similar (clonal): 19, 20; 14, 18; 9, 10; 11, 13; 2, 4, 6; 3, 5, 7, 12. Given that these isolates are identical according to their ribosomal genomics, which are highly conserved, it may be assumed that differences exist between isolates at a chromosomal level, which was shown true by both PFGE dendrograms. Four of the six clonal isolate groupings contained isolates from the same farm, with three of the four being from the same sampling period.

The *Asc*I and *Apa*I PFGE analysis only clustered six and three of the 15 grouped isolates, respectively. Although most of the isolates were differentiated by PFGE, each PFGE derived grouping included another isolate into a grouping that RiboPrinting™ did not include. Both PFGE analyses and RiboPrinting™ with *Eco*RI showed internal reliability given both sets of duplicate samples (TN2SSwab1 and TN2DSwab10) were 100% clonal, each stemming from separate isolation plating media.

RiboPrinting™ with *Eco*RI and PFGE with *Apa*I did not consider the third season California beef swabs (isolates 2, 3, 4), clonal (100% similar), yet reported similarities 91% and 94% respectively. PFGE with *Asc*I (Fig. 2) showed these isolates clonal. Both PFGE analyses identified isolates 16, 17, 18 as clonal, where RiboPrinting™ with *Eco*RI showed these isolates 94% similar.

The PFGE *Asc*I analysis produced 16 isolate grouping at 100% similarity and 15 at 90.5% similarity. The PFGE *Apa*I analysis produced 18 clusters at 100% similarity and 15 groupings at 90.5% similarity. *Apa*I is a more frequent genomic cutter than *Asc*I for *L. monocytogenes*, which probably resulted in it having more groupings at the 100% similarity level. The Simpson's Index of Diversity for RiboPrinting™ with *Eco*RI of the isolates at 100% genetic similarity was 0.9316, for PFGE with *Asc*I 0.9684 and PFGE with *Apa*I 0.9842 and at the 90.5% genetic similarity level was 0.7737 for RiboPrinting™ with *Eco*RI, 0.9526 for PFGE with *Asc*I and 0.9632 for PFGE with *Apa*I.

Both RiboPrinting™ with *Eco*RI and the two PFGE analyses showed isolates from different states being 100% similar. PFGE, with *Asc*I digestion, was able to identify the clonal relationship between isolates located on the same farm (California Beef cattle, swabs 8, 9 and 10), yet identified clonal isolates from three different states (TN, AL, CA), with each from three different animal species during three different seasons.

Isolates 9 and 10 (Alabama dairy feed samples) were further differentiated by PFGE characterization, with 74% and 55% percent similar with *Asc*I and *Apa*I characterization, respectively. This same observation was made for isolate 19 and 20 (Washington chicken feed samples), with 79% similarity seen with *Asc*I analysis and 97% with *Apa*I. Isolates 19 and 20 were from sequential seasons from the same farm and sample type and seem to be highly related, unlike isolates 6 and 9 (Alabama dairy feed samples) were also from the same farm and sample type, yet were not closely related. Given the differences between

samples taken from the same farm in sequential seasons may indicate either a high diversity amongst *L. monocytogenes* on the farm or an environment on these farms that are conducive for growth of *L. monocytogenes*.

Discussion

The diversity amongst RiboPrinted™ isolates was moderate, with one cluster being only 80% similar. Similar to this finding, Arimi and others (1997) reported isolating 6 *L. monocytogenes* isolates from silage, with each exhibiting a distinct ribotype. There were more genetic groupings, 11 at 100% similarity, (Fig. 1) seen with the dendrogram for the RiboPrinted™ isolates than the number of DuPont identified isolates (eight), yet fewer groupings at the 90.5% genetic similarity level (six). The dendrograms created were more sensitive to band position differences than the RiboPrinter's® assignment of Dupont ID numbers. The six sets of clonal isolates (Fig. 1) from very diverse sources indicate a diverse dispersion of related species, which agrees with previous findings that very similar strains of *L. monocytogenes* may be found in different geographical regions (Jaradat and others 2002). The high number of DID groups (eight) amongst number of isolates (20) is more diverse than previous findings given their higher numbers of isolates (Gendel and Ulaszek 2002; Arimi and others 1997; Swaminathan and others 1996).

The number of isolate groupings for the PFGE and RiboPrint™ analyses, especially at the 90.5% similarity level, indicate an overall greater degree of differentiation by PFGE analysis with *Asc*I and *Apa*I compared to *Eco*RI digestion with RiboPrinting™. The ability of RiboPrinting™ with *Eco*RI to not group

isolates that were grouped by PFGE (clonally) supports the continued use of this technique for discerning relationships amongst isolates (Jeffers and others 2001). Each analysis provided some differences in how similarity clusters were formed, yet the more significant difference was seen between PFGE and RiboPrinting™. The genetic similarity of the PFGE derived clusters ranged between 62 and 79% for *Asc*I digestion and 54% and 73% for *Apa*I digestion.

The ability of the PFGE and RiboPrinting™ analyses to identify the duplicate isolates as clonal indicates these technologies are reliable. The grouping of isolates 16-18 as clonal by both PFGE digestions that were not clustered by the RiboPrinter® (isolate 17 being 94% similar to 16 and 18) indicates a difference in how these technologies function. PFGE analysis with both restriction enzymes exhibited a greater diversity for the isolates at both the 100% and 90.5% genetic similarity level, as determined by Simpson's Index of Diversity.

Similar to this, Kerouanton and others (1998) reported that PFGE provided the greater discriminatory power (discriminatory index (D.I.) = 0.886) than RiboPrinting™ (D.I. = 0.849) for distinguishing between strains of *L. monocytogenes*. Kerouanton and others (1998) noted that the combined discrimination power of PFGE and RiboPrinting™ increased (D.I. = 0.978). RiboPrinting™ was unable to distinguish between strains of *L. monocytogenes* that had different sources, yet does provide a reliable and automated procedure for the identification of *L. monocytogenes*. The use of a second restriction

enzyme (*Pvu*II) could provide additional discrimination, as is the case with the use of the PFGE analysis in this analysis (Gendel and Ulaszek 2000).

The high degree of diversity seen amongst isolates is consistent with the overall diverse nature of the samples analyzed. The near non-existent clustering for the *Apa*I PFGE analysis provided further evidence of the diverse nature *L. monocytogenes*. *Asc*I analysis, unlike *Apa*I, did provide a greater agreement with the RiboPrinting™ analysis (DID groups), indicating that *Asc*I may be superior for characterizing *L. monocytogenes*. The unusual relationship between *L. monocytogenes* isolates from geographically and etiologically distinct sources warrants further study. Both automated RiboPrinting™ and PFGE provided evidence that both are effective means for characterizing *L. monocytogenes* from diverse sources.

References

Arimi, S.M., Ryser, E.T., Pritchard, T.J., and Donnelly, C.W.. 1997. Diversity of *Listeria* ribotypes recovered from dairy cattle, silage, and dairy processing environments. J. Food Protect. 60(7): 811-816.

Buchanan, R.L. and Whiting, R.C.. 1998. Risk assessment: a means for linking HACCP plans and public health. J. Food Prot. 61(11):1531-1534.

CDC. 2002a. Centers for Disease Control and Prevention. FoodNet 2002 annual report. www.cdc.gov/foodnet/annual/2002/2002executive_summary.pdf

CDC. 2002b. Centers for Disease Control and Prevention. Public health dispatch: Outbreak of Listeriosis - northeastern United States, 2002. Morbidity and Mortality Weekly Report. 51(42):950-951.

FDA. 2003. United States Food and Drug Administration. Bacteriological Analytical Manual. www.cfsan.fda.gov/~ebam/bam-10.html

Graves, L.M. and Swaminathan, B.. 2001. PulseNet standardization protocol for subtyping *Listeria monocytogenes* by macrorestriction and pulsed-field gel electrophoresis. Intl. J. Food Micro. 65:55-62.

Jaradat, Z.W., Schutze, G.E., Bhunia, A.K.. 2002. Genetic homogeneity among *Listeria monocytogenes* strains from infected patients and meat products from two geographic locations determined by phenotyping, ribotyping and PCR analysis of virulence genes. Intl. J. Food Microbiol. 76:1-10.

Jeffers, G.T., Bruce, J.L., McDonough, P.L., Scarlett, J., Boor, K.J., Wiedmann, M.. 2001. Comparative genetic characterization of *Listeria monocytogenes* isolates from human and animal listeriosis cases. Microbiol. 147:1095-1104.

Jensen, N.E., Aarestrup, F.M., Jensen, J., Wegener, H.C.. 1996. Listeria monocytogenes in bovine mastitis. Possible implication for human health. Intl. J. Food Microbiol. 32:209-216.

Kerouanton, A., Brisabois, A., Denoyer, E., Dilasser, F., Grout, J., Salvat, G., Picard, B.. 1998. Comparison of five typing methods for the epidemiological study of *Listeria monocytogenes*. Intl. J. Food Microbiol. 43:61-71.

Qualicon, Inc.. 1999. RiboPrinter® Microbial Characterization System: Operations User's Guide. Wilmington, DE., USA. 4:1-58.

Simpson, E. H. 1949. Measurement of diversity. Nature (Lond.) 163:688.

Skovgaard, N. and Morgen, C.. 1988. Detection of Listeria spp. in faeces from animals, in feeds, and in raw foods of animal origin. Intl. J. Food Microbiol. 6(3):229-242.

USDA. 2002. United States Department of Agriculture. Food Safety and Inspection Service. Microbiology Laboratory Guidebook. www.fsis.usda.gov/ophs/microlab/mlg8.0 3.pdf

Wiedmann, M., Bruce, J.L., Knorr, R., Bodis, M., Cole, E.M., McDowell, C.I., McDonough, P.L., Batt, C.A.. 1996. Ribotyping diversity of *Listeria monocytogenes* strains associated with outbreaks of listeriosis in ruminants. J. Clin. Microbiol. 34(5):1086-1090.

Appendix III: Tables and Figures

Table 1. Type and frequency of Dupont ID formed clusters amongst isolates of *L. monocytogenes*.

