CHARACTERIZATION OF VIRULENCE IN ADHERENT PHENOTYPES OF *LISTERIA MONOCYTOGENES*

By

KALPANA KALPANA-KUSHWAHA

Bachelor of Science in Agriculture
University of Agricultural Sciences
Bangalore, India
1995

Master of Science in Agricultural Microbiology
University of Agricultural Sciences
Bangalore, India
1997

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CHARACTERIZATION OF VIRULENCE IN

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MONOCYTOGENES

Dissertation Approved

Dr. Peter Muriana
Dissertation Adviser

Dr. Stanley Gilliland

Dr. Christina Dewitt

Dr. William McGlynn

Dr. A. Gordon Emslie
Dean of the Graduate College

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ABBREVIATIONS AND SYMBOLS

1. RTE……..… Ready-To-Eat
2. FDA……...……Food and Drug Administration
3. CFU…….Colony Forming Units
4. MLST……..……. Multilocus Sequence Typing
5. PFGE……...…Pulsed-Field Gel Electrophoresis
6. UPGMA…….……Unweighted Pair Group Method using Arithmetic Averages
7. MOI…… Multiplicity Of Infection
8. LLO……...……Listeriolysin
9. PI-PLC…….……PhosphatidylInositol-specific PhosphoLipase C
10. PC-PLC……PhosphatidylCholine-specific PhosphoLipase C
11. PrfA……..…….Pleiotropic Virulence Regulator
12. I.G…….……IntraGastric inoculation
13. I.P……..……..IntraPeritoneal
14. Mpl…….……. MetalloProtease
15. LRR…….……. Leucine-Rich Repeats
16. HGFR……. Hepatocytes Growth Factor
17. LAP……...…….*Listeria* Adhesion Protein
18. BHI…….Brain Heart Infusion
19. ATCC……American Type Culture Collection
20. EMEM……. Eagle's Minimum Essential Medium

CHAPTER I

INTRODUCTION

Listeria monocytogenes is a Gram-positive, facultative, intracellular foodborne pathogenic bacterium known to cause listeriosis in humans. Listeriosis occurs when the bacterium is ingested via contaminated foods and 99% of the listeriosis cases are known to be foodborne (Mead et al., 1999). Infection with *L. monocytogenes* can also lead to septicemia, possibly followed by meningitis, meningoencephalitis, and other central nervous system disorders (Armstrong and Fung, 1993). It is a major threat to immunocompromised patients, pregnant women and newborns, resulting in high mortality rates. *L. monocytogenes* is capable of causing substantial problem to the food industry by contaminating the foods including raw produce, poultry, meat and dairy products leading to food recalls. Reportedly, an approximate of 2,500 human illness cases and 500 deaths occur annually in the United States (Mead et al., 1999). Because of its high fatality rates, U.S regulatory agencies have established a "zero tolerance" for the species in ready-to-eat (RTE) foods (FDA, 2008). Therefore, it is important to check the contamination and prevent human illness. The identification of atypical, virulent cell forms of *L. monocytogenes* in clinical or food samples is of paramount importance due to the severity of listeriosis in predisposed individuals and the uncertainty as to the infectious dose of this pathogenic bacterium (McLauchlin, 1997; Rowan and Anderson, 1998). To prevent contamination and track transmission of sources of *L. monocytogenes*

within food-processing plants it is important to differentiate the strains of *L. monocytogenes* by subtyping. To characterize this foodborne pathogen several methods have been developed. The current methods to differentiate strains include multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE) and ribotyping (RT). These DNA-based methods differentiate *L. monocytogenes* at the subspecies or strain level which target nucleotide variations at endonuclease restriction or define bacterial subtypes by using either PCR amplification or by defining *L. monocytogenes* strains by their unique banding patterns.

Listeria is often present on raw meat ingredients and has been a recurring problem in meat processing facilities. Some strains of *L. monocytogenes* have shown to attach to abiotic surfaces with different levels of adherence, regardless of surface (glass, rubber and stainless steel) encountered in meat processing facilities, resulting in the formation of biofilms (Borucki and Call, 2003; Gamble and Muriana, 2007). Based on their ability to attach to abiotic surfaces *Listeria* can be classified as strong or weak which can be quantified using microplate fluorescent assay (Gamble and Muriana, 2007). In the biotic systems virulent nature of *L. monocytogenes* is determined by their ability to adhere, invade and multiply in the nonprofessional phagocytes which depend upon the production of virulence factors.

The two most important steps in the pathogenesis of *L. monocytogenes* are adhesion and invasion to host tissues. Adhesion helps in colonizing non-professional phagocytic cells, whereas invasion allows bacteria to gain entry into cells. Invasion of intestinal epithelial is the first step in the establishment of infection by *L. monocytogenes* (Racz et al., 1972) and degree of invasiveness in the epithelial cells can be correlated

with its virulence potential (Finlay et al., 1988; Moulder, 1985). Since cellular adherence is the first stage of infection with *L. monocytogenes* we were interested to see whether the strong adherence observed with abiotic surfaces would also facilitate cellular adherence and aid virulence.

However, correlation between abiotic adherence and cellular adherence as well as invasion is not well studied. Virulence of different strains of *L. monocytogenes* can be compared by using various virulence models which include cell-line based assays and various animal models. More recently, several cell lines such as human epithelial HEp-2, HeLa and Caco-2 have been considered suitable for the evaluation of adherence, invasion and virulence of *L. monocytogenes* (Gaillard et al., 1987; Kathariou et al., 1990). *In vivo* studies done by infecting mice following intravenous (i.v.) intraperitoneal (i.p.) injections and oral route of inoculation are considered to be highly sensitive assays for evaluating the pathogencity of *L. monocytogenes* (Audurier et al., 1980; Hof and Hefner, 1988; Lammerding et al., 1992).

The major route of invasion of *L. monocytogenes* following intragastric inoculation is through the Peyer's patches and other gut-associated lymphoid tissues (MacDonald and Carter, 1980). *L. monocytogenes* will then translocate to the spleen and liver from there it will disseminate to different organs via lymphatic pathways (Marco et al., 1992). Replication of *L. monocytogenes* mainly occurs in hepatocytes and spread cell-to-cell forming infectious foci. Development of infectious foci in liver and spleen depended on the virulence of the strain, amount of inoculum and sensitivity of the mice strain (Cheers and McKenzie, 1978). In the hepatocytes >90% of bacteria are cleared by neutrophils during the first 24 hours of infection (Conlan and North, 1991). The

remaining bacteria which are not killed by neutrophilic attack are internalized by hepatocytes where they undergo intracellular replication. However, it is known that all strains of *L. monocytogenes* are not equally virulent and their virulence can be determined by their invasiveness and ability to grow *in vivo* (Barbour et al., 2001; Larsen et al., 2002; Roche et al., 2003; 2005). Thus, it is essential to study the *in vivo* virulence of *L. monocytogenes* originated from raw meat sources. Information on *in vivo* studies indicating the virulence of *L. monocytogenes* isolated from various meats and adhering to abiotic surfaces is limited. Considering the significance of *L. monocytogenes* as a food borne pathogen, it is important to investigate the effect of high dose of *L. monocytogenes* following oral inoculation of mice.

The purpose of this study was to examine strains of *Listeria* isolated for 13 months from three meat processing facilities for adherence and molecular typing by PFGE to assess if the recurrence of isolates correlates to adherence properties using our microplate adherence assay. In this study we examined the phylogenetic relatedness of strains of *L. monocytogenes* isolated from various sources using DNA sequencing-based subtyping methods. These DNA-based methods define bacterial subtypes by PCR amplification, sequence analysis or restriction digestion of bacterial DNA to generate DNA fragment banding patterns. Typing pathogenic bacteria from environmental sources involved in food processing may help establish strains that are persistent and may have harborage sites within the processing facility.

Examining the correlation between adherence and virulence for strong and weakly adherent strains of *L. monocytogenes* will help to assess the real risk posed by this

pathogen found in foods.Thus, it is essential to study the virulence of *L. monocytogenes* originated from raw, RTE meat and meat processing facilities.

CHAPTER II

REVIEW OF THE LITERATURE

Listeria monocytogenes: foodborne pathogen

Listeria monocytogenes is a Gram-positive, facultative, intracellular, pathogenic bacterium capable of causing severe invasive disease known as 'listeriosis' in humans. Consumption of foods contaminated with *Listeria* is a dominant cause of listeriosis. According to the Centers for Disease Control and Prevention (CDC), 28% of the annual death toll caused by known foodborne pathogens includes nearly 2,500 cases of listeriosis and about 500 deaths in the United States (Mead et al., 1999). Although the U.S. Food and Drug Administration (FDA) recently proposed an acceptable 'defect level' of 100 cfu/gm for this organism in foods, there still remains a 'zero-tolerance' for *L. monocytogenes* in ready-to-eat (RTE) foods (FDA, 2008). *L. monocytogenes* is one among the six species of genus *Listeria* that includes *L. ivanovii, L. innocua, L. seeligeri, L. welshimeri* and *L. grayi* species (Vazquez-Boland et al., 2001). Out of these, *L. monocytogenes* is the major human pathogen and *L. ivanovii* is an animal pathogen for sheep and cattle (Schmid et al., 2005).

This review is to highlight the key research findings about listeriosis, the role of *Listeria* as a food pathogen, the occurrence of *L. monocytogenes*, distribution and transmission in meat processing plants, attachment on abiotic surfaces, subtyping by

different phenotypic and genotypic methods, virulence factors contributing to pathogenesis, and methods for evaluating virulence of strains by *in vitro* and *in vivo* approaches.

Listeriosis

Sporadic and epidemic listeriosis in humans may show mild symptoms in healthy individuals, yet show serious infections in immunocompromised people (Roberts and Wiedmann, 2003). The majority of infections remain asymptomatic, or present as very mild non-invasive forms of intestinal infections (Roberts and Wiedmann, 2003; Vazquez-Boland et al., 2001). People at risk include pregnant women, newborns, elderly people, persons with weakened immune systems, as well as cancer, diabetes, kidney disease, HIV patients on immunosuppressive drugs during organ transplantation and those undergoing chemotherapy. Early symptoms of listeriosis in humans are usually associated with flu-like symptoms, fever, headache, muscle aches, and occasional gastrointestinal symptoms such as nausea or diarrhea. During later stages of infection the symptoms of listeriosis may show as stiff necks, meningitis, meningoencephalitis and septicemia resulting in high mortality rates due to bacteria crossing blood brain barrier (Gray and Killinger, 1966; Macdonald and Carter, 1980; Marco et al., 1991).

Occurrence of *Listeria monocytogenes* in meat

Various environmental sources can harbor *L. monocytogenes* including food and food processing environments. Within a food processing plant, it usually can be found in

a wide variety of reservoirs potentially contaminating finished products and RTE foods (Fenlon, 1999). The occurrence of *L. monocytogenes* in common RTE foods may vary, however several studies have indicated that 1 to 5% of commonly consumed RTE foods may contain *L. monocytogenes* (Pinner et al., 1992; Soriano et al., 2001; Wilson, 1995). The prevalence of *L. monocytogenes* contamination in a Danish turkey processing plant was reported to be 7.3% in RTE and 17.4% in raw products (Ojeniyi et al., 2000). In the United States, approximately 50% of raw beef and pork was reported to contain *L. monocytogenes* and *Listeria* spp. (Ryser et al., 1996). A survey showed an overall prevalence rate of *L. monocytogenes* in various RTE foods to be 1.82% (Gombas et al., 2003). The bacterium has the ability to survive for long periods of time under adverse environmental conditions, such as low pH, high salt concentration and will even grow under refrigeration temperatures in lightly salted and chilled food products (Seeliger and Jones, 1986). This organism can also survive freezing and dry conditions (Dickson, 1990).

Several studies have shown that RTE food products can be cross-contaminated with *L. monocytogenes* in a processing plant environment from a variety of sources (air, drains, raw materials, workers) (Gahan and Collins, 1991; Wendtland, 1994). The consumption without cooking and the limited shelf life of RTE foods under refrigeration allow *Listeria* to grow in high numbers and make such products frequent vehicles of infection (Rocourt et al., 2003; Vazquez-Boland et al., 2001).

Distribution of *L. monocytogenes* in the food processing plants

Distribution of this pathogen within a food processing plant is mainly due to the persistent nature of certain strains of *L. monocytogenes* leading to cross contamination within the environment of food processing areas and ultimately to food products. Sites of contamination within food processing plants include conveyors, coolers, and freezers along with machinery used for slicing, packaging, and dicing (Autio et al., 1999; Miettinen et al., 1999b; Tompkin et al., 1999). Non-food contact surfaces within the processing environment such as floors, walls, trucks, drains, shoes, doors and door handles, sanitizing floor mats, and foot baths have also been shown to be positive for *L. monocytogenes* (Hood and Zottola, 1995). Some complex surfaces of processing machines are difficult to sanitize and may end up sustaining contamination for several months or years (Autio et al., 1999; Miettinen et al., 1999a). Thus, strains of *L. monocytogenes* strains causing persistent contamination that may not be found in raw materials can be repeatedly recovered in RTE meats (Nesbakken et al., 1996).

Transmission of *L. monocytogenes* in meat processing plants

Contamination of finished product with *L. monocytogenes* in various RTE processing plants as well as their dissemination pattern has been reported in several studies (Lappi et al., 2004b; Thimothe et al., 2004). The presence of persistent strains was reported to be the primary reason for finished product contamination (Lappi et al., 2004a). As demonstrated in several studies, the major contamination sources for finished products include the processing environment, raw materials, and food handling practices and cross contamination by employees (Autio et al., 1999; Dauphin et al., 2001; Lappi et

al., 2004a; Norton et al., 2001b; Thimothe et al., 2004; Vogel et al., 2001). The transmission of L. monocytogenes can also occur through water and transporting raw materials such as raw shrimp in and out of a processing plant (Destro et al., 1996). Tracing the dissemination of Listeria, Nesbakken et al, (1996) characterized 133 strains of *L. monocytogenes* in five meat processing plants using Multilocus Enzyme Electrophoresis (MEE) method and isolated the bacteria from deboned fresh meat, the production environment, wastes from slicers, cold cuts and cured dried sausages. They observed that the strains from one of the five plants producing cold cuts were of the same electrophoretic types (ET) found in fresh meat, along the processing chain and the end product stressing the importance of disinfecting the whole processing chain.

Disinfecting *L. monocytogenes* contaminated surfaces can be a difficult task when the bacterium is present at every step of processing for over a year despite cleaning and disinfection efforts as seen in a pork processing plant by Giovannacci et al. (1999). Similarly (Lappi et al., 2004a) reported difficulty in removing *L. monocytogenes* in a meat-bone separator using routine disinfection procedures. It's occurrence in raw meats and prevalence both in slaughter houses and in the processing environment makes it almost impossible for the total removal of this organism (Doyle, 1988; Johnson et al., 1990). Thus the prevention of *L. monocytogenes* in products and processing equipment should be based on avoiding the colonization and further persistence on processing equipment. Also, the presence of non-pathogenic *Listeria* spp. in meat processing plant environments has been reported by various studies. For example, during 1987, USDA-FSIS tested 2,300 environmental samples from 40 meat processing facilities and reported 21% of the samples being positive for *Listeria* spp. (Tompkin et al., 1992). However,

their presence in foods can still be of major concern as it may indicate the possible presence of *L. monocytogenes* (Curiale and Lewus, 1994; Duffy et al., 2001). Frequent isolation of *Listeria* spp. including *L. monocytogenes* from floor drains and floors in food processing facilities suggest that these areas could serve as reservoirs for *Listeria* (Norton et al., 2001a; Rorvik et al., 1997; Thimothe et al., 2004). Cleaning and disinfecting of these areas should be done thoroughly but care should be taken to avoid using high-pressure hoses since such practices readily promote the spread of *Listeria* to nearby areas through splashing and the generation of aerosols (Gravani, 1999). In the meat industry, L. *monocytogenes* is therefore regarded as the most troublesome microorganism to be controlled during processing. Persistent strains adhere more efficiently to abiotic surfaces of stainless steel even after short contact times than the non-persistent strains (Lunden et al., 2000).

Attachment of bacteria to abiotic surfaces

The persistence of *L. monocytogenes* in a processing plant is often due to their ability to form biofilms on a variety of surfaces allowing for resistance to sanitizing agents and increased survivability (Aase et al., 2000; Blackman and Frank, 1996; Borucki and Call, 2003). Several strains of *L. monocytogenes* can adhere to almost all food contact surfaces with the involvement of the polysaccharide and protein matrices. The ability to adhere to various abiotic surfaces such as glass, polypropylene, rubber, and stainless steel common in food processing plants may also be mediated by non-specific (hydrophobic) interactions (Chae and Schraft, 2000; Frank and Koffi, 1990; Kalmokoff et al., 2001; Mafu et al., 1990). The level of adherence however, varies regardless of the

type of surface (glass, plastic, rubber, stainless steel) or temperature and different strains of *L. monocytogenes* have been shown to attach to abiotic surfaces with different levels of adherence (Gamble and Muriana, 2007). Within a processing facility, *Listeria* strains may find harborage sites and adhere to food surfaces such as tables, utensils, steel pipes and vessels resulting in biofilm formation that poses a high risk of contaminating food products (Arnold and Bailey, 2000).

Molecular subtyping of *L. monocytogenes* and methods used

The persistence of *L. monocytogenes* strains in various sites of a food processing facility can be established by genotyping methods. Several DNA based molecular methods have been used for genotyping of *L. monocytogenes* and such methods usually define bacterial subtypes by PCR amplification, sequence analysis, restriction digestion of genomic DNA or DNA fragment banding patterns. This molecular subtyping approach can differentiate bacterial isolates at sub-species or strain level in epidemiological studies. It also helps to understand the source of transmission during an outbreak or to identify the relationship between isolates implicated in an outbreak, and differentiation of strains within a food processing industry (Fenlon, 1999; Revazishvili et al., 2004; Swaminathan et al., 2001). Many molecular methods have been developed including, serotyping, multilocus sequence typing (Maiden et al., 1998), pulsed-field gel electrophoresis (Brosch et al., 1996; Graves and Swaminathan, 2001) and ribotyping (Bruce et al., 1995a; Wiedmann et al., 1997).

Serotyping

Serotyping involves differentiating strains based on their antigenic determinants and identifying each strain serologically. The antigens produced are lipoteichoic acids, membrane proteins, and extracellular organelles such as fimbriae and flagella (Seeliger and Hohne, 1979). There have been at least 13 serotypes of *L. monocytogenes* identified but three serotypes (1/2a, 1/2b and 4b) are responsible for nearly 95% of all reported cases of human listeriosis (Gellin and Broome, 1989).

Multilocus sequence typing

Multilocus sequence typing (MLST) is a DNA sequence-based subtyping method, developed and used by Maiden et al. (1998) to characterize the naturally transformable Gram-negative pathogen, *Neisserria meningitides*. In this subtyping method, DNA sequencing of multiple housekeeping genes or virulence genes is used to differentiate bacterial subtypes and to determine the genetic relatedness of isolates (Spratt, 1999; Zhang et al., 2004). Ability to detect all genetic variations within an amplified gene is the major advantage of MLST and the sequence data obtained is less ambiguous and easier to interpret via this approach (Spratt, 1999). Data obtained by MLST is portable through web-based databases thereby making it easier to compare results of different research groups for phylogenetic analyses (Salcedo et al., 2003; Zhang et al., 2004). The use of MLST for subtyping foodborne pathogens, including *L. monocytogenes* has been reported in several studies (Cai and Wiedmann, 2001; Meinersmann et al., 2004; Revazishvili et al., 2004; Salcedo et al., 2003; Zhang et al., 2004). Due to conserved nature of housekeeping genes of *L. monocytogenes*, the MLST schemes may not provide

satisfactory discriminatory power to differentiate *L. monocytogenes* strains that are closely associated with food contamination and human listeriosis (Meinersmann et al., 2004; Salcedo et al., 2003). Therefore, virulence or virulence associated genes can be used that provide improved discriminatory power of MLST-based subtyping of *L. monocytogenes* (Zhang et al., 2004). Salcedo et al. (2003) showed that analysis of seven housekeeping genes was required to differentiate fifteen epidemiologically unrelated *L. monocytogenes* strains and Cai et al. (2002) achieved the same resolution by using a single virulence gene *act*A.

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) is a typing method used for comparative typing in molecular epidemiology of bacterial pathogens especially outbreak studies and hospital epidemiology (Struelens, 1998; Tenover, 1995). PFGE was developed by Schwartz and Cantor (1984) based on the lysis of agarose plugs containing genomic DNA of *L. monocytogenes* which is digested with selected restriction enzymes recognizing few sites along the chromosome, generating large fragments of DNA (10-800 Kb) that cannot be separated effectively by conventional electrophoresis. The plugs containing the digested DNA are transferred into an agarose gel and electrophoresed for 16-18 hr with alternating currents. The orientation of the electric field across the gel is periodically changed (pulsed), allowing DNA fragments on the order of megabase pairs to be effectively separated according to size (Lai, 1989). On the basis of distinct DNA band patterns, PFGE classifies *L. monocytogenes* into subtypes or pulsotypes, providing sensitive subtype discrimination (Brosch et al., 1996; 1994; Graves et al., 1994).

Restriction enzymes *Asc*I and *Apa*I are often used for *L. monocytogenes* (Brosch et al., 1994) which cut DNA yielding between 8 and 25 large DNA bands respectively ranging from 40 to 600 kb (Wiedmann, 2002). Fingerprinting by PFGE has been very useful for the precise characterization of *L. monocytogenes* (Brosch et al., 1991a; Brosch et al., 1994; Buchrieser et al., 1993; Carriere et al., 1991). A national network known as 'Pulsenet' has been developed by CDC and health departments in the United States to rapidly exchange standardized PFGE subtype data for isolates of foodborne pathogens (Swaminathan et al., 2001). It is considered as the current gold standard method for molecular subtyping of most foodborne bacterial pathogens, including *L. monocytogenes* because of its high discriminatory power and reproducibility (Gerner-Smidt et al., 2006). The main disadvantages of PFGE are with regard to the technical demands and the long time (>30 h) required for performing the procedure itself (Tenover et al., 1995). Usually, PFGE requires two to four days before results are available and also needs specialized equipments that are more expensive than those required for PCR or Southern hybridization. The results produced are suboptimal for inter -laboratory comparisons, and can be subjective because it is based on banding patterns (Noller et al., 2003). Also, it is difficult to compare PFGE banding patterns among research groups due to usage of different experimental protocol and analytical tools.

Ribotyping

Of all the subtyping methods available today ribotyping is considered as standardized subtyping method. Since ribosomal RNA is present and highly conserved in all bacteria, the method is commonly used for subtyping of different bacterial species.

Discrimination of ribotyping depends on species and on choice and number of restriction endonucleases used. In this subtyping method genomic DNA of *Listeria* is extracted and digested using restriction enzymes *Eco*RI, *Pvu*II or *Xho*I into many pieces generating fragments of approximately 1-30kb size. These DNA fragments are then separated by gel electrophoresis, transferred to a nylon membrane and hybridized with an appropriately labeled copy DNA (cDNA) probe derived from the *Escherichia coli* gene that encodes ribosomal RNA (rRNA) by reverse transcriptase. Thus, the resulting DNA banding patterns are based on only those DNA fragments that contain the rRNA gene (Grimont and Grimont, 1986). An automated ribotyping system has been developed by DuPont Qualicon™ (Wilmington, DE, USA) in 1995 that can process eight samples simultaneously. The automated device creates riboprints that are matched or compared to those of known strains stored on computer software (Bruce, 1996). Automated ribotyping has been used for subtyping *L. monocytogenes* but it is expensive and not as discriminatory as PFGE (Inglis T.J., 2002).

Comparison of PFGE, MLST and RT

For subtyping *L. monocytogenes*, choosing a method of MLST, PFGE or RT depends on several factors including the ability of the method to give desired results. For example, PFGE can differentiate strains of *L. monocytogenes* that are indistinguishable by housekeeping-loci-based MLST (Revazishvili et al., 2004; Salcedo et al., 2003; Zhang et al., 2004) as the nucleotide conservation in housekeeping genes limits the discriminatory power of MLST. The restriction enzymes *Asc*I and *Apa*I used in PFGE generate 8-14 fragments and 11-23 fragments respectively (Brosch et al., 1991b) whereas

*Eco*RI enzyme used in ribotyping generates only 7-9 fragments (Bruce et al., 1995b). This indicates that enzymes used in PFGE have more cutting sites in the DNA of *L. monocytogenes* than *Eco*RI has in the rRNA genes. It has thus been reported that the discrimination power of ribotyping has not been adequate in epidemiological cases especially among serotype 4b isolate of *L. monocytogenes* (Louie et al., 1996). Moreover, ribotyping has lower resolving power than PFGE because ribosomal operons cover less than 0.1% of the chromosomal DNA and tend to cluster in one particular region of the genome. The resulting DNA banding patterns are thus based on only those DNA fragments that contain rRNA genes (Tenover et al., 1995). Compared to ribotyping, PFGE is less automated and labor intensive requiring greater experimental skill. In addition, the interpretations of the PFGE banding patterns differ between researchers (Gravesen et al., 2000). Even recently developed protocols take approximately 30 hours to perform (Graves and Swaminathan, 2001; Graves et al., 1999). However, Graves and Swaminathan (2001) reported that PFGE is the standard subtype method for *L. monocytogenes* and it provides sensitive subtype discrimination. On the other hand, compared to PFGE and RT, MLST is less ambiguous and easier to interpret as this method is sequence based (Ward et al., 2004). The advantage of DNA sequencing-based methods over DNA fragment size-based typing methods is low cost of sequencing and hence MLST is being used more for subtyping and phylogenetic studies. Although each of the subtyping procedures has its own approach to the tracking of *L. monocytogenes* strains, the combined use of two or more procedures may provide more discrimination than any single subtyping method.

Genetics of listeriosis pathogenesis and virulence of *L. monocytogenes*

Factors that are involved in pathogenic mechanism of *L. monocytogenes*, entry into the host cell, lysis of phagocytic vacuoles, intracellular movement in the cytoplasmic environment and cell-to cell spread and evade immune system, have been well documented (Roberts and Wiedmann, 2003). The surface associated proteins and secreted proteins of *L. monocytogenes* are involved in interacting with the host and nearly 4.7% of all its genes encode surface proteins which have been recognized as important virulence factors (Cabanes et al., 2002). All of the virulence determinants in *L. monocytogenes* are chromosomally encoded.

Role of PrfA

Positive regulatory factor A (*Prf*A) is the sole regulator of the virulence genes identified in *L. monocytogenes*. PrfA is a 27 kD protein encoded by the *prf*A gene. The *prf*A gene is situated immediately downstream of, and sometimes co-transcribed with, the *plc*A gene.It regulates genes within the virulence gene cluster that harbors *prf*A itself, *plc*A, *hly*, *mpl*, *act*A and *plc*B. *Prf*A is also known to regulate cell-wall associated internalins (*inl*A and *inl*B), secreted internalins (*inl*C) and activates the transcription of genes involved in hexose phosphate uptake and bile salt hydrolases. The virulence genes of *L. monocytogenes* (*Prf*A, *Plc*A, *hly*A, *Mpl*, *Act*A and *Plc*B) are clustered in a 9-Kbp fragment of the bacterial chromosome referred to as the *Listeria* pathogenicity island (Fig. 2) (Chakraborty et al., 2000). All of these genes are co-ordinately regulated by PrfA, the transcriptional activator encoded by the *prf*A gene (Portnoy et al., 2002; Vazquez-Boland, 2001).

All of these virulence factors participate in specific ways in the infection process, and in addition each may also affect host cell signal transduction in ways that enhance the spread of infection (Kuhn and Goebel, 1999).