Isolate #	Dupont ID	% Similarity	Dupont ID Grouping	*Eco*RI Cluster	*Asc*I Cluster	*Apa*I Cluster
1	1061	0.95	1	A	A	C
2	1061	0.80	1	A	A	A
3	1061	0.87	1	A	A	A
4	1061	0.94	1	A	A	A
5	1061	0.88	1	A	A	A
6	1061	0.87	1	A	A	B
7	1061	0.96	1	A	A	C
8	14003	0.86	2	B	B	C
9	14003	0.95	2	B	B	D
10	14003	0.95	2	B	B	C
11	1059	0.91	3	B	B	A
12	1061	0.94	1	A	C	B
13	1059	0.89	3	B	C	C
14	1062	0.97	4	C	D	A
15	1024	0.93	5	C	D	A
16	1042	0.95	6	C	D	A
17	1042	0.87	6	C	D	A
18	1042	0.93	6	C	D	A
19	1039	0.90	7	C	E	A
20	1030	0.89	8	C	E	A

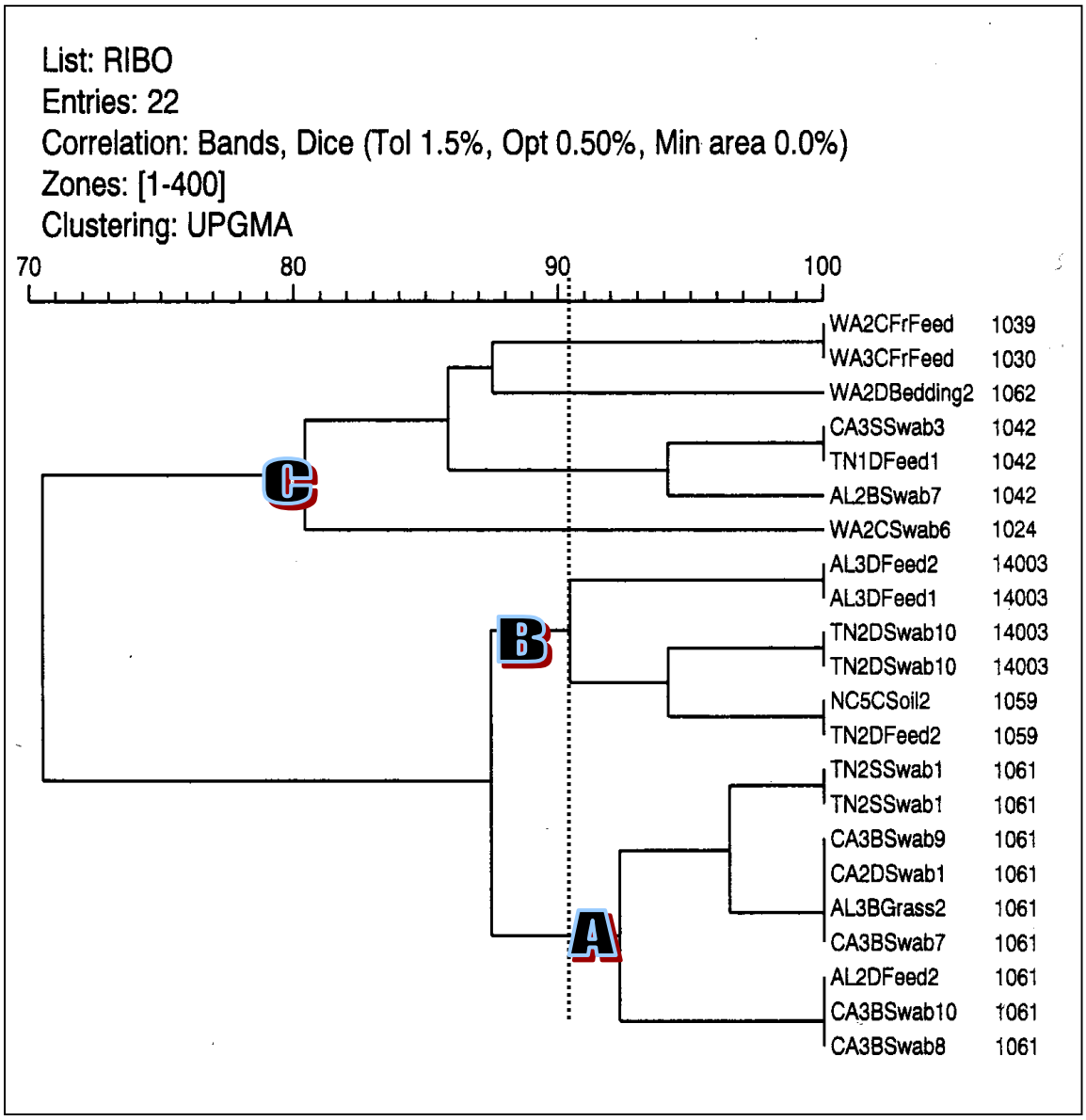

Figure 1. Dendrogram of *EcoR*I digested and fingerprinted isolates of *L. monocytogenes* characterized by automated RiboPrinting™. Numbers identify each isolate. The dotted line represents average genetic similarity (90.5%) amongst all groups of isolates based upon the DuPont identification number. The block letters indicate the genetic cluster the isolates are grouped into based on the genetic similarity.

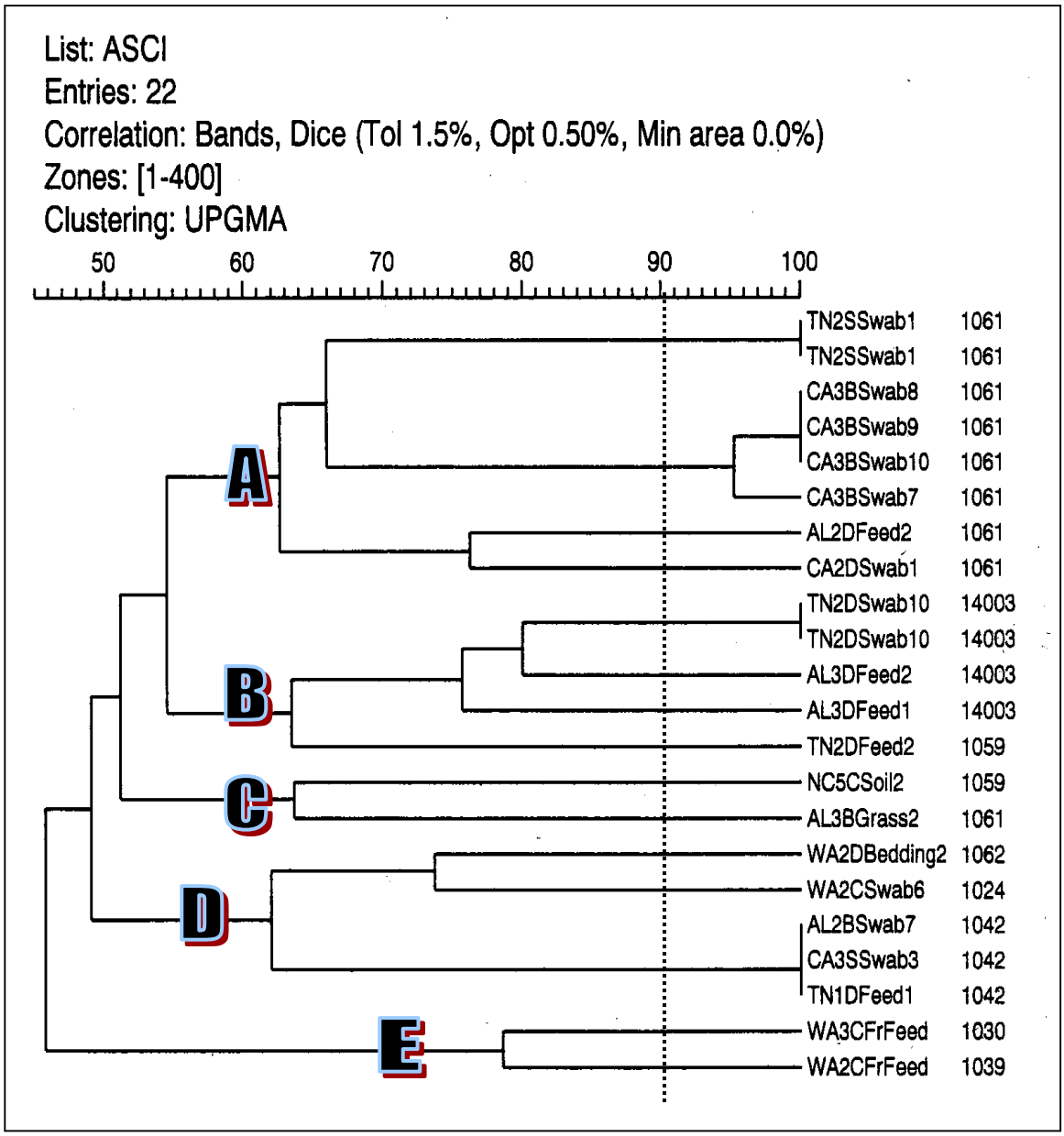

Figure 2. Dendrogram of *Asc*I digested and fingerprinted isolates of *Listeria monocytogenes* characterized by pulsed-field gel electrophoresis. Numbers identify each isolate. The dotted line represents average genetic similarity (90.5%) amongst all groups of isolates based upon the DuPont identification number. The block letters indicate the genetic cluster the isolates are grouped into based on the genetic similarity.