Role of *inl*A

Internalin A is part of the internalinmultigene family consisting of InternalinA, B, C, D, E, F, G, H and J. The *inlA* and *inlB* are located in the same locus while the remainders are on different loci.Internalin A is 88 kD surface proteins and is involved in the entry of *L. monocytogenes* into mammalian cells. Evidence shows that *inl*A is involved in entry of the pathogen into epithelial cell line (Caco-2 cells) whereas *inl*B promotes the entry of noninvasive bacterial cells into mammalian cells and cause internalization of inert particles. Dramsi et al. (1995) suggested that inlA mediates entry into Caco-2 cells by introducing the *inl*A gene into the non-invasive *L. innocua* which was capable of invading Caco-2 cells. The *inl*A surface protein belongs to a large family of leucine-rich repeats (LRR) proteins and is covalently anchored to the cell wall. The receptor present on cell wall is E-cadherin, which is a transmembrane glycoprotein normally involved in cell-cell interactions (Schubert et al., 2002). The amino acid proline in position 16 of E-cadherin of humans, guinea pigs, and rabbits is crucial for interaction with the leucine-rich repeat of inlA. In mice, the proline residue in the position 16 is replaced with Glutamic acid thus, InlA-mediated invasion does not occur in this speces. Entry of *Listeria* into cells involves interaction between the LRR region of internalin and the first ectodomain of human E-cadherin. E-cadherins interact at adherence junctions of polarized epithelial cells (Cossart and Lecuit, 1998). The terminal 35 amino acids of E-

cadherin are required for entry of *Listeria* into cells. The latter portion binds to β-catenin which recruits α-catenin that in turn interacts with actin. Actin polymerization during internalin -mediated entry is Rac- dependent and mediated by Arp2/3.

Role of *inl*B:

Internalin B (InlB) is another surface protein that interacts with three cellular ligands. It possess N-terminal signal sequence and LRR repeats, and the C-terminal domain carries three repeats of 80 amino acid long starting with GW (Gly-Trp) repeats (called GW module). The GW module acts as an anchor and remains attached to the membrane lipoteichoic acid of the cell wall (Portnoy et al., 1988). The 69 kD inlB protein helps *Listeria* to invade epithelial cells (Vero, HEp-2, HeLA), endothelial cells and hepatocytes (Bierne, 2002; Cabanes, 2002; Parida, 1998). InlB has two mammalian receptor proteins; gC1q-R which is the binding partner for the globular heads of the complement and hepatocytes growth factor (HGFR), Met, a family of tyrosine kinase (Shen et al., 2000). When Met receptor binds to inlB on the host cell surface, it then leads to a signaling cascade causing activation of several proteins. Among these activated proteins, phosphoinositide (PI) 3-kinase is known to cause changes in the actin cytoskeleton of the host cells and has been shown to be directly responsible for entry of *L. monocytogenes* into cells by zipper mechanism (Ireton et al., 1996). Cytochalasin D inhibits actin recruitment and prevents bacterial entry.

Listeriolysin O (LLO)

Listeriolysin O (LLO) is a sulfhydryl (SH)-activated pore-forming hemolysin with a molecular mass of 58-60 kD secreted protein which allows the bacterium to escape from a phagocytosis vacuole (Berche et al., 1988). LLO is encoded by *hly* gene, which is under direct control of *prf*A. LLO is a member of the cholesterol –dependent pore-forming toxin which is very similar to perfringolysin O (PFO) from *Clostridium perfringens*, ivanolysin O from *L.ivanovii*, pneumolysin from *Streptococcus pneumoniae* and streptolysin O (SLO) from *Streptococcus pyogenes* (Gilbert, 2002). LLO molecule oligomerizes in the membrane forming a pore that resulting in the cell lysis. Mutations within the LLO gene (*hly*) results in mutants that is unable to escape from phagosomes (Gedde et al., 2000; Michel et al., 1990). Thus, loss of LLO production is correlated with avirulence (Portnoy et al., 1988). Active at pH 5.5 and having undetectable hemolytic activity at neutral pH, LLO is a toxin which acts only on cholesterol containing membranes in which cholesterol is considered the receptor (Tilney and Portnoy, 1989). *L. monocytogenes* has evolved such that its hemolysin is most active at a pH which would be encountered within the acidic phagolysosomal vacuoles. Expression of optimal activity upon acidification of the vacuoles would allow *L. monocytogenes* to disrupt the phagolysosomal membrane and escape into the cytoplasm. *L. monocytogenes* disrupts the phagosomal membrane within 30 min after it has been internalized (Gaillard et al., 1987), and within 2 h half of the internalized bacteria can usually access the host cell cytoplasm (Tilney and Portnoy, 1989). To survive and maintain its intracellular life style, *L. monocytogenes* must escape from the phagosome and evade the adverse effect of the phagolysosomal environment.

ActA

Actin polymerization protein (ActA) is a 90 kD surface protein required for actin polymerization and allows intracytoplasmic movement of *L. monocytogenes* and is under direct control f *prf*A (Cossart, 1995). It is polarly distributed on the bacterial surface and controls the actin polymerization (Domann et al., 1992). It functions to recruit and activate host proteins necessary for polymerization of F-actin assembling actin as an "actin tail" which allows movement of bacterial cells within the host cell cytosol. Although actin polymerization is initiated at the bacterial surface, depolymerization at the distal end of the actin tail limits the length of this structure (Tilney et al., 1990). The mutant strains with defective *act*A expression are unable to accumulate actin, and thus fail to infect adjacent cells.

Phospholipases

L. monocytogenes secretes two types of phospholipase C which are phosphatidylinositol-specific phospholipase C (PI-PLC) and phosphatidylcholine-specific phospholipase C (PC-PLC) which are responsible for membrane disruption. PC-PLC is a 29 kD enzyme that require zinc as a cofactor and is active at a pH range of 6-7. PI-PLC is a 33-36 kD enzyme, encoded by the *plc*A gene which is regulated by *prf*A and is present only in *L.* monocytogenes and *L.ivanovii*. PI-PLC aids *L. monocytogenes* in escape from the primary vacuole by destroying the lipid bilayer membrane of phagosome whereas PC-PLC aids in destruction of the double membrane vacuole during cell-to-cell spread of *Liseria* (Smith et al., 1995). PI-PLC is synthesized in an active form whereas PC-PLC is produced as an inactive precursor. PC-PLC is synthesized as a pro-enzyme and is

activated by a secreted *L. monocytogenes* metalloprotease (Mpl) (Vazquez-Boland et al., 2001). Mpl is a zinc-dependent metalloprotease which is a co-factor for PC-PLC. *L. monocytogenes* cells that are unable to express both PLCs demonstrate a marked defect in vacuolar escape from human epithelial cells such as HeLa cells.

Role of other molecules involved in adhesion and invasion

One of the new protein Vip is required for the invasion of Caco-2 and L2071 cell lines (Cabanes et al., 2005). It is LPXTG-anchored cell wall protein and Gp96 is the cellular receptor. Several autolysins including amidase Ami and Auto are shown to be involved in virulence. Ami exhibits lytic activity on *L.monocytogenes* cell walls and mutants are attenuated in a mouse model of infection, indicating that Ami plays an important role in virulence (Milohanic et al., 2001). Auto is another *L.* monocytogenes GW anchored autolysin that is necessary but not sufficient for invasion (Cabanes et al., 2004). Another surface protein known as p104 has been indentified and shown to play a role in adhesion to intestinal cells (Pandiripally et al., 1999).

Virulence of *L. monocytogenes* and Cell culture virulence assays

Incidences of listeriosis outbreak due to ingestion of contaminated foods strongly suggest that the infection is initiated via the intestinal route (Farber and Peterkin, 1991; Gaillard et al., 1987). As the ability of strains of *L. monocytogenes* to invade epithelial cells correlates with their virulence (Finlay et al., 1988; Moulder, 1985), the investigations of virulence are mainly studied *in vitro* by their invasion of human colon

carcinoma cell line Caco-2, and by *in vivo* infection of either immunocompromised or immunocompetent mice by following the strain's subsequent growth in spleen or liver tissues (Gaillard et al., 1987; Roche et al., 2001; Stelma et al., 1987; Van Langendonck et al., 1998). When virulent strains of *L. monocytogenes* cultures are inoculated orally or intragastrically they may either survive in liver and spleen and cause localized tissue damage or death of the mice and these virulent strains have shown to invade mammalian cells *in vitro* (McLauchlin et al., 2004). However, various strains of *Listeria* have been reported to show variable degrees of virulence (Brosch et al., 1993) and correlation between invasion of tissue culture and the mouse models has been established by several studies (Conner et al., 1989; Pine et al., 1991; Roche et al., 2001; Van Langendonck et al., 1998; Vonkoenig et al., 1983). Mice infected with oral inoculation of *L. monocytogenes* suggests that the small intestine is the primary site of invasion and an inflammatory reaction of phagocytic cells in the underlying lamina propria of the caecum and colon can be observed (Vazquez-Boland et al., 2001).

Virulence assay using cell culture was used to measure the ability of *L. monocytogenes* to adhere, invade and cause cytopathogenic effects in the enterocyte-like cell line Caco-2 (Pine et al., 1990) and to form plaques in the human adenocarcinoma cell line HT-29 (Roche et al., 2001). The pathogenic potential of *L. monocytogenes* can be evaluated using different cell lines (e.g., hepatocyte Hep-G2, macrophage-like J774, epithelial Henle 407 and L2), the human colonic carcinoma cell line Caco-2, the most widely used cell line to study analysis of *L. monocytogenes* virulence (Gaillard et al., 1987). This cell line has been used in various attachment studies due to their ability to exhibit spontaneous *in vitro* enterocytic differentiation characteristics and appears to be

most susceptible to *L. monocytogenes* infection (Conte et al., 2000; Milohanic et al., 2001; Mounier et al., 1990; Pandiripally et al., 1999; Pinto et al., 1983). Pine at al. (1991) observed that Caco-2 cells were at least 10-fold more efficient at mediating internalization which is mainly due to enterocyte-like cells being the most probable initial site of entry in patients with foodborne listeriosis. It has been observed that virulent strains are more capable of adhering and entering Caco-2 and other cells, and they are also more efficient in escaping from vacuoles, undergoing intracellular growth, and spreading to neighboring cells (Liu et al., 2006).

The initial step in pathogenesis is the adhesion of *L. monocytogenes* to intestinal epithelial cells. Proteins involved in the adhesion of *L. monocytogenes* to Caco-2 cells are encoded by the *ami* gene (Milohanic et al., 2001) and the *Listeria* adhesion protein (LAP) (Pandiripally et al., 1999). Other *Listeria* spp. has shown adherence capabilities without invading human epithelial cells (Meyer et al., 1997). The presence of LAP protein in *L. innocua* suggested that even non- pathogenic *Listeria* spp. have adherence capabilities to cells (Pandiripally et al., 1999).

Cell culture assay is mainly performed to examine the ability of the pathoen to attach and invade the cell line. Adhesion assay is performed by growing confluent cell monolayer in the wells of a cell culture plates. Cell monolayers are infected with different ratios of the pathogen and incubated for different time points at 37^{o} C. After incubation the loosely adherent cells are removed by wasing the cell monolayer for three times using phosphate-buffer saline (PBS). The monolayer is treated with Triton-X 100 (0.1-1%) to release surface attached bacteria which are then plated out on agar plates by serial dilution .For invasion assay monolayer preparation and infection is similar to adhesion

assay. After washing the monolayer with buffer to remove loosely attached cells the monolayer is treated with gentamicin antibiotic to kill extracellular bacteria.The monolayer is lysed with Triton-X 100 and serially diluted and plated to determine the intracellular bacteria counts (Gaillard et al., 1987; Bhunia and Wampler, 2005).

The main advantages of *in vitro* cell culture models include their relatively low cost and ease of use. In addition, these models allow us to study microbial adhesion, invasion, and cell-to cell movement. However, the drawbacks of cell culture model are that it is time consuming, and are occasionally variable (especially with isolates whose virulence lies between the virulent and avirulent extremes). Animal models are used to verify the pathogenic mechanism that has been established in an *invitro* cell culture model (Bhunia and Wampler, 2005).

Mouse virulence assay

The mouse virulence assay is regarded as the gold standard for any newly developed test for *L. monocytogenes* virulence because it is capable of providing an *in vivo* measurement of all virulence determinants (Liu et al., 2003; Nishibori et al., 1995; Pine et al., 1991; Roche et al., 2001). In this method, the virulence assay is conducted by inoculating groups of mice with various doses of *L. monocytogenes* bacteria via the oral, nasal, intraperitoneal, intravenous, or subcutaneous routes. Infectivity or lethality of a pathogen may vary depending upon the route of administration used i.e., oral, intragastric (ig), intravenous (iv), intraperitoneal (ip) or subcutaneous (sc). Oral or i.g. routes are often used for assessing the infective dose for foodborne pathogens. Measurements are expressed as infectious dose (ID) or as lethal dose (LD). Virulence of a given *L.*

monocytogenes is determined by the number of bacteria reaching the spleen and liver, after oral inoculation. These organs are harvested and, homogenized and bacterial counts are estimated by plating methods. Differences in counts for strains in target organs and tissues are indicative of their respective virulence (Liu et al., 2006). This type of experiment provides data on the virulence traits for adhesion, invasion and translocation to distant organs (Bhunia and Wampler, 2005). The main disadvantage of animal experiments is the high cost of mice and associated maintenance fees and therefore this virulence assay is not routinely used for determining *L. monocytogenes* virulence.

Variation in the response in the infected animals depends upon the susceptibility of the animals and route of infection used. Variations in infectivity among *L. monocytogenes* strains has been reported when animals are challenged orally or by i.g. route (Barbour et al., 2001; Jaradat and Bhunia, 2003; Roche et al., 2003). The particular strain, immune status of the mice, and route of infection of mice affects the outcome of disease. A/J mice manifest listeriosis upon i.g. inoculation while C57BL/6 mice do not (Czuprynski et al., 2003). The oral route is established as a major portal of entry of *L. monocytogenes* causing disease. This is also supported by various outbreaks of human listeriosis after consumption of contaminated foods (Dalton et al., 1997; Fleming et al., 1985).

In mouse and guinea pig models, it has been shown that *L. monocytogenes* penetrates the intestinal epithelium covering the Peyer's patches, infects them and then extends further rapidly to the mesenteric lymph nodes and then reaches the liver and spleen (Macdonald and Carter, 1980) . Within six hours of infection in the liver, 90% of the bacteria are killed by the resident macrophages (Kupffer cells) (Lepay et al., 1985).

Remaining surviving bacteria then infect adjacent hepatocytes leading into a systemic infection and internalization of *L. monocytogenes* into a vacuole of the host cell (Conlan and North, 1992). To promote its further replication in the cytoplasm of the host cell, it is released from the vacuole via listeriolysin O. In murine models it was shown that listeriolysin O is expressed in the acidified vacuole but not in the cytosol, thus, preventing the destruction of the host cell and providing a safe shelter for survival and replication of the bacterium (Decatur and Portnoy, 2000). Shortly after escape from the vacuole, *L. monocytogenes* multiplies intracellularly with a generation time of 40-60 minutes, compared to approximately 40 minutes in rich broth culture (Portnoy et al., 1988). Once inside the cytoplasm *L. monocytogenes* can move rapidly at a rate of 1.5µm/sec (Robbins and Theriot, 2003) by translocating and polymerizing with the help of host cell actin in combination with the bacterial *Act*A protein. This motility is mediated by actin polymerization which provides the propulsive force for intracellular movement (Sanger et al., 1992). The *Act*A protein (639 amino acids) is encoded by *act*A (Vazquezboland et al., 1992). *L. monocytogenes* spreads to the neighboring cells by forming microvilli-like protrusions. These are then phagocytosed by the adjacent cells and a secondary vacuole with a double membrane is formed (Tilney et al., 1990). Release of *L. monocytogenes* from the secondary vacuole is promoted by LLO and the *Listeria*l phospholipase PC-PLC, and the cycle repeats (Vazquez-Boland et al., 2001).

Histopathological changes in Liver and Spleen of mice

Racz et al., (1972) suggested that *L. monocytogenes* may pass from one host cell to another without traversing the intestinal space. The liver is one of the major organs

showing characteristic lesions, both on gross and microscopic examination. Lesions are in the form of focal patches of hepatic cell necrosis. The histopathological changes in the liver of pregnant mice were described by Siddique et al. (1978) demonstrating the multiple pale to gray foci of hepatic cell necrosis. Numerous short bacterial rods are generally seen in the necrotic foci and in hepatic cells peripheral to the primary lesions. Schlech et al. (1983) described that animals developed liver and spleen infection after oral inoculation using an inoculums of 10^8 - 10^9 CFU/ml. After infection with *L. monocytogenes*, colonization and localized cell damage is determined by collecting intestinal sections and staining histological sections which are then examined under a microscope. The histopathological changes appear in the form of focal areas of patchy necrosis in organs like spleen and liver which is helpful in the final diagnosis (Siddique et al., 1978).

Conclusion

Overall, it is imperative to realize that ready-to-eat (RTE) food processing plants can become contaminated with *L. monocytogenes* that can further cross -contaminate products from a variety of sources such as air, drains, raw materials, and workers within a processing plant. RTE foods are generally consumed with no further heating and the growth of *L. monocytogenes* at refrigerated temperatures is important factors contributing to *Listeria* outbreaks. Plant contamination can become persistent due to the ability of the organism to form biofilms attaching either strongly or weakly to abiotic surfaces commonly encountered in a processing plant. Elucidating the sources of *Listeria* contamination by comparison of DNA profiles generated via DNA macrorestriction

analysis in pulsed-field gel electrophoresis (PFGE) is a highly discriminatory and reproducible approach. As the cell attachment forms the first stage of infection, information from *in vivo* studies concerning the virulence of abiotic surface -adhering *L. monocytogenes* isolated from various meats and plant sources is limited. Examining the correlation between adherence and virulence for strong and weak adherent strains of *L. monocytogenes* may help to assess the real risk posed by this pathogen found in foods. Considering the significance of *L. monocytogenes* as a foodborne pathogen, it is important to obtain quantitative data on the effect of oral inoculation of strong and weakly adherent strains of *L. monocytogenes* in mice. The following objectives were laid out in this study to address similar issues.

Objective 1: PFGE typing and adherence characteristics of *Listeria* strains isolated from RTE meat processing plants.

Objective II: Comparison of virulence of strong and weakly adherent strains of *L. monocytogenes* using Caco-2 cells for adhesion and invasion assays.

Objective III: Comparison of virulence for strong and weakly adherent strains of *L. monocytogenes* isolated from raw and RTE meats by oral inoculation of A/J mice.

Objective IV: Subtyping of strains of *Listeria monocytogenes* by multilocus sequence typing, pulsed-field gel electrophoresis and ribotyping.

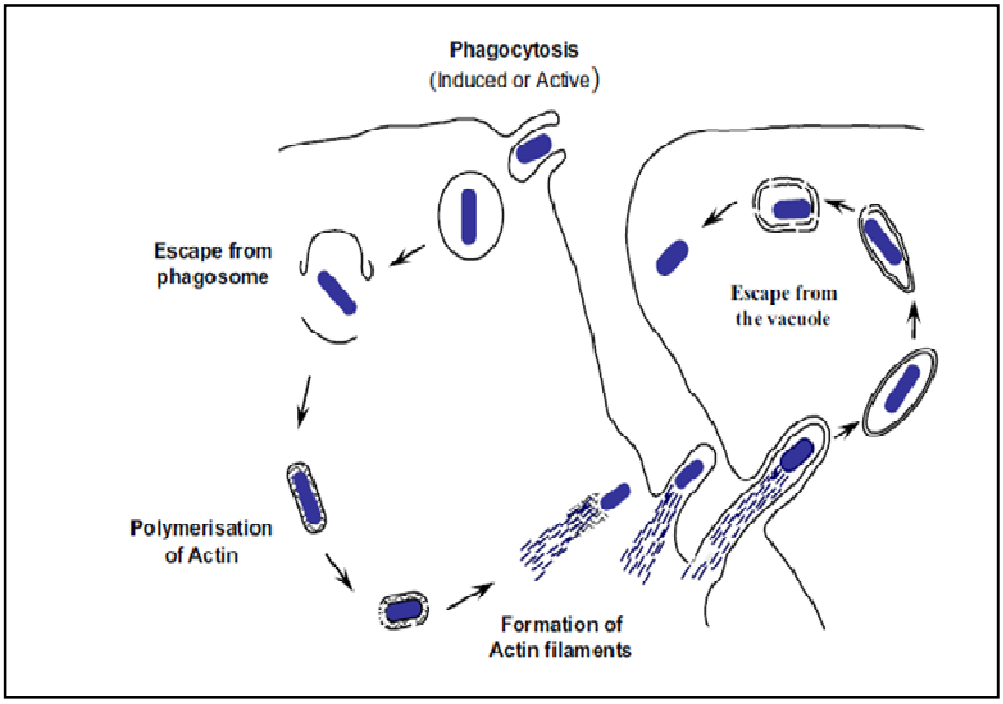

Figure1. Successive steps in the infectious cycle of *Listeria monocytogenes*. The figure was adapted from Tilney and Portnoy, (1989). The figure shows the entry, escape from a vacuole, actin nucleation, actin-based motility, and cell-tocell spread.

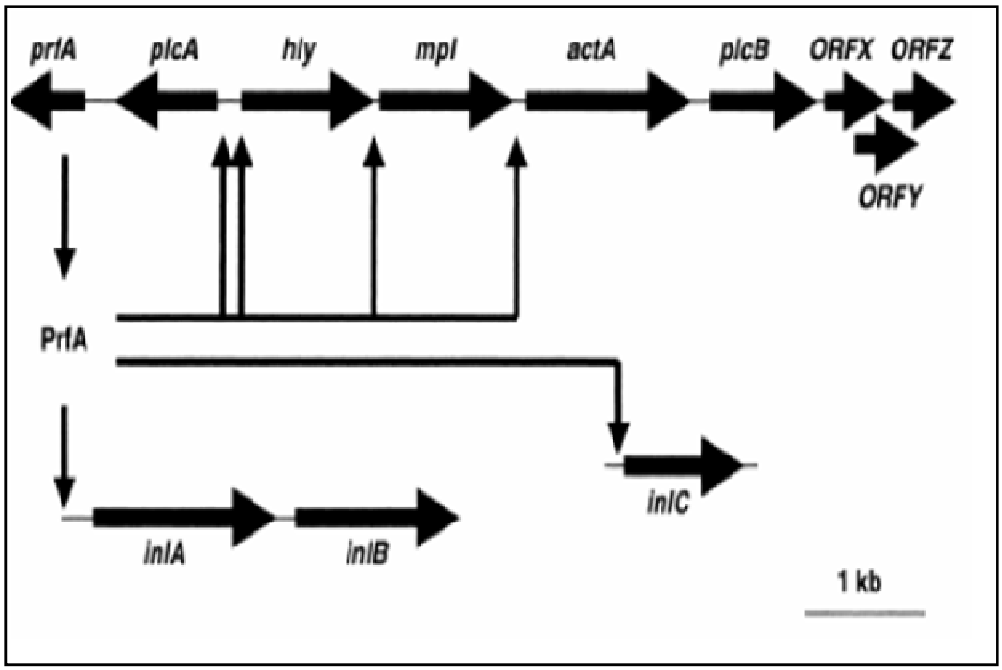

Figure 2. Virulence gene cluster in 9kb *L .monocytogenes* Pathogenicity Island and the internalin genes (Cossart and Lecuit, 1998)

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CHAPTER III

PFGE TYPING AND ADHERENCE CHARACTERISTICS OF *LISTERIA* STRAINS ISOLATED FROM RTE MEAT PROCESSING PLANTS

Kalpana Kushwaha[1] and Peter M. Muriana[1,2] ‡

[1]Department of Animal Science &

[2]The Robert M. Kerr Food & Agricultural Products Center,

Oklahoma State University

Stillwater, Oklahoma 74078

INTRODUCTION

Listeria monocytogenes is a facultative, intracellular, Gram-positive bacterium that can cause severe foodborne illness (listeriosis) in immunocompromised patients, pregnant women, and neonates. Listeriosis often occurs as a sporadic disease, but can also occur as large outbreaks with fatality rates of 25-30% (Farber and Peterkin, 1991). Several studies have shown that ready-to-eat (RTE) food products can be cross-contaminated with *L. monocytogenes* in a processing plant environment from a variety of sources (air, drains, raw materials, workers) (Gahan and Collins, 1991; Wendtland, 1994). The presence of *L. monocytogenes* is significant for RTE foods because they are usually consumed with no further heating/cooking and the organism is capable of growing at refrigerated temperatures. RTE products have been frequent vehicles of infection because these products have a limited shelf life at refrigeration temperatures which may go overlooked and allow *Listeria* to grow to high numbers (Rocourt et al., 2003; Vazquez-Boland et al., 2001). Although the U.S. Food and Drug Administration (FDA) recently proposed an acceptable 'defect level' of 100 cfu/gm for this organism in foods in which it is not expected to survive, there still remains a 'zero-tolerance' for *L. monocytogenes* in RTE foods (FDA, 2008).

L. monocytogenes is able to reside and persist in food processing environments for long periods of time (Autio et al., 1999; Norton et al., 2001). Some reasons for their persistence in food processing plants are the complex surfaces of processing equipment

that are difficult to sanitize and results in numerous harborage points (Autio et al., 1999; Miettinen et al., 1999; Norwood and Gilmour, 1999). In one dairy plant, contamination lasted several years with one particular clone (Unnerstad et al., 1996; Wulff et al., 2006) also found several molecular subtypes recurring in food processing facilities they tested. Harborages for *L. monocytogenes* are not only found among environmental surfaces, but also within numerous crevices on equipment used for slicing, dicing, and packaging (Autio et al., 1999; Miettinen et al., 1999; Tompkin, 2002). Lunden et al. (2000) correlated the persistence of strains with their ability to adhere to food contact surfaces, even after short contact times. Different strains of *L. monocytogenes* have been shown to attach to abiotic surfaces with different levels of adherence, regardless of surface (glass, plastic, rubber, stainless steel) or temperature (Gamble and Muriana, 2007). Their persistence in a plant may be related to their ability to form biofilms, which can provide resistance to sanitizing agents and lead to increased survival of these bacteria in food processing facilities (Aase et al., 2000; Blackman and Frank, 1996; Borucki and Call, 2003; Frank and Koffi, 1990).

Molecular typing by pulsed-field gel electrophoresis (PFGE) using DNA macrorestriction analysis is a highly discriminatory and reproducible method that has proven to be very useful in elucidating sources of *Listeria* contamination in food processing environments by comparison of DNA profiles (Brosch et al., 1996; Kerouanton et al., 1998). The purpose of this study was to examine strains of *Listeria* isolated for over 1 year from 3 RTE meat processing facilities for adherence characteristics using our microplate assay in combination with PFGE molecular typing to assess if the recurrence of isolates correlates to adherence properties.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Bacterial strains were cultured by transferring 100 μl of thawed frozen culture suspension into 9 ml of brain heart infusion broth (BHI; Difco, Becton-Dickenson, Franklin Lakes, NJ), incubated overnight (18-24 hrs) at 30ºC, and subcultured again before use. Frozen culture stocks were prepared by centrifuging 9 ml of culture and resuspending the pellet in 2 ml of sterile BHI broth (containing 10% glycerol) and storing at -76^{o}C.

Collection of swab samples in three food processing plants

Environmental and non-food contact surfaces were sampled in order to identify areas where sanitation could be improved. During each sampling, approximately 50% of the sample sites were selected objectively based on a random number generator where numbers correlated to specific sites; the remaining 50% of sample sites were sampled subjectively based on areas not yet tested, or, the retesting of areas that previously provided positive *Listeria* samples. Swab samples were collected from non-food contact surfaces of food processing equipment, from wheels (garbage bins, hand trucks, dollies, and racks), floors, tables, pole holders, windows, walls, carts, hoses, drains, motor housings, standing water, equipment/machines, casing waste, hanging chute. A limited

number of raw meat ingredient samples (pork, chicken) were also taken. Over the course of 13 months, approximately 1560 swab and raw ingredient samples were tested.