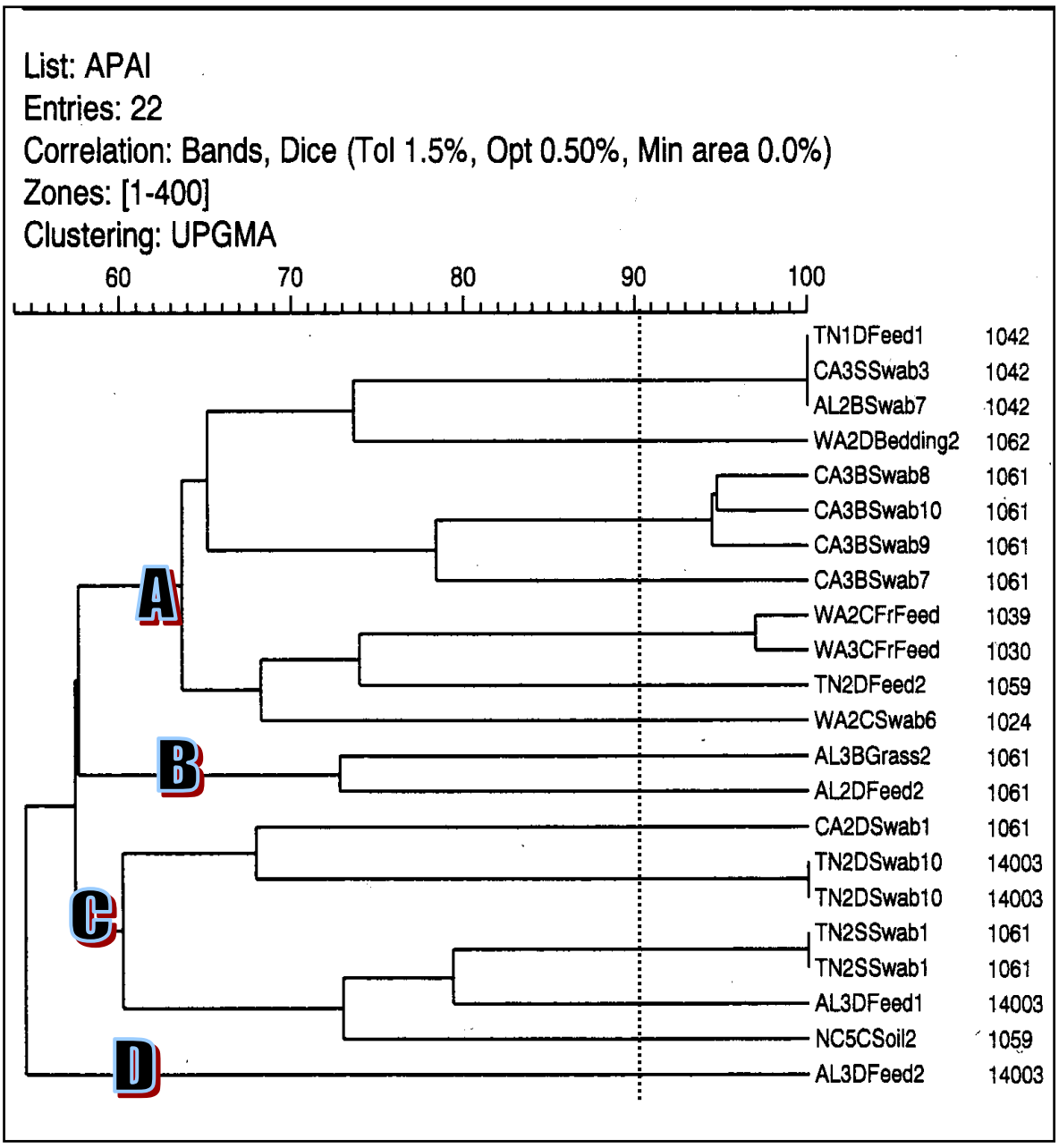

Figure 3. Dendrogram of *Apa*I digested and fingerprinted isolates of *Listeria monocytogenes* characterized by pulsed-field gel electrophoresis. Numbers identify each isolate. The dotted line represents average genetic similarity (90.5%) amongst all groups of isolates based upon the DuPont identification number. The block letters indicate the genetic cluster the isolates are grouped into based on the genetic similarity.

PART IV: A MULTI-STATE SURVEY OF 16 BEEF, DAIRY, POULTRY AND SWINE FARMS FOR *LISTERIA MONOCYTOGENES*

Abstract

Listeria monocytogenes is an intracellular pathogen that poses serious risks for immuno-compromised individuals. On the farm risk assessment is needed to reduce exposure of such individuals to *L. monocytogenes*. Base line epidemiological surveys for this pathogen are needed to identify animal and ecological reservoirs and sources. A 21 month (September 2002 to May 2004) survey of four animal farm types for *L. monocytogenes* in five different states was conducted to determine the level of occurrence in various environments. An overall occurrence of 1.4% (20/1432 samples) for *L. monocytogenes* was observed in this 21 month survey. Each sample type (soil, bedding/litter, feed/grass and animal rectal swabs) produced at least one isolate of *L. monocytogenes*. The Winter and Spring of 2003 produced the greatest number of isolates (18 of 20 total isolates). Feed and animal rectal swabs exhibited the highest number of isolates, eight and 10, respectively, compared to environmental samples.

Introduction

The United States Department of Agriculture's (USDA) Food Safety and Inspection Service (FSIS) is "committed to improving public health through food safety." (FSIS 2004). One of the major activities of this objective is the use of risk assessment to evaluate risks in farm-to-table food safety strategies, as well as the evaluation of surveillance programs for the ability to provide accurate scientific knowledge for the establishment and improvement of public health programs.

The Centers for Disease Control and Prevention (CDC) reported in 2002 that *Listeria monocytogenes* had the highest hospitalization rate (86.7% of reported cases) and the highest mortality rate (18.0%) for all food-borne pathogens tests (CDC 2002a). Listeriosis incidence has declined over the past 14 years and in 2002 was 0.26 cases per 100,000 population, near the Healthy People 2010 objective of 0.25 cases per 100,000 population, yet the mortality rate of this foodborne pathogen requires a comprehensive study for the prevention of this bacterium's propagation in the U.S. food chain (CDC 2002a).

L. monocytogenes exhibits several physiological characteristics that provide for greater survival on the farm and in food processing plants. The ability of *L. monocytogenes* to grow at refrigeration temperatures (1˚C) and as high as 42˚C extends the possible habitats for growth and survival beyond most food borne pathogens (Junttila and others 1988). Soft cheese, as well as raw and pasteurized milk have been shown to be adequate environments for the survival and growth of *L. monocytogenes* (Beckers and others, 1987; Fleming and others

1985; Linnan and others 1988). The largest food recall in the USA concerned further processed turkey meats, which was due to contamination with *L. monocytogenes* (CDC 2002b).

Schlech and others (1983) reported that manure from sheep infected with *L. monocytogenes* was used to fertilize soil used to grow cabbage, which was eventually used to produce coleslaw that was consumed, resulting in an outbreak of human listeriosis. *L. monocytogenes* has been isolated from various farm environments, including dairy feed (silage), soil and dairy cattle (Arimi and others 1997). The etiology of *L. monocytogenes* on the farm is unknown, yet a further understanding of other sources or reservoirs of *L. monocytogenes* may provide probable sources for continued contamination of the farm. Weis and Seeliger (1975) did note that the lowest prevalence for *L. monocytogenes* was in land used for current agricultural use.

Silage has been implicated as a reservoir of *L. monocytogenes*, even showing the ability to survive below a pH of 4.00 (Arimi and others 1997). Skovgaard and Morgen (1988) reported that *L. monocytogenes* was isolated from 6 of 7 dairy farms in fecal and feed samples. The percentage of feed samples tested containing *L. monocytogenes* isolates ranged between 20 and 100%.

Van Renterghem and others (1991) reported that 9 out of 10 *L. monocytogenes* isolates taken from 82 environmental samples were from fecal sources (4 from swine and 5 from cattle feces). Eighteen percent of beef cattle from Rio de Janeiro were shown to be infected with *L. monocytogenes*, as

determined by positive isolation from stool samples (Hofer 1983). Hofer (1983) also noted that 9 of the 11 infected beef cattle were in the greater than 5 years of age category, which might indicate the significance of age to harboring *L. monocytogenes*.

The feces from 6 out of 73 herds of swine in Denmark collected during slaughter (March and April of 1988) exhibited *Listeria* isolates (n=172), three of seven being *L. monocytogenes* (Skovgaard and Nørrung 1989). Chicken carcasses have been known to harbor *L. monocytogenes*, including on skin of necks. Materials (feces) from transport cages for chickens have also been found to be positive for *L. monocytogenes* (Skovgaard and Morgen 1988).

The overall objective of this research project is to determine the occurrence of *L. monocytogenes* in environmental samples from 16 farms using classical microbiological and molecular tools to facilitate risk assessment.

Materials and Methods

Sample collection

Samples were collected from four different animal farm types located across five different states (Alabama, California, North Carolina, Tennessee, and Washington) from September of 2002 until May of 2004 (6 seasons) for a total of 16 farms (not all states contained all animal farm types). The farm types consisted of beef cattle, dairy cattle, swine, and chicken/turkey farms. Four sample types were collected from each farm type, including four animal rectal swabs for the 1st season and twenty for the 2nd through 6th seasons (combined to analyze in pairs), two soil samples, two bedding/litter/fecal samples, and two

feed/grass/hay samples from each farm. Samples were collected by trained farmers (Appendix IV) who then shipped samples overnight to The University of Tennessee's Food Safety Center of Excellence (Knoxville, TN.). Upon receiving, samples were refrigerated until analyzed.

Isolation and Confirmation of L. monocytogenes

Twenty five grams of each environmental farm sample was analyzed, being removed from a larger sample container and immediate placed within an enrichment broth. Rectal swabs (Becton Dickson™ Culture Swabs™ with Cary-Blair medium (Sparks, MD.)) were placed into Universal Preenrichment Broth (Difco™, Beckton Dickson, Sparks, MD.), shaken and then refrigerated until sampling occurred, at which point 1 ml of broth was removed from each of combined samples and placed into 6 ml of enrichment broth. All environmental samples were manually stomached in sterile filter bags (Labplas, Inc., Québec, Canada) for one minute and incubated at 30˚C for 24h or 48h.