Isolation of *Listeria* spp. from environmental swabs

Swab samples were collected using either sterile cotton swabs or sponges moistened with neutralizing broth and placed into 10 ml of Difco UVM Modified *Listeria* Enrichment Broth (Becton Dickinson, Sparks, MD, USA) and incubated at 30^{o} C for 24 h. No attempt was made to swab a standardized-size surface area as the intention was merely for positive/negative detection of *Listeria* spp. Subsequently 1.0 ml of UVM broth was inoculated into 9 ml of Difco Fraser broth (Becton Dickson) supplemented with 0.05% ferric ammonium citrate (Sigma-Aldrich, St Louis, MO, USA) and incubated at 35^{o}C for 48 hr. A loopful from black Fraser broth was streaked onto Difco Modified Oxford Agar (MOX; Becton Dickson) and incubated at 35^{o}C for up to 48 hr. For each tentatively-positive Fraser broth sample, four black concave colonies from MOX were tested by Gram stain, catalase test (3% H_2O_2), hemolytic reaction on Horse Blood Agar, and the API-*Listeria* assay. All tentative *Listeria*-positive isolates were stored at -76^{o}C as noted earlier**.**

PCR confirmation of *Listeria spp.* as *L. monocytogenes*

Aside from the API/hemolytic reactions, *Listeria spp.* were confirmed as *L. monocytogenes* using primers specific to portions of the hemolysin (*hly*A) and internalin A (*inl*A) genes unique to *L. monocytogenes*. PCR confirmation was obtained for *hly*A

(560-bp) using the forward primer 5'-TGAACCTACAAGACCTTCCA-3' and the reverse primer (5'-CAATTTCGTTACCTTCAGGA-3') and for *inl*A (575-bp) using the forward primer 5'-GCTTCAGGCGGATAGATTAG-3' and reverse primer 5'-AACTCGCCAATGTGCC-3'. PCR amplification was performed with an initial 4 min denaturation step at 95^{o}C, followed by 40 cycles of 95^{o}C for 15 sec (denaturation), 51^{o}C for 18 sec (annealing), 72^{o}C for 40 sec (extension), with a final step holding at 4^{o}C. Amplification was confirmed by visualization of the PCR product of the expected size on agarose gels. *L. monocytogenes* Scott-A served as the positive control and *L. innocua* ATCC 33090 and *L. ivanovii* ATCC 19119 as the negative controls during PCR reactions.

Pulsed-field gel electrophoresis

Genomic DNA from individual isolates of *L. monocytogenes* was prepared in agarose plugs as described by Graves and Swaminathan (2001). Briefly, isolates were grown on BHI agar plates at 37^{o}C overnight to obtain a lawn. The cells were harvested from the BHI agar plates by adding 1 ml of TE buffer and using a 'hockey stick' to suspend the cells. The absorbance measured at 610 nm (OD_{610}) for the cell suspension was adjusted to 1.3 using a Spectronic 20 spectrophotometer (Thermo Electron Corporation, West Palm Beach, FL). After adjusting the OD_{610}, 240µl of the cell suspension was transferred to a microcentrifuge tube and 60 µl of lysozyme solution (10 mg/ml) was added and mixed by gently aspirating the solution. The lysozyme/cell suspension was incubated at 37^{o}C for 10 minutes and then embedded in 1% agarose plugs (Seakem Gold agarose; Cambrex, Rockland, ME). Plugs were washed twice with 15 ml

of pre-heated (50°C) sterile water for 10 min with shaking and then twice with 15 ml of preheated (50°C) TE with shaking at 50°C and twice with room temperature TE solution. The plugs could then be used immediately or stored at 4°C until needed. Plug slices (2.0-2.5 mm) containing lysozyme-treated cells lysed *in situ*, were digested with *Apa*I at a concentration of 160 U/plug for 5 hr at 30° C. Marker DNA was obtained from *L. monocytogenes* H2446. The DNA restriction fragments in plugs were electrophoresed through 1% (wt/vol) SeaKem Gold Agarose prepared in 0.5X Tris-borate-EDTA at 6V/cm on a CHEF DR III system (Bio-Rad Laboratories, Hercules, CA). A linear ramping factor with pulse times from 4.0 to 40.0 sec at 14° and 120° were applied for 20 hr. After electrophoresis, PFGE gels were stained for 15-20 min in 250 ml of deionized water containing 25 µl of ethidium bromide (10 mg/ml) and destained by three washes of 20-30 min each using 400 ml of deionized water and photographed with Gel-Doc 2000 using the Quantity One software (Bio-Rad).

Computer analyses of PFGE data

Images generated by Quantity-One software on a Gel-Doc 2000 (Bio-Rad) were saved in TIFF format and transferred to BioNumerics software (Applied Maths Sint-Martens-Latem, Belgium) for computer analyses. DNA banding patterns were analyzed with BioNumerics version 4.5 (Applied Maths) with optimization set at 0.5% and position tolerance set at 5%. The Dice coefficient of similarity was calculated, and the unweighted pair group method with arithmetic averages (UPGMA) was used for cluster analysis. A cut-off at 80% similarity of the Dice coefficient was used to indicate similar PFGE types. This corresponds to approximately one band difference, and this degree of

similarity also allows for minor technical errors that frequently occur (Salamon et al., 1998).

Microplate adherence assay

Isolated strains were characterized for their adherence as described by Gamble and Muriana (2007). Briefly, *Listeria* strains were cultured in BHI broth at 30°C and diluted 10^5-fold (i.e., from 10^9 CFU/ml to 10^4 CFU/ml) in fresh BHI broth, and 200 µl was transferred to designated wells of a 96-well black microwell plate with a clear lid (Nunc, Denmark), wrapped with Parafilm, and incubated for 24 hr at 30°C. After incubation, the microplate was washed three times with Tris buffer (pH 7.4; 0.05 M) in a Biotec Elx405 Magna plate washer (Ipswich, Suffolk, United Kingdom) to remove loosely adhered cells and sanitized with 200 ppm sodium hypochlorite (pH 6.5) after each use. Washing was followed by the addition of 200 µl of fresh (sterile) BHI and another cycle of incubation followed by washing. After the final incubation and washing, 200 µl of 5,6-CFDA (5,6-carboxyfluoresecein diacetate; Molecular Probes/Invitrogen, Carlsbad, CA) fluorescent substrate solution was added to each microplate well, incubated at 25^{o}C for 15 min, and then washed three times with Tris buffer (pH 7.4; 0.05 M), ending with 200 µl of the Tris buffer solution. The plate was then read from above in a Tecan GENios fluorescent-plate reader (Phoenix Research Products, Hayward, CA) within 5 min using a fixed signal gain of 75% with excitation at 485 nm and detection at 535 nm.

Fluorescence microscopy

The fluorescence microplate assay described above was modified for visualization by fluorescence microscopy (Gamble and Muriana, 2007). Briefly, 8-compartment CultureSlides^{TM} (Falcon, Becton-Dickenson, Bedford, MA) were used for incubation of representative weak, medium, and strongly-adhering strains. After incubation and adherence, the chamber wells were washed and disassembled and the bottom of the wells becomes a microscope slide, allowing direct side-by-side comparison of the attached cells. Cultures were incubated under the same conditions as the microplate assay (48 hrs, 30^{o}C), rinsed by manual pipette aspiration using Tris buffer (pH 7.4, 0.05M), and incubated with CFDA-based substrate as previously described. Chambers were removed using the manufacturer's tool and the bottom slides were examined by fluorescence microscopy using a Nikon Eclipse E400 fluorescent microscope (excitation @ 450-490 nm, detection @ 500nm) using a BA 515 B-2A filter. Pictures were taken with a digital camera attachment.

RESULTS AND DISCUSSION

During the initial year of the implementation of HACCP, we started to sample 3 RTE meat processing plants to help identify the incidence of *Listeria* spp. and typical harborages in postprocess areas where the emphasis is for a '*Listeria*-free' environment. We examined doors, windows, walls, carts, wheels of carts, brooms, hoses, ladders, equipment surfaces (non-food contact), floors and drains to help establish points of emphasis for sanitation crews. This allowed the sanitation managers in the various facilities to understand typical places where *Listeria* can be found so that they may increase their vigilance, improve their sanitation regimens, and drastically reduce the subsequent incidence of *Listeria*. Food contact surfaces were not tested because that posed a potential problem with the regulatory authority (USDA-FSIS) in that if a food contact surface were found to be contaminated with *L. monocytogenes*, any exposed RTE food that could have come in contact with that surface would then be considered contaminated. Early-on, the RTE meat industry was able to abate that regulatory hurdle by choosing not to differentiate isolates according to species, simply allowing for identification as "*Listeria* spp.". However, USDA-FSIS subsequently evolved away from this position and started to establish that even the presence of undifferentiated "*Listeria* spp." could represent the presence of *L. monocytogenes*, since both share the same habitat and contamination sources. The issue of testing food contact surfaces directly for *L. monocytogenes* has always been a sensitive bottleneck in RTE processing areas, however,

even the presence of *Listeria* spp. in the vicinity of the RTE processing environment also has significant implications for plant sanitation and food safety.

During our sampling of 3 processing facilities, we recovered 259 *Listeria*-positive samples from 1560 samples taken (17% overall incidence rate). A breakdown of the isolates of *Listeria* spp. obtained from each of the plants demonstrates the specific sampling areas they were associated with, showing a 9.0%, 8.7%, and 32% incidence rate in Plants A, B, and C, respectively, for the 520 samples taken at each plant (Table 1). The incidence of *Listeria* spp. was found to be rather similar in two modern plants (Plants A and B), yielding a 9% and 8.7% incidence rate, respectively (Table 1). However, in the third, and older plant we tested, the incidence of *Listeria* spp. was 32% of samples taken, significantly higher than that found in the other plants. This could be a combination of several factors such as the older plant having outdated construction (at that time) that may have impacted sanitation efforts and it's design may have presented limitations in restricting traffic patterns through postprocess areas whereas the newer plants more effectively segregated raw preprocess from cooked postprocess plant traffic. Even so, *Listeria* contaminants still made their way into the postprocess areas of all plants and our data brought attention to the areas where they were found (Table 1).

Recently, we developed a convenient microplate fluorescence adherence assay that proved effective in distinguishing different adherence phenotypes in strains of *L. monocytogenes* isolated from RTE meats, raw ground beef, and meat processing facilities (Gamble and Muriana, 2007). In that study, we showed that the degree of cellular attachment of the highly adherent strains was significantly higher than weakly adherent strains, i.e., 10^8 cfu/well vs 10^3 cfu/well, respectively (Gamble and Muriana, 2007). The

degree of adherence demonstrated by the strongly adherent strains could easily be hypothesized as a possible factor responsible for retention and persistence of *L. monocytogenes* in meat processing environments. We applied this adherence assay to 246 of the 259 strains isolated in Table 1 and arranged the strains in order of fluorescence signals (RFU) obtained with the microplate assay in order to standardize the range of adherence of all *Listeria* strains from weakest to strongest (Fig. 1). Using the entire range of RFU adherence signals, we qualitatively classified the degree of adherence as weak, moderate, or strongly adherent based on how the slope of the RFU 'curve' broke in its entirety to assign the 3 adherence classifications used in this study (Fig. 1). As observed previously (Gamble and Muriana, 2007), when representative isolates were selected from each of the 3 phenotypic adherence classifications and examined by fluorescence microscopy, the cell distribution of a strongly adherent strain occluded the slide surface, whereas moderately or weakly adherent strains showed significantly fewer attached cells (Fig. 1 inset). Such strong adherence events could readily initiate the start of biofilm formation that may lead to foci of contamination and persistence in plant environments if not eliminated (Costerton, 1999; Wong, 1998). Differentiation of the strains based on adherence phenotype and identification of those strains that are *L. monocytogenes* provides a better picture of the significance of adherence (Table 2).

The presence of generic *Listeria* spp. is significant as it represents a failure of sanitation hurdles to eliminate these organisms from the processing environment, and even more so if they are *L. monocytogenes,* as they are human pathogens. The data shows that Plant C had almost 4x the prevalence of *Listeria* isolates as either Plants A or B (164

vs. 39 or 43, respectively) and from that perspective, had a greater potential to generate contaminated products (Table 2).

The predominance of moderate and strongly adherent strains of *L. monocytogenes* that may have found their way into an older processing facility may have presented an impossible situation to eliminate and resulted in persistent retention as environmental contaminants. Similarly, Plant B demonstrated a high proportion of *L. monocytogenes* among the *Listeria* isolates (41 of 43) of which 32 were moderately adherent (Table 2). Of the 164 *Listeria*-positive samples recovered from Plant C, 80 (49%) were *L. monocytogenes* of which 76% were moderately or strongly adherent (Table 2). The predominance of moderate and strongly adherent strains of *L. monocytogenes* that may have found their way into an older processing facility may have presented a difficult situation to rectify and resulted in persistent retention as environmental contaminants. Eventually, Plant C was involved in a recall due to detection of *L. monocytogenes* on RTE product during routine USDA-FSIS sampling. Although Plant B had approximately ¼ the amount of total *Listeria* isolates as Plant C (43 vs 164), it demonstrated an unusually high proportion of *L. monocytogenes* among its *Listeria* isolates (41 of 43, 95%) of which 32 (78%) were moderately adherent (Table 2). A situation noticed at Plant B during one or more sampling visits was the presence of a large pool of 'standing water' noticed in the post process area which may have allowed for widespread distribution of one or more isolates.