Samples were enriched in Buffered Listeria Enrichment Broth ((BLEB), Oxoid Ltd., Basingstoke, Hampshire, England) for the recovery of *L. monocytogenes* (FDA 2003). PALCAM agar (Polymixin B, Acriflavin, Lithium chloride, Ceftazidime, Aesculin, d-Mannitol) (Difco™, Beckton Dickson, Sparks, MD.) was used for isolating *L. monocytogenes* from the enrichment broth, incubated at 35˚C for 24 to 48h (FDA 2003). Several suspected colonies were selected and re-streaked onto Trypticase Soy Agar with 0.6% Yeast Extract ((TSAYE), Difco™, Beckton Dickson, Sparks, MD.) for purification and incubated at 30˚C for 24 to 48h. Motility was observed using a phase-contrast microscope

to ensure tumbling motility was present. Biochemical testing for identification was done using fermentation of rhamnose, mannitol and xylose (Difco™, Beckton Dickson, Sparks, MD.) and hemolysis testing using sheep's blood agar plates (TSAYE with 5% blood), using the CAMP (Christie, Atkins, and Munch-Peterson) test utilizing beta-lysin disks (Remel, Inc., Lenexa, KS.) (FDA, 2003).

Aerobic Plate Count

Aerobic plate count (APC) was prepared according to Feldsine and others (2003) using SimPlate® (Biocontrol, Bellevue, WA). Fecal swabs were diluted in 10ml of lactose broth. All other samples (25g) were diluted with 225ml 0.1% peptone water (w/v) and ten-fold dilutions were utilized to gain countable range for plating. Samples were plated on SimPlates® and mixed with provided indicator solution, which were incubated at 35˚C for 24h. Color changes in wells indicated a positive, which were counted. Most probable number (MPN) of organism were counted by counting number of positive wells and calculating to gain CFU/ml using a provided conversion table (Biocontrol, Bellevue, WA.), which was multiplied by the dilution being considered.

Total Coliforms/Escherichia coli

Total coliforms (TC) and *E. coli* (EC) populations were enumerated using SimPlates® as was done with APC, yet followed instructions provided by the manufacturer for TC and EC (Biocontrol, Bellevue, WA.). SimPlates® were incubated at 35˚C for 24h. TC was determined by counting total wells with color change and EC by observing fluorescing wells as seen under UV light. The total MPN for TC and EC was calculated using the number of positive wells with a

provided conversion chart (Biocontrol, Bellevue, WA.), multiplied by the dilution used for the reading.

Fecal Streptococcus

Fecal Streptococcus were counted as described by Downes and Ito (2001). Fecal swabs were diluted in 10ml lactose broth, yet further dilutions were carried out in 0.1% peptone water. All other samples (25g) were diluted initially with 225ml 0.1% peptone water and diluted further with 9ml tubes of 0.1% peptone water (decimal dilutions). Pour plates with KF Streptococcus Agar were utilized to gain plate counts after incubation at 35˚C for 48h.

Results

L. monocytogenes was isolated twenty times out of 1432 samples or 1.4% over 21 months. This percentage of positive samples was significantly lower than other studies (Table 1). A visual representation of the distribution of *L. monocytogenes* across the five states during the 21 month survey is presented in Figure 1.

The range of occurrences of *L. monocytogenes* isolates was 0.35 to 2.84%, with North Carolina exhibiting the lowest (0.35%) and Alabama the highest isolation percentage (2.84%). Given the low number of isolates for the overall project, there does not seem to be a correlation between occurrence *L. monocytogenes* and geography (states) (Figure 1).

The occurrence of *L. monocytogenes* among animal farms was highest among dairy farms (2.27%), followed by beef (1.27%), avian (1.09%) and swine farms (0.55%) (Table 1). Concerning samples types, 4.17% of feed samples

(isolated only from dairy and chicken feed), 1.09% of animal rectal swabs, 0.52% of soil samples, and 0.52% of bedding samples contained *L. monocytogenes*.

The highest number of *L. monocytogenes* isolates, 9 isolates per season, the Winter of 2003 and Spring of 2003 (seasons 3 and 4) (Fig. 2). Tables 2 to 5 provide the distribution of *L. monocytogenes* per sample types per animal farm type in each state over the six seasons surveyed.

Tables 6 to 9 provide background bacterial counts for the samples collected, which are the normative bacterial types present in these types of samples. Aerobic plate counts (APC), total coliform (TC), *Escherichia coli* counts (EC) and fecal Streptococci (FS) are reported, along with the number of positive *L. monocytogenes* isolates for samples tested.

Discussion

Skovgaard and Nørrung (1988) reported that 1.7% of swine feces were positive for *L. monocytogenes* and Van Renterghem and others (1991) reported 16% of swine feces being positive. Although no feces samples were positive for *L. monocytogenes*, the 0.55% frequency of isolation of swine farm environmental samples in this study is not far different that the 1.7% occurrence seen in Denmark. They also reported 6 occurrences of *L. monocytogenes* from 51 minced pork samples (11.8%) in Denmark, leading the authors to conclude that fecal contamination may be a significant source for *L. monocytogenes*. In this study both *L. monocytogenes* isolates were from rectal swabs, which may also relate to a higher risk for contaminating swine meat during slaughter. Contamination from other environmental samples was not observed in this study

for swine farms, yet the contamination of swine at the Tennessee (Winter 2003) and California (Spring 203) farms must have a source, which may be from an earlier contamination source, which was removed before sampling and the animal was a carrier. There is a possibility that a contaminated environmental source was present, yet had a low enough prevalence that our sampling did not detect it.

Dairy cattle farms exhibited the highest occurrence during this study (2.27%). Jensen and others (1996) reported a mean of 0.04% of dairy cows infected with *L. monocytogenes* and a 1.2% mean for herds containing an infected cow, where Perry and Donnelly (1990) found 2.9% of silage samples positive for *L. monocytogenes*. Although this study examined milk samples and not rectal or farm environmental samples, milk sampling from each dairy cow was used to infer bovine mastitis from *L. monocytogenes*. The majority of *L. monocytogenes* isolates found on dairy cattle farms were from feed sources, which for dairy cattle were farm made silage or TMR (Total Mixed Ration). Arimi and others (1997) reported silage samples having the same ribotype as those found in dairy cattle and dairy processing environments, indicating a possible relationship between silage being consumed, contamination of dairy cattle and contamination of milk, which possibly lead to contamination of the dairy processing environments.

Beef cattle feces have been shown to harbor *L. monocytogenes*, even as high as 20% (Van Renterghem and others 1991). Beef cattle feces were not positive for *L. monocytogenes*, yet the majority of isolates from this study were

from rectal swab samples. This indicates that beef cattle feces are not a suitable environment for the survival of *L. monocytogenes*. The only other isolate came from an Alabama feed-grass sample.

There was one occurrence of *L. monocytogenes* found on poultry rectal swabs (Washington, Winter 2003). This isolate was not closely related to the poultry feed samples found on this same farm during Winter of 2003 or Spring of 2003, ranging from 45 to 76% similarity on dendrograms produced with automated RiboPrinting™ (*Eco*RI) and pulsed field gel electrophoresis (PFGE) with two different restriction endonucleases (*Apa*I and *Asc*I). Poultry feces have been shown to harbor *L. monocytogenes* (Skovgaard and Morgen 1988). These authors provided further evidence of poultry contaminated with *L. monocytogenes* from slaughterhouse sampling of chicken neck skins and transport cages in Denmark.

The poultry feed samples were commercial and pelletized. The Washington poultry farm feed samples found positive in Winter and Spring of 2003 were closely related, ranging from 78% similarity (*Asc*I) to 97% similarity (*Asc*I), indicating these isolates are likely related. Pelletized feed has been of concern for contamination with Salmonella species, yet should also extend to contamination with *L. monocytogenes*. Poultry litter was not shown to contain *L. monocytogenes* and for the first three seasons rhamnose positive *L. innocua* was not isolated either.

Soil from a North Carolina poultry farm produced an isolate of *L. monocytogenes*. This was the only soil sample that was positive for *L.*

monocytogenes in this survey. Weis and Seeliger (1975) observed soil from various environments being a sufficient environment for *L. monocytogenes*, yet the low frequency of isolation may indicate this is not a primary source for *L. monocytogenes*. Given raw (uncooked) poultry has been shown to harbor *L. monocytogenes*, the risk of finding *L. monocytogenes* on processed poultry may be increased due to it's presence in environmental sources on the farm (Tiwari and Aldenrath 1990).

The Winter and Spring of 2003 produced 18 of 20 isolates, 9 in each season. This agrees with Fernandez-Garayzabal and others (1987), in that the colder seasons provided a more conducive environment for growth. *L. monocytogenes* has been shown to have a minimum growth temperature of +1.1 $\pm$ 0.3°C., which was 0.6°C lower than non-pathogenic strains of Listeria. A decrease in growth of *L. monocytogenes* was seen at 2.9 $\pm$ 0.4°C (Junttila and others 1988).

Table 2 to 5 provide a visual representation of the seasonal distribution of *L. monocytogenes* for the four animal farm types observed in four different states. Winter 2003 was the only season that observed multiple sample types being positive for *L. monocytogenes* on the same farm, which occurred twice. The Winter of 2004 did not produce any isolates, unlike the previous Winter. Surveys of the farmers who participated in this survey of *L. monocytogenes* are being conducted to help determine changes that might have occurred during sampling that could have lead to this result.

Season influences the isolation of *L. monocytogenes*, yet contradicting results indicate climate is only an associated factor and is not the cause of the isolation event. In Mexico, *L. monocytogenes* was isolated from raw dairy cattle milk significantly more often during the Spring and Summer than the Fall and Winter ($p<0.05$) (Carlos and others 2001). This indicates that the season is not as important as the climate of the location throughout the year. Fernandez-Garayzabal and others (1987) reported more *L. monocytogenes* isolates from raw milk from November to March (78.3%) than the warmer months of April until October (30.2%) in Spain. Rea and others (1992) report a rise in the isolation of *L. monocytogenes* from raw milk samples from 0 to 5% during the Spring and Summer to a baseline range of 35 to 37% during the Winter in Ireland. These results reinforce the belief that colder temperatures provide a favorable environment for growth of *L. monocytogenes*.

Tables 6 through 9 provide the background bacterial ecology in the samples collected. Table 6 indicated that a feed sample positive for *L. monocytogenes* from the beef cattle farm exhibited higher APC, TC and FS than those samples not positive for *L. monocytogenes* from other states. Conversely, dairy bedding samples in Washington, which contained a positive isolate of *L. monocytogenes*, exhibited a lower APC than the other states. Poultry rectal swabs from North Carolina and Washington, which contained *L. monocytogenes*, exhibited a higher EC count than those from Tennessee by at least one log. These are possible indicators for increased risk of harboring *L. monocytogenes.*

The identification of various risk factors may provide direction for reducing the probability of the risk of the presence of *L. monocytogenes*. Sanaa and others (1993) assessed suspected risk factors on dairy farms, determining that poor quality silage, poor hygiene and improper disinfection of towels between milking are amongst some of the risks associated with the presence of *L. monocytogenes*. On the farm risk assessment has served to increase knowledge of microbial risks associated with fresh produce for growers and improved on the farm practices (Powell and others, 2002). The integration of hazard analysis critical control point (HACCP) and quantitative microbial risk assessment on the farm could lead to a more effective risk management plan for the reduction of the introduction of *L. monocytogenes* into food producing animals and their food processing environments (Buchanan and Whiting 1998). Incorporating economic analysis may also provide a realistic approach to risk assessment on the farm, emphasizing a prioritization for what steps the farmer could afford (Morales and McDowell 1998). Blaha (1999) recommends the use of epidemiological data collection methods, processing and analysis to assist in on-farm pathogen control and on-farm residue avoidance programs and implementation of HACCP.

Integrating the ability to quantify risk within hazard analysis critical control points (HACCP) programs enables risk managers the ability to connect food safety programs to public health impact (Buchanan and Whiting 1998). Dose-response relationships and pathogen baseline data are needed to develop risk assessments concerning *L. monocytogenes* on the farm. Baseline date provides

the normative level for *L. monocytogenes* in the environment, which enables future survey a baseline to compare against. Dose-response relationships provide a risk level for those animals that are exposed to *L. monocytogenes*. Identification of risk factors, such as nutrition, climate, hygiene, housing conditions and animal management practices, must be integrated with data of baseline pathogen prevalence for common animal and foodborne pathogens (Noordhuizen and Welpelo 1996).

References

Arimi, S.M., Ryser, E.T., Pritchard, T.J., and Donnelly, C.W.. 1997. Diversity of *Listeria* ribotypes recovered from dairy cattle, silage, and dairy processing environments. J. Food Protect. 60(7): 811-816.

Beckers, H.J., Soentoro, P.S.S. and Delfgou-van Asch, E.H.M.. 1987. The occurrence of *L. monocytogenes* in soft cheeses and raw milk and its heat resistance to heat. Int. J. Food Microbiol. 4:249-256.

Blaha, Th.. 1999. Epidemiology and quality assurance application to food safety. Prevent. Vet. Med. 39:81-92.

Buchanan, R.L. and Whiting, R.C.. 1998. Risk assessment: a means for linking HACCP plans and public health. J. Food Prot. 61(11):1531-1534.

Carlos, V.S., Oscar, R.S. and Irma, Q.R.E. 2001. Occurrence of Listeria species in raw milk in farms on the outskirts of Mexico City. Food Micobiol. 18:177-181.

CDC. 2002a. Centers for Disease Control and Prevention. FoodNet 2002 annual report. http://www.cdc.gov/foodnet/annual/2002/2002executive_summary.pdf

CDC. 2002b. Centers for Disease Control and Prevention. Public health dispatch: Outbreak of Listeriosis - northeastern United States, 2002. Morbidity and Mortality Weekly Report. 51(42):950-951.

Downes, F.P. and Ito, K.. 2001. Compendium of methods for the microbiological examination of foods. 4th Ed. American Public Health Association. Washington DC. pp 83-87.

Feldsine, P.T., Leung, S.C., Lienau A.H., Mui L.A., Townseud D.E.. 2003. Enumeration of total aerobic microorganisms in foods by SimPLate Total Plate Count-Color Indicator methods and conventional culture methods: Collaborative study. AOAC 83: 257-274.

Fernandez-Garayzabal, J.F., Dominguez, L., Vazquez, J.A., Gomez-Lucia, A., Rodriguez Ferri, E.R. and Suarez, G.. 1987. Occurrence of Listeria monocytogenes in raw milk. Vet. Rec. 120:258-259.

Fleming, D.W., Cochi, S.L., MacDonald, K.L., Brondum, J., Hayes, P.S., Plikaytis, B.D., Holmes, M.B., Audurier, A., Broome, C.V., Reingold, A.L.. 1985. Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. N. Engl. J. Med. 312:404-407.

FDA. 2003. United States Food and Drug Administration. Bacteriological Analytical Manual. www.cfsan.fda.gov/~ebam/bam-10.html

FSIS. 2004. Food Safety Inspection Service. www.fsis.usda.gov/PDF/Fulfilling_the_Vision.pdf

Hofer, E.. 1983. Bacteriologic and epidemiologic studies on the occurrence of *Listeris monocytogenes* in healthy cattle. Zentral. fuer Bakteriol. Mikrobiol. und Hyg. Series A. 256(2):175-183.

Jensen, N.E., Aarestrup, F.M., Jensen, J., Wegener, H.C.. 1996. *Listeria monocytogenes* in bovine mastitis. Possible implication for human health. Intl. J. Food Microbiol. 32:209-216.

Junttila, J.R., Niemelä, S.U., and Hirn, J.. 1988. Minimum growth temperatures of *Listeria monocytogenes* and non-haemolytic *Listeria*. J. Appl. Bacteriol. 65:321-327.

Linnan, M.J., Mascola, L., Lou, X.D., Goulet, V., May, S., Salinen, N.C., Hird, D.W., Yonekura, M.L., Hayes, P., Weaver, R., Audurier, A., Plikaytis, B.D., Fannin, S.L., Kleks, A., Broome, C.V.. 1988. Epidemic listeriosis associated with Mexican style cheese. N. Engl. J. Med. 319:823-828.

Morales, R.A. and McDowell, R.M.. 1998. Risk assessment and economic analysis for managing risks to human health from pathogenic microorganisms in the food supply. J. Food Prot. 61(11):1567-1570.

Noordhuizen, J.P.T.M. and Welpelo, H.J.. 1996. Sustainable improvement of animal health care by systematic quality risk management according to the HACCP concept. Vet. Quart. 18(4):121-126.

Perry, C.M. and Donnelly, C.W.. 1990. Incidence of *Listeria monocytogenes* in silage and its subsequent control by specific and nonspecific antagonism. J. Food Prot. 53(8):642-647.

Powell, D.A., Bobadilla-Ruiz, M., Whitfield, A., Griffiths, M.W., Luedtke, A.. 2002. Development, implementation, and analysis of an on-farm food safety program for the production of greenhouse vegetables. J. Food Prot. 65(6):918-923

Qualicon, Inc.. 1999. RiboPrinter® Microbial Characterization System: Operations User's Guide. Wilmington, DE., USA. 4:1-58.

Rea, M.C., Cogan, T.M., and Tobin, S.. 1992. Incidence of pathogenic bacteria in raw milk in Ireland. J. Appl. Bacteriol. 73:331-336.

Sanaa, M., Poutrel, B., Menard, J.L., Serieys, F.. 1993. Risk factors associated with contamination of raw milk by *Listeria monocytogenes* in dairy farms. J. Dairy Sci. 76:2891-2898.

Schlech, W.F.III, Lavigne, P.M., Bortolussi, R.A., Allen, A.C., Haldane, E.V., Wort, A.J., Hightower, A.W., Johnson, S.E., King, S.H., Nicholls, E.S., Broome, C.V.. 1983. Epidemic listeriosis-evidence for transmission by food. N. Engl. J. Med. 308:203-206.

Skovgaard, N. and Morgen, C.. 1988. Detection of Listeria spp. in faeces from animals, in feeds, and in raw foods of animal origin. Intl. J. Food Microbiol. 6(3):229-242.

Skovgaard, N. and Nørrung, B.. 1989. The incidence of Listeria spp. in faeces of Danish pigs and in minced pork meat. Int. J. Food Microbiol. 8:59-63.

Tiwari, N.P. and Aldenrath, S.G.. 1990. Occurrence of *Listeria* species in food and environmental samples in Alberta. Can. Inst. Food Sci. Technol. J. 23(2/3):109-113.

Van Renterghem, B., Huysman, F., Rygole, R., and Verstraete, W.. 1991. Detection and prevalence of Listeria monocytogenes in the agricultural ecosystem. J. Appl. Bacteriol. 71:211-217.

Weis, J. and Seeliger, H.P.. 1975. Incidence of Listeria monocytogenes in nature. Appl. Microbiol. 30:29-32.

Appendix IV: Tables and Figures

Table 1. The occurrence of isolation of *L. monocytogenes* per animal and sample type in five states from September 2002 to May 2004.

	TN	NC	AL	CA	WA	#isolates/ #samples (% isolates)
Beef	**0/88**	**N/A**	**2/88**	**4/88**	**0/88**	**6/352 (1.7%)**
Soil	0/12	N/A	0/12	0/12	0/12	
Bedding	0/12	N/A	0/12	0/12	0/12	
Grass	0/12	N/A	1/12	0/12	0/12	
Animal Rectal Swabs	0/52	N/A	1/52	4/52	0/52	
Dairy	**3/88**	**N/A**	**3/88**	**1/88**	**1/88**	**8/352 (2.27%)**
Soil	0/12	N/A	0/12	0/12	0/12	
Bedding	0/12	N/A	0/12	0/12	1/12	
Feed	2/12	N/A	3/12	0/12	0/12	
Animal Rectal Swabs	1/52	N/A	0/52	1/52	0/52	
Avian[†]	**0/88**	**1/192**	**N/A**	**N/A**	**3/88**	**4/368 (1.09%)**
Soil	0/12	1/24	N/A	N/A	0/12	
Litter	0/12	0/24	N/A	N/A	0/12	
Feed	0/12	0/24	N/A	N/A	2/12	
Animal Rectal Swabs	0/52	0/120	N/A	N/A	1/52	
Swine	**1/88**	**0/96**	**N/A**	**1/88**	**0/88**	**2/360 (0.55%)**
Soil	0/12	0/12	N/A	0/12	0/12	
Feces	0/12	0/12	N/A	0/12	0/12	
Feed	0/12	0/12	N/A	0/12	0/12	
Animal Rectal Swabs	1/52	0/60	N/A	1/52	0/52	
#isolates/ #samples (% isolates)	**4/352 (1.14%)**	**1/288 (0.35%)**	**5/176 (2.84%)**	**6/264 (2.27%)**	**4/352 (1.14%)**	**20/1432 (1.40%)**

[†] Avian: North Carolina includes Turkey along with Chicken samples.