We also examined PFGE for typing strains based on DNA 'fingerprint' patterns. The restriction enzymes *Asc*I and *Apa*I have been commonly used for subtyping *L. monocytogenes* by PFGE (Autio et al., 2000; Bille and Rocourt, 1996; Brosch et al.,

1996; Destro et al., 1996; Giovannacci et al., 1999). We used PFGE *Apa*I macrorestriction pattern and dendrogram analysis to examine strains of *Listeria* described in this study which formed 21 clusters at a similarity level of 80% (data not shown). The use of PFGE fingerprint analysis was informative in suggesting that similar strains were isolated repeatedly within the same facility on the same day or on different dates as recurring isolates (Fig. 3). Since we used only 1 restriction enzyme profile (*Apa*I), although this is usually confirmed from identical profiles obtained with at least 2 or more restriction enzymes, and even then there could be room for ambiguity in exact strain identity. For general sanitation purposes, gross pattern analysis should be sufficient to correlate potentially similar strains with isolation 'hot spots', since neither *L. monocytogenes* nor *Listeria* spp. should be present in an RTE postprocess environment.

Samples were collected mostly from non-food contact sites, however, some samples were initially taken from raw meat ingredients for possible comparison with profiles obtained with isolates from postprocess environmental samples. Raw meat sampling was discontinued early in the study because it was not thought that the limited number of raw meat samples could possibly represent the incoming pool of *Listeria* in the large quantities of raw meat ingredients unless a fair amount of sampling was diverted from the environmental samples. However, even with the few raw meat ingredient samples from which *Listeria* were isolated, we found a major pattern cluster in Plant B that was recurrent for 10 months that had the same profile as that obtained from a sample of raw chicken (Fig. 3). In hindsight, continued testing of incoming raw ingredients for comparison with plant specimens may have given greater insight into possible distribution pathways or harborages (Nesbakken et al., 1996). A number of these isolates

were obtained even before the date that the raw chicken sample was taken, indicating a long term, recurring contamination problem. The isolates within this recurring cluster pattern turned out to be *L. monocytogenes* and demonstrated mostly a 'moderate' adherence phenotype (Fig. 2). It was further noted that during the 3/18 sampling period, a large pool of 'standing water' was visible in the postprocess area. This is one issue of concern in food processing environments because standing water allows for easy distribution of microbial flora by virtue of anything crossing through it (foot traffic, cart wheels, dragged water hoses, etc), and this could be why we found a large cluster of isolates registering with >90% similarity index on that sampling date (Fig. 3). PFGE pattern similarity and ID match also included an isolate from raw pork ingredient used at another plant, but having a weak adherence phenotype (Fig. 3).

There have been occasions when researchers have noticed a higher incidence of *L. monocytogenes* in raw meat plants compared to on-farm animal levels and have attributed the high plant levels to the distribution and persistence of flora in raw meat processing plants (Giovannacci et al., 1999). Raw meat ingredients are an obvious source of contamination in further processing plants (Chasseignaux et al., 2002; Lawrence and Gilmour, 1995).

Although there are no regulatory requirements for presence/absence/levels of *L. monocytogenes* on raw meat products, the influx of such contaminated raw meat ingredients continuously places a tremendous sanitary burden on RTE meat processing facilities where contamination with *L. monocytogenes* poses a serious health risk because many RTE products are consumed without further heating.

Other environmental sampling sites that are often tested are processing drains. The presence of *L. monocytogenes* in drains has often been considered an indicator of facility contamination, but not the source of contamination (Berrang et al., 2002; Hoffman et al., 2003) Berrang et al (2002) also found strains of *L. monocytogenes* from drains on the preprocess (pre-cook) side of a poultry processing plant indistinguishable from those isolated from drains on the postprocess side, suggesting that contamination from the raw ingredients may have breached into postprocess areas. One concern for drain contamination would be that there may be a blockage and backflow into a facility that would carry *Listeria* contamination back into the processing environment. Another interpretation and concern would be that the organisms in the drain originated in the processing environment. One of the problems with cleaning drains is that sanitizing fluids may only occupy the bottom half of a drain tube and never effectively drench the upper, inside surface. However, new designs for drains have incorporated methods allowing sanitizers to access all inside surfaces (Howard, 2007). Other interventions with wide application in a processing plant also include the use of antimicrobial stainless steel impregnated with silver ions (Cowan et al., 2003). Still other interventions can simply be the re-design of existing equipment to be 'sanitation friendly', including minimizing screw holes where bacteria can hide (i.e., equipment footings) or the incorporation of sloped surfaces so water can run off and not pool (i.e., square-sided equipment frames). Isolates were also recovered from floors, which would explain their presence in drains, but also demonstrates the propensity for dispersal by foot traffic, whereby cart wheels, hoses dragged along the ground, broom bristles used to sweep fallen products, and utility ladder steps were all found to be positive at different times (Table 1). The presence of

Listeria on walls and windows could be due to splashing of 'standing water' (or from human contact), a concern for postprocess areas that could assist in widespread distribution of bacteria in a processing environment and was noticed during the 3/18 sampling in Fig. 2. Redistribution of bacterial contamination by high pressure water hoses is one argument used against the practice of a mid-shift cleanup.

Strains possessing strong adherence characteristics (and perhaps even moderate adherence) have a greater likelihood of being more persistent and recurring in a plant environment than weakly adherent strains. Even if all of the strains isolated from any of the plants were simply *Listeria* spp. other than *L. monocytogenes*, it would still be highly significant in that they should not be present at that stage of an RTE processing facility. The presence of such a prevalence of moderate and strongly adherent strains in plant C suggests that these contaminants may not have been as efficiently eliminated by sanitation efforts as the other plants (Fig. 3).

To examine the aspect of strong adherence and plant retention, we examined information for all 37 of the 'strong' adherent isolates from our 3 plant study and found that 92% were isolated from Plant C (Fig. 4). Nine of 34 (26%) of the strongly adherent *Listeria* isolates from plant C were *L. monocytogenes* (Fig. 4). This was the older plant that had a 32% incidence of *Listeria*-positive samples during our testing.

After review of the sites that tested positive, it is easy to formulate the opinion that all exposed surfaces in a process facility can become contaminated at one time or another. An important criterion for preventive maintenance would be to eliminate the entry of contamination into the processing environment in the first place, after which widespread distribution could easily occur during a production run. Intervention

strategies for processing environments including sanitizer foot baths at entry points, frequent hand washing, negative pressurized room, UV lighting in air ducts, drain system sanitation, no mid-shift cleanup using high pressure water hoses, and brine rinse decontamination. One or more recalls have been attributed to plant construction that may have generated dust/bacteria transported via the ventilation system connected to the RTE processing area. The potential influx from the raw meat ingredients during processing is the largest known reservoir of *Listeria* contaminants placing pressure on the sanitation system of any plant. Linear processing whereby the trespass of personnel from raw to post process areas is prohibited, as well as preventive measures to insure contaminants do not breach the postprocess area, and incorporating separate drainage and ventilation systems for raw vs postprocess areas are necessary barriers in preventing postprocess contamination.

Improvements and enhancements in regard to facilities, personnel, and sanitation programs have since resulted in greatly improved conditions in the plants. The comparative analysis demonstrates the utility of microbial analysis and assessment of plant sanitation conditions based on recovery of targeted organisms. Although preservatives and antimicrobials may prevent survival and/or growth of contaminants on food products, they will only work in combination with good plant sanitation as continuous and constant pressure of contaminating microbial flora may eventually overcome even the most promising ingredient interventions.

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Table 1. Environmental and non-food contact sites from which *Listeria* were isolated in 3 RTE meat processing plants.

Source	Plant A	Plant B	Plant C
Drains	10	10	44
Floors	4	4	16
Garbage bins	4	2	5
Wheels (cart, bin, equip)	4	2	11
Wall/window surfaces	1	3	3
Raw meat ingredient	1	3	3
Equipment/table surfaces	15	17	64
Brooms/bristles	2		1
Casing waste chute/bin	6		9
Ladder		2	2
Vacuum line		2	
Pallet, pallet jack			4
Water hose line, hose handle			3
Scale			2
Listeria spp. =	47	45	167
Total samples =	520	520	520
Prevalence	**9.0%**	**8.7%**	**32.1%**

Table 2. Distribution of adherent phenotypes of *Listeria* isolated from RTE meat processing plants.

Adherence phenotype	Plant A	Plant B	Plant C	Total
Weak*:				
Listeria spp.	16 (41%)	9 (21%)	37 (22.6%)	62
*L. monocytogenes***	2 (22.2%)	9 (22%)	19 (23.8%)	30
Medium*:				
Listeria spp.	21 (53.9%)	33 (76.7%)	93 (56.7%)	147
*L. monocytogenes***	7 (77.8%)	32 (78%)	52 (65%)	91
Strong*:				
Listeria spp.	2 (5%)	1 (2%)	34 (21%)	37
*L. monocytogenes***	0	0	9 (11.2%)	9
Total†:				
Listeria spp.	39 (8%)	43 (8%)	164 (32%)	246
L. monocytogenes	9 (23.1%)	41 (95.3%)	80 (49.8%)	130

*Percentage of each phenotype is based on comparison to the same group (total *Listeria* spp. or *L. monocytogenes*) within a given plant.
** Count for *L. monocytogenes* is included in the count for *Listeria* spp.
†For Total *Listeria spp.*, the percentage is relative to total samples taken within a given plant (i.e., 520); for *L. monocytogenes*, the percentage is relative to total *Listeria* isolates.

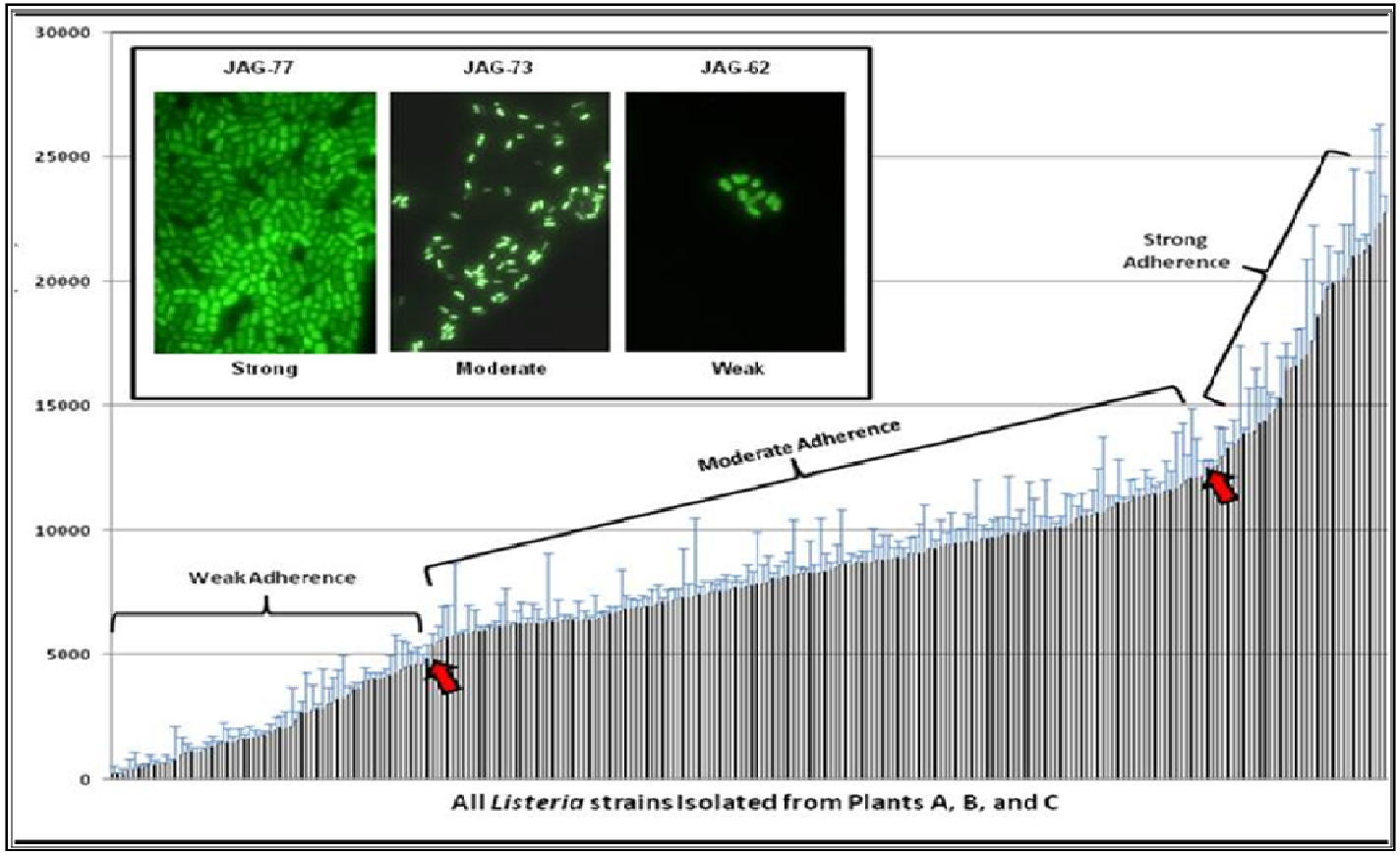

Figure 1. Relative fluorescence of all *Listeria* strains isolated from meat processing plants using the microplate adherence assay, from lowest to highest means of fluorescence signals in order to determine cutoff values (arrows) for weak, moderate, and strong adherence. Error bars represent standard deviation obtained from triplicate replications. Inset: fluorescence microscopy of representative strains considered being weak moderate, and strongly adherent based on the microplate adherence assay.