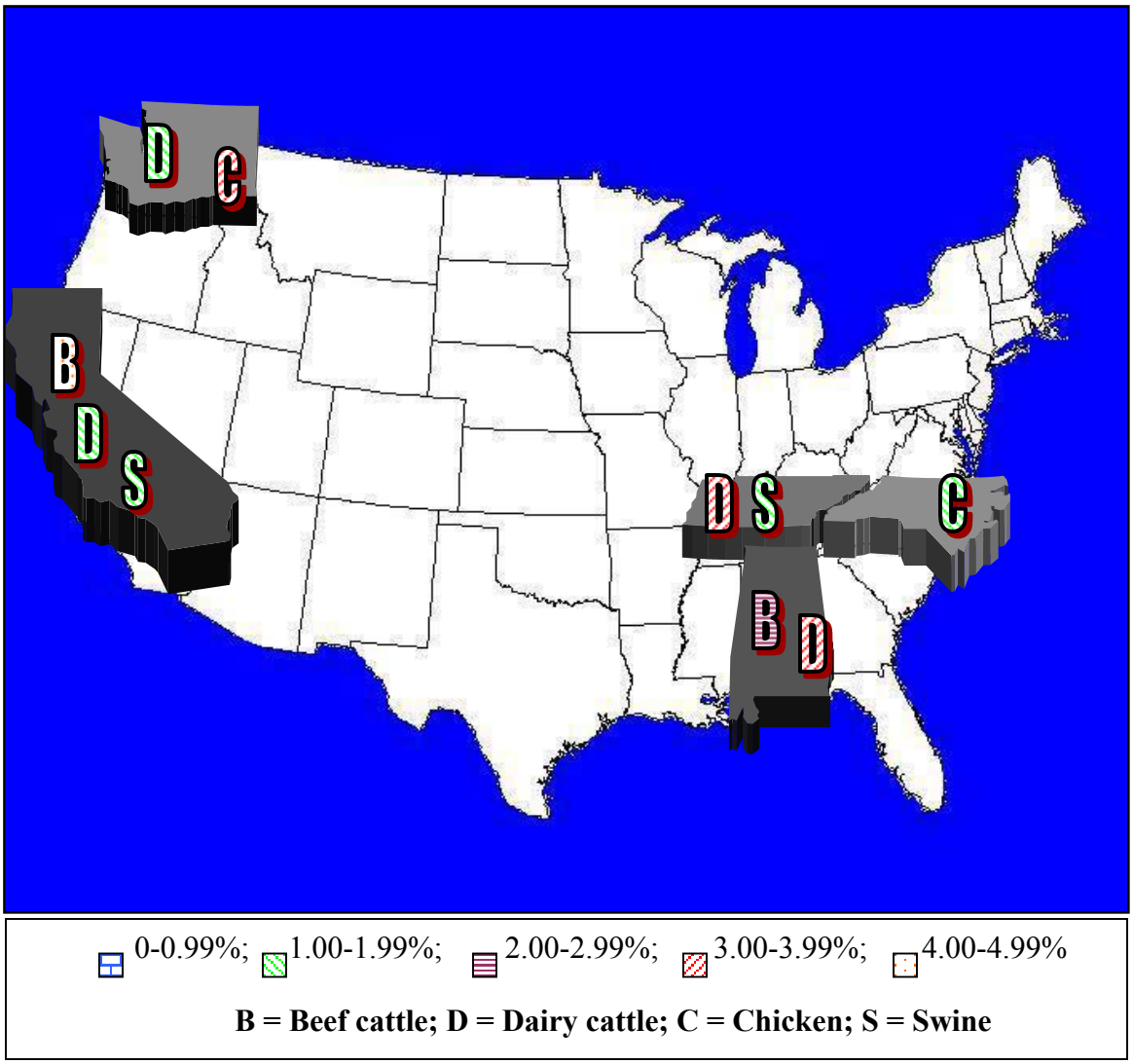

Figure 1. Distribution of *L. monocytogenes* across five states during 21 months

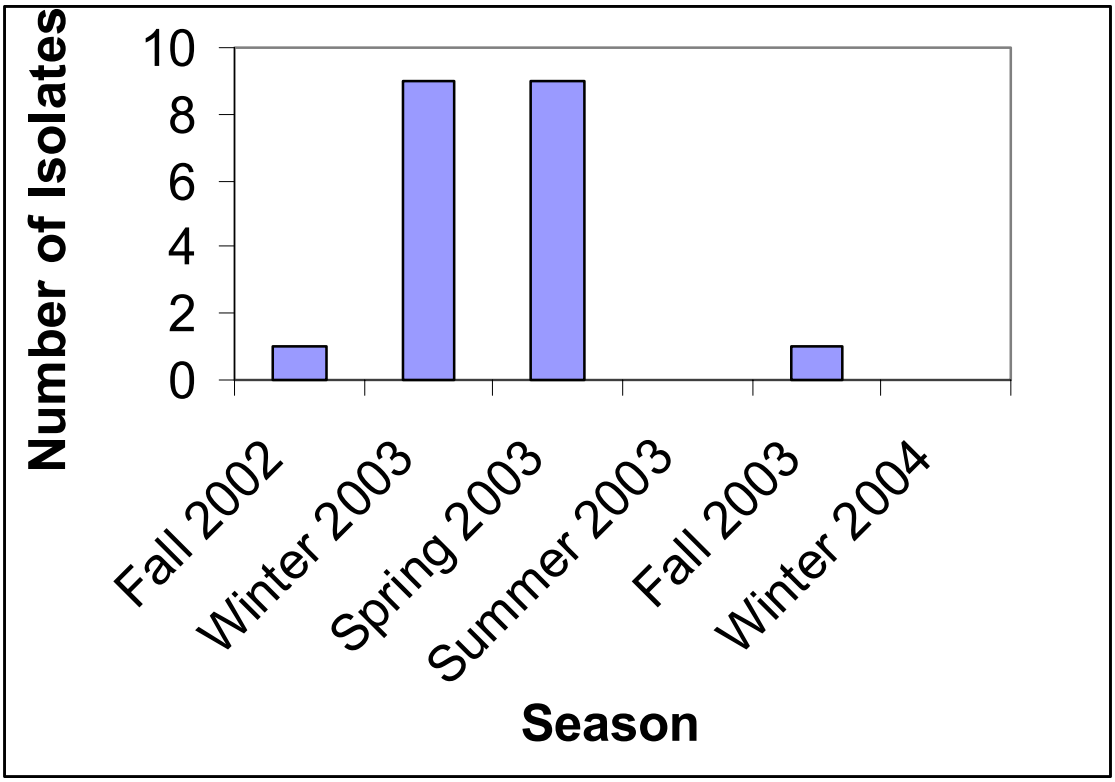

Figure 2. Number of *L. monocytogenes* isolates over 21 months in five states amongst four animal farm types.

Table 2. Seasonal distribution of *L. monocytogenes* by sample type for beef cattle farms over six seasons in four states.

Beef Cattle	**Fall 2002**	**Winter 2003**	**Spring 2003**	**Summer 2003**	**Fall 2003**	**Winter 2004**
TN	-	-	-	-	-	-
AL	-	+	+	-	-	-
CA	-	-	+	-	-	-
WA	-	-	-	-	-	-

(+) one sample type positive; (+ +) two sample types positive.

Table 3. Seasonal distribution of *L. monocytogenes* by sample type for dairy cattle farms over six seasons in four states.

Dairy Cattle	**Fall 2002**	**Winter 2003**	**Spring 2003**	**Summer 2003**	**Fall 2003**	**Winter 2004**
TN	+	+ +	-	-	-	-
AL	-	+	+	-	-	-
CA	-	+	-	-	-	-
WA	-	+	-	-	-	-

(+) one sample type positive; (+ +) two sample types positive.

Table 4. Seasonal distribution of *L. monocytogenes* by sample type for chicken and turkey farms over six seasons in four states.

Avian	**Fall 2002**	**Winter 2003**	**Spring 2003**	**Summer 2003**	**Fall 2003**	**Winter 2004**
TN	-	-	-	-	-	-
NC	-	-	-	-	+	-
CA	-	-	-	-	-	-
WA	-	+ +	+	-	-	-

(+) one sample type positive; (+ +) two sample types positive.

Table 5. Seasonal distribution of *L. monocytogenes* by sample type for swine farms over six seasons in four states.

Swine	**Fall 2002**	**Winter 2003**	**Spring 2003**	**Summer 2003**	**Fall 2003**	**Winter 2004**
TN	-	+	-	-	-	-
NC	-	-	-	-	-	-
CA	-	-	+	-	-	-
WA	-	-	-	-	-	-

(+) one sample type positive; (+ +) two sample types positive.

Table 6. Comparison of isolation of *L. monocytogenes* and background microflora in beef cattle farm samples from September 2002 to May 2004.

Sample Types	Tests	Log CFU/g or Log CFU/swab			
		TN	AL	CA	WA
Bedding	APC	7.1	8.2	7.8	7.8
	Total coliform	5.9	8.1	6.5	5.3
	E. coli	4.8	8.1	6.5	5.3
	Fecal Streptococcus	4.7	7.0	4.5	7.5
	# *L. monocytogenes*[†]	0	0	0	0
Soil	APC	6.7	7.6	7.5	8.3
	Total coliform	5.5	6.3	4.2	5.4
	E. coli	4.6	6.2	3.0	4.7
	Fecal Streptococcus	3.5	4.8	6.8	3.2
	# *L. monocytogenes*[†]	0	0	0	0
Rectal Swab	APC	10.0	9.9	9.8	10.1
	Total coliform	9.2	9.6	9.5	9.7
	E. coli	8.9	9.4	9.1	9.6
	Fecal Streptococcus	7.8	8.0	7.2	7.9
	# *L. monocytogenes*[†]	0	1/52	4/52	0
Feed	APC	8.3	9.0	6.8	6.7
	Total coliform	6.6	8.0	5.3	5.9
	E. coli	5.5	5.7	3.1	4.5
	Fecal Streptococcus	5.8	6.9	4.7	5.6
	# *L. monocytogenes*[†]	0	1/12	0	0

[†]*L. monocytogenes* is reported as # positive isolations/# samples tested.

Table 7. Comparison of isolation of *L. monocytogenes* and background microflora in dairy cattle farm samples from September 2002 to May 2004.

Sample Types	Tests	Log CFU/g or Log CFU/swab			
		TN	AL	CA	WA
Bedding	APC	8.0	9.0	8.6	7.7
	Total coliform	6.3	7.2	7.9	7.4
	E. coli	5.4	6.7	6.7	6.3
	Fecal Streptococcus	5.2	7.0	7.3	6.5
	# *L. monocytogenes*[†]	0	0	0	1/12
Soil	APC	7.0	8.9	7.3	6.8
	Total coliform	4.9	6.6	6.9	3.8
	E. coli	4.1	6.4	5.1	3.6
	Fecal Streptococcus	3.8	4.3	5.0	6.4
	# *L. monocytogenes*[†]	0	0	0	0
Rectal Swab	APC	10.3	9.9	9.9	9.5
	Total coliform	9.4	9.2	9.3	8.8
	E. coli	8.8	9.6	8.8	8.6
	Fecal Streptococcus	7.6	7.7	8.2	7.8
	# *L. monocytogenes*[†]	1/52	0	1/52	0
Feed	APC	6.9	8.1	7.8	6.4
	Total coliform	4.6	7.1	5.7	4.8
	E. coli	3.3	3.5	2.8	2.9
	Fecal Streptococcus	3.8	6.6	6.4	5.3
	# *L. monocytogenes*[†]	2/12	3/12	0	0

[†] *L. monocytogenes* is reported as # positive isolations/# samples tested.

Table 8. Comparison of isolation of *L. monocytogenes* and background microflora in chicken and turkey farm samples from September 2002 to May 2004.

Sample Types	Tests	Log CFU/g or Log CFU/swab		
		TN	NC††	WA
Bedding	APC	7.2	8.0	7.5
	Total coliform	4.8	6.5	5.0
	E. coli	4.0	6.0	3.9
	Fecal Streptococcus	5.6	6.8	6.1
	# *L. monocytogenes*†	0	0	0
Soil	APC	6.3	6.5	6.3
	Total coliform	3.5	4.0	4.1
	E. coli	2.1	2.7	2.7
	Fecal Streptococcus	2.4	3.1	3.6
	# *L. monocytogenes*†	0	0	0
Rectal Swab	APC	9.4	9.3	9.8
	Total coliform	8.7	8.4	9.1
	E. coli	6.9	7.9	8.5
	Fecal Streptococcus	7.3	7.2	7.3
	# *L. monocytogenes*†	0	1/104	1/52
Feed	APC	4.9	5.7	5.8
	Total coliform	3.0	3.2	3.5
	E. coli	1.3	2.5	1.8
	Fecal Streptococcus	3.3	3.5	3.3
	# *L. monocytogenes*†	0	0	2/12

† *L. monocytogenes* is reported as # positive isolations/# samples tested.
††Contains data for Chicken and Poultry samples

Table 9. Comparison of isolation of *L. monocytogenes* and background microflora in swine† farm samples from September 2002 to May 2004.

Sample Types	Tests	Log CFU/g or Log CFU/swab			
		TN	NC	CA	WA
Feces	APC	7.2	9.5	8.9	8.4
	Total coliform	3.9	7.6	8.6	7.0
	E. coli	3.8	7.1	8.5	6.1
	Fecal Streptococcus	5.1	6.9	6.9	7.0
	# *L. monocytogenes*†	0	0	0	0
Soil	APC	6.3	7.4	7.9	6.6
	Total coliform	3.7	5.2	6.1	4.6
	E. coli	2.1	5.1	5.3	6.7
	Fecal Streptococcus	2.8	4.7	6.6	4.5
	# *L. monocytogenes*†	0	0	0	0
Rectal Swab	APC	9.2	9.5	9.6	10.0
	Total coliform	8.9	9.2	9.5	9.6
	E. coli	7.8	9.2	9.3	8.8
	Fecal Streptococcus	7.3	7.0	7.2	6.5
	# *L. monocytogenes*†	1/52	0	1/52	0
Feed	APC	4.9	5.0	5.6	5.5
	Total coliform	3.8	4.1	4.6	4.1
	E. coli	1.0	1.0	2.5	3.9
	Fecal Streptococcus	3.6	4.4	4.9	3.3
	# *L. monocytogenes*†	0	0	0	0

† *L. monocytogenes* is reported as # positive isolations/# samples tested.

Appendix V: Farmer Sampling Protocol

Beef Farms:

It is time for the final sample collection. We are in the process of analyzing the data collection and intend on providing the final report at the end of this year. Thank you for your participation in this project. The sample types are the same as last time, and are listed below. As a reminder, the animals to be swabbed should be randomly selected. We would still like diverse soil samples from around the farm. We leave the sample choices to your discretion. If you have any questions, let me know.

Samples

1. 20 rectal swabs from 20 different cows (label C 1-20)
2. Two Ziploc bags of grass clippings from the cattle's grazing area
3. One soil sample from the grazing area.
4. One soil sample from any other location on the farm
5. Two soil samples from the animals resting or watering area.

Remember to record the GPS location for each sample collected (except the swabs). Place the samples, trowel, and GPS unit back into the kit and its ready to be sent back by FedEx. If you need anything, please call. Harry Richards

Dairy Farms:

It is time for the final sample collection. We are in the process of analyzing the data collection and intend on providing the final report at the end of this year. Thank you for your participation in this project. The sample types are the same as last time, and are listed below. As a reminder, the animals to be swabbed should be randomly selected. We would still like diverse soil samples from around the farm. We leave the sample choices to your discretion. If you have any questions, let me know.

Samples

1. 20 rectal swabs from 20 different cows (label D 1-20)

2. One fresh feed sample and one trough feed sample (Large containers).

3. Two separate soil samples from different areas of the farm (at least 100' apart). Use the trowel provided and wash it between sample collections.

4. Fill two Ziploc bags of bedding material from the holding area floor. Take the samples from different locations within the holding area.

Remember to record the GPS location for each sample collected (except the swabs). Place the samples, trowel, and GPS unit back into the kit and its ready to be sent back by FedEx. If you need anything, please call. Harry Richards

Swine Farms:

It is sample collection time again. The sample types are the same as last time, and are listed below. We are still looking for diverse samples; we leave it at your discretion. If you have any questions, let me know.

Samples

1. 20 rectal swabs from 20 different swine. (label S 1-20)

2. One fresh feed sample and one trough feed sample (fill the containers provided).

3. Two separate soil samples from different areas of the swine farm (at least 30' apart). Use the trowel provided and wash it between sample collections.

4. Fill the two containers provided with material from the swine holding pen floor. If possible, collect the samples from different sides of the holding pen. (large containers)

Remember to record the GPS location for each sample collected (except the swabs… only one number for the swine and cattle will be sufficient for all of them). Place the samples, trowel, and GPS unit back into the kit and its ready to be sent back by FedEx. If you need anything, please call. Thanks again. Harry Richards

Poultry Farms:

Here is the sampling kit for the USDA risk assessment project. There are four types of samples that we require. We need 20 rectal swabs from twenty different chickens. Do not worry about marking the animals because we will select new ones for the next sample period. Label the swabs with the letter and a number (P 1 – 20).

We also need two types of feed samples, one from before the food is distributed (a fresh sample) and one from after distribution (an old sample). Filling the labeled containers provided will be sufficient. We would like two soil samples from outside the hen houses. Collect from random locations at least 30 feet apart. We are looking for a diversity of samples, and leave it at your discretion. At least one sample from a chicken yard would be nice. Again, filling the marked containers will do. Use the trowel in the kit to collect soil, and wash off the tool between sample collections. Finally, we need two bags of chicken litter. We require 200 g, and filling the Ziploc bags provided should do it.

The final aspect of the project is to couple sample collection with global positioning data. This will allow us to access all of the relevant climatic data at the time of collection. We will be able to determine the soil and air temperature, humidity, and other data with the numbers you provide. Included in the kit is a hand held GPS unit. To activate the unit, press and hold the lower button on the right side of the device. You will see a picture of the earth being circled by satellites. Allow the device to find its satellites (it will take a few minutes, and must be outside to do this). When the top the screen says “Ready to Navigate”, the device is activated. Each time you collect a sample, write down the numbers appearing in the “Location” window at the bottom of the screen. The numbers are latitude and longitude. For instance, my office location is N 35^{o} 56.894’ W083^{o} 56.575’. We only need one of the rectal swabs to be label this way. Feel free to play with the unit’s features for your own amusement. I have had great fun with it. When you are finished with sample collection, put everything back in the kit container (including the GPS unit and trowel), put the container back in the box, and place the included shipping label on it. Call 1-800-GOFEDEX to arrange a pick-up at your location, and I will be happy to set it up if you let me know the package is ready to go.