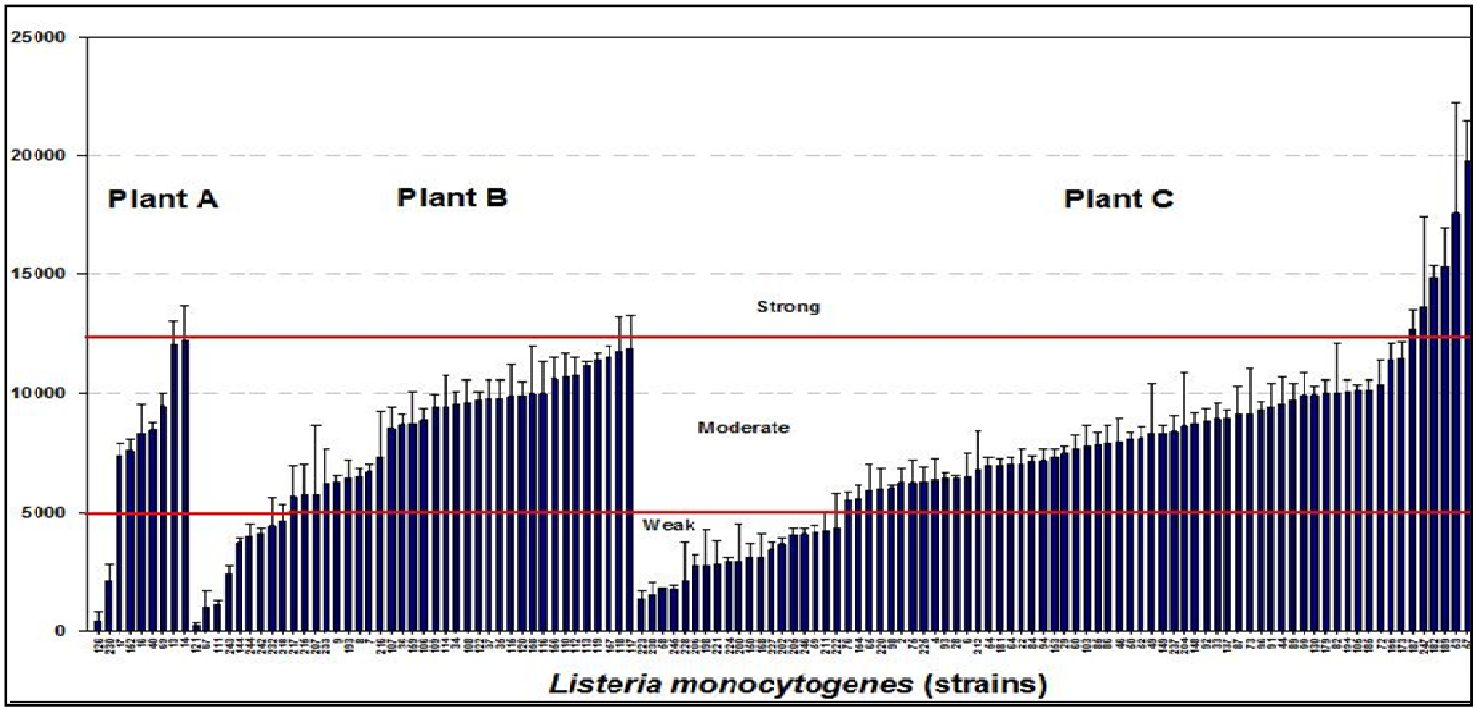

Figure 2. Relative fluorescence of individual strains of *L. monocytogenes* isolated from three RTE meat processing plants and distributed according to plant. Error bars represent standard deviation of triplicate replications. Cutoff values for weak and strong adherence was determined from the entire set of *Listeria* spp. tested

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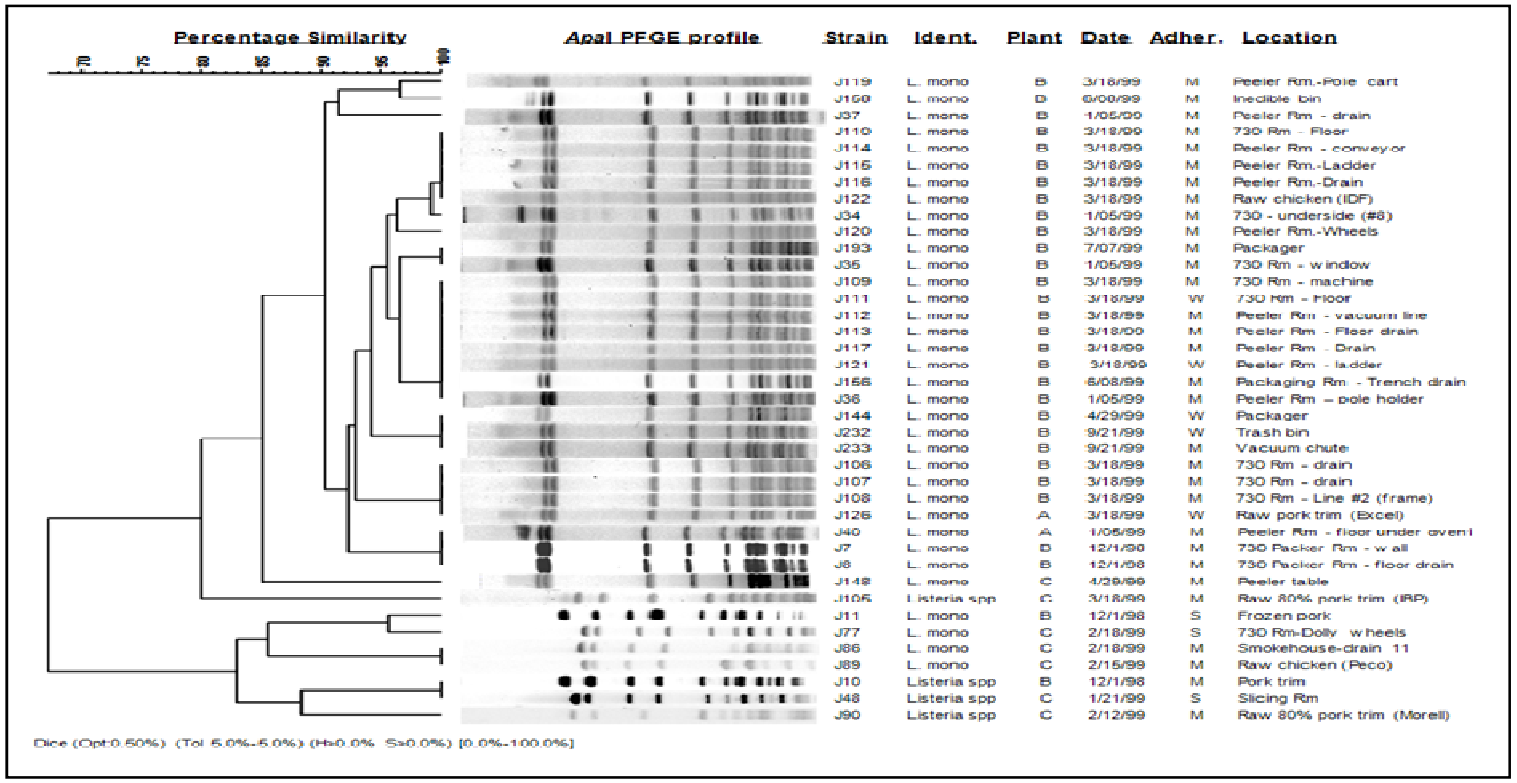

Figure 3. PFGE *Apa*I profile and clustal analysis of raw meat and environmental isolates of a large cluster having closely related PFGE profiles.

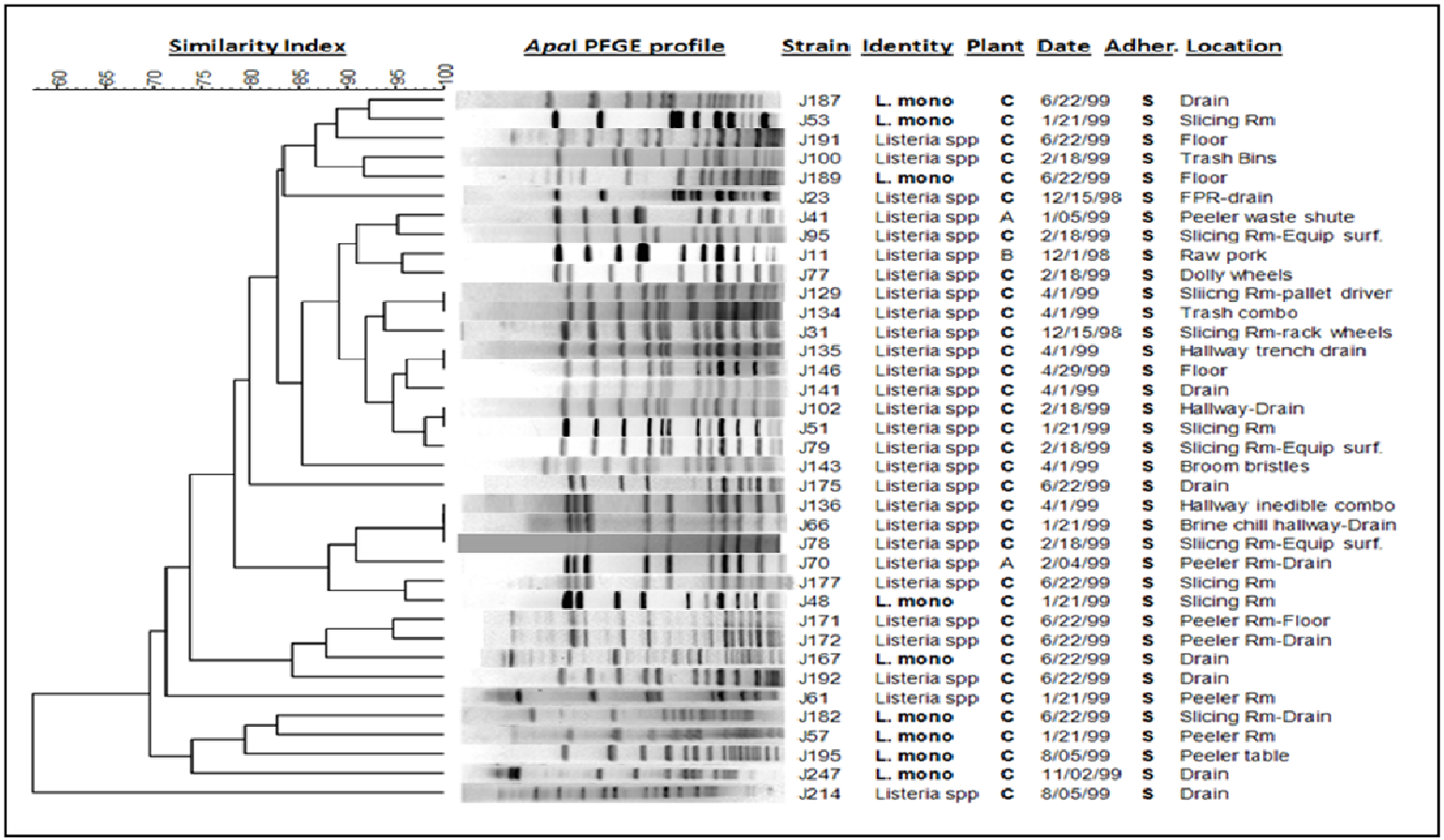

Figure 4. PFGE *Apa*I profile and clustal analysis of all 37 “strongly” adherent *Listeria* isolated from the three processing plants.

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CHAPTER IV

COMPARISON OF STRONG AND WEAKLY ADHERENT STRAINS OF *LISTERIA MONOCYTOGENES* USING CACO-2 CELLS FOR ADHESION AND INVASION ASSAYS

Kalpana Kushwaha[1] and Peter M. Muriana[1,2] ‡

[1]Department of Animal Science &

[2]The Robert M. Kerr Food & Agricultural Products Center,

Oklahoma State University

Stillwater, Oklahoma 74078

INTRODUCTION

Listeria monocytogenes is an intracellular Gram positive bacterium capable of causing severe invasive illness (listeriosis) in humans at high fatality rates (Alberti-Segui et al., 2007). It mainly affects immunocompromised people, the elderly, pregnant women and neonates (Farber and Peterkin, 1991). The vast majority of human *Listeria* infections are thought to be foodborne, and the pathogen has been isolated from various raw and ready-to-eat (RTE) products (Aarnisalo et al., 2003; Gombas et al., 2003). The presence of *L. monocytogenes* in RTE foods is mainly due to contamination during post process procedures rather than survival during the processing itself (McLauchlin, 1987). RTE meat products often have high incidence of contamination because of their long shelf life and may allow *Listeria* to grow to high numbers under refrigeration temperatures (Rocourt et al., 2003; Vazquez-Boland et al., 2001). Although, the U.S. Food and Drug Administration (FDA) recently proposed an acceptable 'defect level' of 100 cfu/gm for this organism in foods in which it is not expected to survive, there still remains a 'zero-tolerance' for *L. monocytogenes* in RTE foods (FDA, 2008).

Several strains of *L. monocytogenes* are able to adhere to various abiotic surfaces such as glass, polypropylene, rubber and stainless steel (Chae and Schraft, 2000; Frank and Koffi, 1990; Gamble and Muriana, 2007; Kalmokoff et al., 2001; Mafu et al., 1990; Sinde and Carballo, 2000). Such surfaces being common in food processing facilities can

harbor the pathogenic bacteria and potentially contaminate food products. Strains adhere to such surfaces and their degrees of adherence can be qualitatively classified as strong or weak based on a microplate fluorescent assay (Gamble and Muriana, 2007). Their ability to adhere, invade and multiply in biotic systems such as phagocytes may identify the virulent nature of the bacteria, however, correlation between abiotic adherence and cellular adherence as well as invasion is not well documented. The invasiveness of *L. monocytogenes* is reported to be affected by variation in strain virulence as well as differences in environmental conditions (Kim et al., 2004; Larsen et al., 2002; Wiedmann et al., 1997). Attachment and invasion are the first steps in the establishment of infection. The ability of bacteria to invade cells, such as epithelial cells often correlates with bacterial virulence (Finlay et al., 1988; Moulder, 1985).

Comparing the pathogenic potential of *Listeria* strains can be done using cell line based assays or animal models. Since animal model studies are expensive and time consuming, several cell lines, such as human epithelial HEp-2, HeLa and Caco-2 have been considered suitable for the evaluation of adherence, invasion, and virulence of *L. monocytogenes* (Gaillard et al., 1987; Kathariou et al., 1990). Environmental conditions such as growth temperature may also determine virulence of *L. monocytogenes* strains showing decreased adherence when grown at higher temperature (37^{o}C) than the ones grown at low temperature (20^{o}C) which was attributed to thermoregulation of virulence genes as reported by Leimeister Wachter (1992) and Dramsi et al. (1993). Growth conditions prior to exposure may also influence the level of cell attachment to surfaces as determined by environmental stress such as pH, temperature, and the hydrophobicity of the surface (Smoot and Pierson, 1998).