If you have any questions, or need anything, please call. Thanks again, and I will talk with you soon, Harry Richards

Feed Samples

Beef Farms:

Feed samples consisted of hay or grass samples that cattle ate. Tennessee Beef provided corn silage and hay, both produced onsite, during the Winter and Fescue-Bermuda grass grazing during the rest of the year. California feed cattle were fed alfalfa hay and some given cottonseed meal besides grazing in pasture. Washington beef cattle were fed hay during December through March and grazed in pasture the rest of the year.

Dairy Farms:

Feed samples consisted of a mix of commercial feeds (TMR and other ingredients) and on-farm produced silage. Tennessee dairy cattle were given a Forage ration including corn silage, whole cottonseed (blended), alfalfa-orchard grass hay free choice. From October to April wet brewers grains were added to the Tennessee dairy feed. Tennessee dairy cattle were also pastured on small grains, orchard grass-clover or pearl millet.

Alabama dairy cattle were fed “TMR” and a corn silage base (grass, hay, ground corn, commercial protein and mineral mix). California dairy were fed “TMR” only. Washington dairy cattle were fed corn silage, alfalfa hay, distillers, canola, soymeal, potatoes and rolled corn.

Poultry Farms:

Feed samples were from commercial sources (pellets). North Carolina and Tennesee poultry were fed a started feed until 14 days of age, a grower diet from 15 to 34 days of age, and a finisher diet from 35 to 42 days of age. Washington poultry were fed “layer mash”.

Swine Farms:

Feed samples were from commercial sources (pellets). Tennessee swine were fed a commercially produced pellet diet for those under 40 lbs. and a “meal” diet when over 40 lbs. (all animals swine swab on this farm were over 40 lbs.). California swine were fed corn and soybean meal. Washington swine were fed barley and peas.

Appendix VI: PFGE Protocol

PFGE Protocol

A rapid Pulsed-Field Gel Electrophoresis protocol for the typing of *Listeria monocytogenes* in one day. This adapted protocol is compliments of Stephen P. Oliver's Mastitis Research Group, which is adapted from Gautom (1997), yet follows the PulseNet standardized protocol set by Graves and Swaminathan (2001).

A. Preparation of Gel Plugs

1. Bacterial colonies incubated overnight at 30°C on BHI agar are directly suspended using sterile cotton swabs, in 3 ml of Cell Suspension TE buffer (100 mM Tris and 100 mM EDTA pH 7.5). (Note: Swab the colonies gently without disturbing the agar surface.) [10 mM Tris, 1 mM EDTA, pH 8.0]
2. Adjust the turbidity of bacterial cells to an O.D. of 1.40 (1.3) using a spectrophotometer at 610 nm using a Vitek colorimeter.
3. Transfer 240 μl of each bacterial suspension to 1.5 ml microcentrifuge tube.
4. Add 60 μl (10 mg/ml) lysozyme (sigma), and incubated for 10 min for 37°C, in each tube and mix gently 5-6 times. To use, thaw one tube of lysozyme and keep on ice, discard after use. (Note: In steps 5-7, specimen tubes must be individually processed. Finish making plugs from one specimen tube before proceeding to the next.)

5. Add 300 μl of 1.2% InCert/SDS agarose mix (SeaKem Gold agarose, w/1% SDS and 0.2 mg/ml Proteinase K (Roche)). Agarose should be held at 55°C for 10-15 minutes before mixing with cell suspension.
6. Immediately mix by pipetting up and down 5-6 times, avoid creating air bubbles in mixture (NO VORTEXING).
7. Following mixing, immediately dispense the bacteria and agarose mixture into the wells of the BioRad disposable plug molds. Make two plugs per specimen, and 4 plugs for the control isolate.
8. Allow the plugs to solidify either at room temp for 15 minutes or at 4°C for 5-10 minutes.
9. Transfer the plugs to 50 ml polypropylene conical tubes (Beckton Dickinson).
10. Add 4 ml of ES (lysis) buffer (0.5 M Tris, 0.5 M EDTA, pH 8.0: 1% sodium-lauryl-sarcosine) and 40 (?) μl of Proteinase K (0.15 mg/ml stock solution). Add proteinase K immediately before use.
11. Incubate plugs in a 53 °C shaker water bath for 2 h, 200 rev/min. Tubes with plugs should be completely immersed in water and be shaking horizontally. (Note: In case a shaker water bath is not available, gently shaking the tubes manually to mix every 15-20 minutes works well.)

B. Washing of the Plugs (PFGE GNR)

1. After incubation, remove ESP buffer and transfer plugs to 50 ml tubes. (Note: Washing should be done in 50 ml tubes. These tubes should be shaking at an angle in a rack to get maximum washing effect.)

2. Wash the plugs in 10 ml of sterile distilled water (A. bidest.) (preheated to 50°C ± 2) for 15 minutes at 50°C (53 °C) in a shaker water bath. If shaker water bath in not available, gently shaking the tubes manually two to three times in the step.
3. Replace the water with 10 ml Plug Wash TE buffer (pH 8.0) (preheated to 50°C ± 2). Incubate/wash the plugs at 50°C (53 °C) in a shaker water bath for 15 minutes (if shaker water bath in not available, gently shake the tubes manually two or three times in this step.)
4. Repeat step 3 two additional times, 10-15 minutes each wash.
5. Store plugs in 2 ml of Plug Wash TE buffer at 4°C until ready for restriction digestion. (Plug remain useable for several months if stored in Plug Wash TE buffer at 4°C.)

C. Alternate Plug Washing Procedure

1. Load pre-numbered RioRad screen caps with the two plugs from each specimen. Cap number and corresponding lab number should be written beforehand in laboratory notebook. Assemble caps into a column by screwing threaded caps together, use an empty cap for the top of the column.
2. Insert column into PVC washing tube.
3. Pour pre-heated (50°C ± 2) (53°C) wash reagent into tube.
4. Screw end-cap on PVC tube.
5. Place sealed PVC tube on platform. (Platform should have clamps to hold tube submerged in water).

6. Place platform in 50°C ± 2 (53°C) shaking water/bath, and turn shaker on.
7. Do 5 washes. First wash in sterile distilled water (A.bidest.) for 15 minutes, subsequent washes are Plug Wash TE buffer for 10-15 minutes each.
8. Store plugs in 1.5 mls of Plug Wash TE buffer at 4°C until ready for restriction digestion.

D. Restriction Endonuclease Digestion (PFGE GNR)

1. Remove one plug of storage tube and place onto a clean sterile glass slide (with ethanol on it). With the help of a razor blade, cut three 2mm wide slices of the plugs and transfer them to the same labeled 1.5 ml tube (with DNA water). Save rest of the plug in Plug Wash TE buffer at 4°C for future use. (Note: Use 70% isopropyl alcohol to sterile glass slide and razor blade after every 3 specimens.)
2. Add 86 μl of sterile distilled water. 10 μl of 10X appropriate restriction enzyme buffer. 1 μl of BSA, and 2.5 μl (25 Units) of restriction enzyme *Asc*I or 18 μl (180 Units) of *Apa*I. Mix gently with the pipette. Master mix of water, buffer, BSA, and enzyme can be made on the basis of total number of specimens in a particular run.
3. Incubate the *Asc*I plugs in a 37°C water bath for 3 hours or *Apa*I plugs in a 30 °C water bath for 5 hours.

E. Pulsed-Field Gel Electrophoresis

1. After the incubation, aspirate the enzyme mix from the tube with a P-200 pipette and replace it with 0.5 ml of Plug Wash TE buffer.

2. Prepare 1% SeaKem Gold Agarose using 0.5 X TBE buffer.
3. Align the plug slices on the teeth of the comb in the appropriate order and allow them to stick to the comb for approximately 15-20 minutes before proceeding to step 4. Save unused plug slice in Plug Wash TE buffer at 4^{o}C for future use.
4. Set the comb in the gel casting mold and pour the agarose. (Note: Save 2-3 mls agarose to seal wells.)
5. Allow the gel to harden for about 20 minutes.
6. Remove the comb and seal the wells with saved agarose.
7. Perform electrophoresis with 2 liters of 0.5 X TBE running buffer using the following conditions for *L. monocytogenes*:
    a. Initial switch time: 4.0 seconds
    b. Final switch time: 40.0 seconds
    c. Run time: 22 hours
    d. Angle: 120^{o}
    e. Gradient: 6.0 V/cm
    f. Temperature: 14^{o}C
    g. Ramping factor: linear
8. Stain gel in 500 ml of distilled water with 1 drop (25 μl) of 10 mg/ml of Ethidium Bromide for 20 minutes (if possible on a rocker in the dark). Follow with three 30 minute washings with 500 ml distilled water.
9. Take picture of stained gel using Polaroid camera and digitize picture for analysis.

10. Restricted *Asc*I were sized against lambda DNA ladder (Bio-Rad) and a high-molecular weight DNA marker (Life Technologies). Strain H2446 (*L. monocytogenes*) acts as the reference (standard) strain.

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Vita

David Dean Rasmussen was born April 22, 1973 in Manchester, Connecticut. He graduated from East Windsor High School in June of 1991, having been inducted into the National Honor Society for Secondary Schools while attending. David then graduated from Virginia Tech May 1996 with a Bachelor of Science in Psychology and a Bachelor of Science in Biology, with a concentration in microbiology and immunology and a minor in chemistry. David then returned to Virginia Tech August of 1997 to pursue a Masters of Science, majoring in Food Science and Technology. David successfully defended his thesis in September of 1999, then graduated in December of 1999. David accepted a research and development position as a Food Scientist with Perdue Farms, Inc. in Monterey, Tennessee in June of 1999 and started work in October of 1999. David left his position with Perdue Farms, Inc. in June 2001 to pursue a Ph.D., majoring in Food Science and Technology, at The University of Tennessee. During his time at The University of Tennessee David taught a laboratory section of Food Microbiology lecture, twice coached the college bowl team, was the Food Science Club's fundraising chairperson and served in many other volunteer capacities. David also maintained a 4.0 grade point average during his time at The University of Tennessee, which lead to his induction in the Gamma Sigma Delta (agriculture honor society) and Phi Kappa Phi (national multi-disciplinary honor society). David has accepted an offer to work with Nestlé USA in Solon, Ohio as an Associate Food Technologist.