In this study we compared the virulence potential of strong and weakly adherent strains of *L. monocytogenes* isolated from raw, RTE meats and meat processing facilities using human intestinal epithelial cell line, Caco-2. Virulence was tested *in vitro* by the adhesion and invasion assay of these strains using human cell line Caco-2. Examining the correlation between adherence and invaion for strong and weakly adherent strains of *L. monocytogenes* may help to assess the real risk posed by this pathogen found in foods.

MATERIALS AND METHODS

Bacterial strains and growth media

The *L. monocytogenes* strains used in this study were from raw, RTE meat and meat processing facilities. Several strains of *L. monocytogenes* (CW50, 99-38, CW77, SM5, CW62, CW34, CW35, CW52, CW72, SM3, J7 and J126) were chosen for further experiments after screening for their adherence using microplate fluorescence assay. All CW strains were originated from RTE retail frankfurter products whereas 99-38, SM3, and SM5 were isolated from retail ground beef. Strains designated as 'J' were isolated from RTE meat processing facilities. Bacterial strains were cultured by transferring 100 µl of thawed frozen culture into 9 ml of brain heart infusion (BHI) (Difco, Becton-Dickenson, Franklin Lakes, NJ) broth, incubating them overnight (18 to 24 h) at 30°C, and subculturing the bacteria twice before use. Frozen culture stocks were prepared by centrifuging 9 ml of culture, resuspending the pellet in 2 ml of sterile BHI broth (containing 10% glycerol), and storing it at -76°C.

Fluorescent microplate assay for adherence

These strains were characterized for their adherence as described in Gamble and Muriana (2007). Briefly, strains were sub cultured in BHI broth held at 30°C and diluted to 10^5-fold in fresh BHI broth, and 200 µl was transferred to designated wells of a 96-

well black microwell plate with a clear lid (Nunc, Denmark). After incubation, the microplate was washed three times with Tris buffer (pH 7.4; 0.05 M) to remove loosely adhered cells. The washing was followed by the addition of 200 µl of fresh (sterile) BHI broth to each experimental well, incubated at 30°C, and washed three times with Tris buffer (pH 7.4; 0.05 M) after another 24 h. After the final washing, cells are incubated with the fluorescent substrate solution (5, 6-CFDA) for 15 min. Following incubation with the 5, 6-CFDA substrate, the plates were washed three times with Tris buffer (pH 7.4; 0.05 M) in the plate washer, and the medium was replaced with 200 µl of the same medium. The plate was then read from above in a Tecan GENios fluorescent-plate reader (Phenix Research Products, Hayward, CA) using a fixed signal gain of 75% with excitation at 485 nm and detection at 535 nm. Based on the level of fluorescence signals obtained with our microplate assay for the strains screened, the strains were categorized as weak and strong adherent strains.

PCR confirmation of *L. monocytogenes*

The conformation of strains as *L. monocytogenes* was done by PCR amplification of the hemolysin gene (*hly*A) using specific primers. The primers used were 5'-TGAACCTACAAGACCTTCCA-3' (For_{560}) and 5'-CAATTTCGTTACCTTCAGGA-3' (Rev_{560}) for generation of a 560 bp amplimer. PCR amplification was performed with an initial 4 min denaturation step at 95^{o}C, followed by 40 cycles of 95^{o}C for 15 sec (denaturation), 51^{o}C for 18 sec (annealing), 72^{o}C for 40 sec (extension), with a final step holding at 4^{o}C. Amplification was confirmed by visualization of a DNA band of the expected size on agarose gels.

Growth curve

A growth curve was developed for select strains of *L. monocytogenes* by measuring the optical density (OD) at 600 nm every hour for 10 hours growing at 37^{o} C in brain heart infusion (BHI) broth. From overnight grown culture, 30µl was inoculated in 3 ml of BHI broth, and incubated at 37°C with shaking until mid-log growth phase was reached. The optical density of the bacterial suspension was read with a spectrophotometer, and the actual number of CFU in the inoculums was verified by plating on tryptic soy agar plates.

Cell culture

The human colon carcinoma cell line Caco-2 (ATCC HTB-37) was obtained from American Type Culture Collection, Rockville, MD**.** Cells were maintained in Eagle's minimum essential medium (EMEM) supplemented with 20% fetal bovine serum (Manassas, Virginia) grown in 75 cm^2 flasks at 37^{o} C in a humid atmosphere of 5% CO_2 in air. Sub culturing was done twice weekly by treating the monolayer with 0.5mM ethylenediaminetetraacetic acid (EDTA) - trypsin.

Adhesion and invasion assays

For adhesion and invasion assays, Caco-2 cells were trypsinized and seeded into 24-well tissue culture plates at a cell concentration adjusted to 3x10^5 Caco-2 cells/well. Cells were grown without gentamicin antibiotic to form a monolayer in Eagle's MEM supplemented with 20% heat inactivated fetal bovine serum in a 24-well tissue culture

plate by incubation for 16-18 h at 37^{o} C. The medium was changed every 24 h. Before infecting cells, the overnight cultures of *L. monocytogenes* were diluted in fresh BHI media and incubated until reaching mid-logarithmic growth phase. The monolayer was inoculated with bacterial suspensions adjusted to obtain a multiplicity of infection (MOI) of 100 bacteria per cell. Following inoculation, the plates were incubated at 37^{o} C in 5% CO_2 for 2 h. Infected monolayers were washed thrice with phosphate-buffered saline (PBS; Sigma) to remove non-adhering bacteria. Medium containing gentamicin (1000 µg/ml) was added to wells for killing extracellular bacteria before incubating at 37^{o} C in 5% CO_2 incubator for 2 h. After 2 h of incubation, the monolayer was washed twice with phosphate buffered saline (PBS) and 0.1 ml of Triton X-100 added to lyse cells and release internalized bacteria.

Quantification of surviving bacteria was done by determining colony forming units (CFU) obtained from appropriate dilutions. Each strain was measured in duplicate on three separate experiments. *L. innocua* ATCC 33090 and *L. innocua* F2411KA was included as a control in the experiments. The number of bacteria adhered to cell lines was determined by subtracting the CFU derived from the gentamicin treated wells from those of total associated bacteria (invaded plus adhered bacteria). Invasion index was calculated by dividing the number of bacteria invading the cells (gentamicin treated) with the total number of associated bacteria.

Time of incubation

Adhesion and invasion was examined for two strong and two weakly adherent strains at different incubation time. Infected Caco-2 monolayers were incubated for 15, 30, 60, 90 and 120 minutes with a constant MOI of 100:1.

Infection of Caco-2 cells with strains grown at 20^{o} C

Adhesion and invasion for strong adherent strains (CW77, and 99-38) and weakly adherent strains (CW34 and SM3) along with *L. innocua* 33090 as a control was tested by growing strains at 20^{o}C with one hour of incubation time and MOI of 100:1.

Statistical analysis

Adhesion data was reported as the average of three independent experiments each performed in duplicate. Statistical analysis was performed using one-way analysis of variance (ANOVA) Tukey's HSD test to determine differences in mean of strong and weakly adherent strains of *L. monocytogenes.* All statistical significance was reported for $p<0.05$ using SAS 9.1 (SAS Institute, Inc, Cary, NC, USA).

RESULTS

Microplate fluorescence assay

Using a microplate fluorescence adherence assay previously developed in our lab (Gamble and Muriana, 2007), different strains of *L. monocytogenes* isolated from raw ground beef, RTE meats and RTE meat processing facilities were qualitatively classified as weak or strong adherent based on the level of fluorescence obtained with the microplate assay. Strains CW50, 99-38, CW62, SM5 and CW77 were categorized as strongly adherent and strains CW34, CW35, CW52, CW72, SM3, J7 and J126 as weakly adherent (Fig. 1).

Adhesion and invasion of strong and weakly adherent strains in Caco-2 cell line with high MOI and long incubation time

Virulence potential of *L. monocytogenes* can be assessed by its ability to invade human intestinal epithelial cells. For our study, we evaluated strong and weakly adherent strains of *L. monocytogenes* using an *in vitro* model with human enterocyte-like Caco-2 cells as these types of cells are considered to be most likely entry point for the bacteria in food-borne human listeriosis (Gaillard et al., 1987). As indicated in Fig. 2, the adhesion and invasion profiles of *L. monocytogenes* strains at high MOI (100:1) and long

incubation time (2 hrs) showed higher adhesion and extensive invasion for both strong and weakly adherent strains. Though the adhesion was exhibited by all the strains, the degree of invasion by strong adherent strains (CW50 and 99-38) was highest. The adhesion of strongly adherent CW62 and weakly adherent CW34 strains differed significantly. Control strains *L. innocua* 33091 and *L. innocua* F2411KA showed invasion with high level of MOI (100:1) and long incubation time (2 hrs). Invasion index was calculated for all the strains (Fig.3). Among the weakly adherent *L. monocytogenes* strains, CW34 and CW35 had invasive index of 0.71 and 0.72 respectively whereas among strong adherent strains CW50 and 99-38 had invasive index of 0.89 and 0.79 respectively. Strongly adherent CW50 strain with highest invasion index differed significantly from CW77, SM5 and CW62 strains and also from majority of the weakly adherent (CW34, CW52, SM3, J7 and J126) strains. The invasion index of weakly adherent strains (CW52, SM3, J7 and J126) was comparable to control strains with no significant difference. The adhesion profiles of all the strains were compared with their invasion indices and it was found that there was no correlation between these parameters (Fig.4).

Adhesion and Invasion at High MOI and different incubation times

The variation in adhesion and invasion among strong and weakly adherent strains was examined using two strong and two weakly adherent strains infected with 100 bacterial cells per Caco-2 cells. The incubation time points of 15, 30, 60, 90 and 120 minutes were tested. At this level of infection, the adhesion to the cell line was mostly

similar among strong and weak adherent strains irrespective of incubation time. However, at 30 minutes of incubation time, the strong adherent CW77 strains showed significantly different adhesion when compared with weakly adherent CW34 strain. The invasion of strongly adherent strains was higher and differed significantly with weak adherent strains only at longer incubation time of 90 and 120 minutes. Evidently, the weak adherent strains showed comparable degree of invasiveness at different incubation times indicating that these strains may also be potentially virulent (Fig.5).

Adhesion and Invasion at optimized conditions of low MOI and reduced incubation time

After initial optimization of MOI and incubation times, the strongly adherent strains were examined for their invasiveness at lower MOI (10:1) and reduced incubation time of 15 minute. It was observed that all the strongly adherent strains were able to invade the Caco-2 cells at a 1:10 MOI and 15 minutes of incubation time with no significant difference among themselves but differed significantly from control strain. Adhesion of weakly adherent strains showed similar trend not differing significantly from strong adherent strains. One of the weakly adherent strains J7 showed significantly low adhesion when compared to control strain. With these minimal conditions, the weakly adherent strains did not show invasion except CW52 strain and the invasion of most of the strong adherent strains was similar (Fig.6).

DISCUSSION

Some studies have used tissue culture assays to determine differences between virulent and non- virulent strains of *Listeria monocytogenes* (Bhunia et al., 1994; Pine et al., 1991). In this study, we have compared strains of *L. monocytogenes* isolated from raw, RTE meat and RTE meat processing facilities to assess if the adherences of *L. monocytogenes* strains influence the invasiveness of human epithelial cell line Caco-2. We report that *L. monocytogenes* strains irrespective of their origin or source showing strong adherence on abiotic surfaces may have greater potential invasiveness of epithelial cell lines. Our results also indicate that the multiplicity of infection (MOI) and incubation time play key role in adhesion and invasion that may be correlated to virulence of *L. monocytogenes* strains. Though the strong adherent and weakly adherent strains both could adhere to Caco-2 cells irrespective of MOI and incubation times, the longer contact time (2hrs) and high MOI (100:1) can help even the weakly adherent strains to invade Caco-2 cells. Whereas, the low MOI (10:1) and shorter incubation time (15 min) are sufficient enough for the strong adherent strains to invade the epithelial cells. Our results were in agreement with findings of Gaillard et al (1987) and Vesikari et al (1982) who also reported enhanced invasion efficiencies of *L. monocytogenes* due to higher MOI and longer incubations of the bacteria onto the cell monolayers. Francis and Thomas (1996) reported similar findings that infection at high MOI results in extensive invasion. We also observed invasion by nonpathogenic controls used in our study *L. innocua* 33091 and

F2411KA which was similar to the findings of Chiu et al. (2006) wherein *L. innocua* strain included as a nonpathogenic control had a relatively high level of attachment to the Caco-2 cells compared with other strains, but displayed low levels of entry and multiplication in these cells. Van Langendonck et al. (1998) found some *L. innocua* strains had a low rate of entry and were able to multiply within Caco-2 cells. As we report here, the control strain *L. innocua* 33091 can adhere and invade at higher MOI (Fig. 2) but may not invade at low MOI as shown by *L. innocua* F2411KA (Fig.6). This further indicates that, MOI play a crucial role in adhesion and invasion of epithelial cells by *Listeria* strains.

As evidenced in our adhesion assay, the attachment was observed for all *L. monocytogenes* strains examined and did not seem to correlate with the source of isolation of these organisms. Del Corral et al. (1990) also reported similar findings of no difference between the isolation source and virulence of *Listeria*. Variation in the adhesion and invasion among strong and weakly adherent strains could be attributed to variation in the expression of different virulence factors and invasion factors such as internalins (Dramsi et al., 1996). The data suggest that adherence by these strains on abiotic surface is not correlated to cellular adherence. A study reported by Del Corral et al. (1990) on adhesion and invasion properties of various food and clinical isolates with the HEp-2 cell line showed that both invasion and adherence of *L. monocytogenes* strains varies, and the degree of invasion and adherence overlaps with those of the non-pathogenic strains. The capacity to adhere to Caco-2 cells is known to vary greatly among *Listeria* species (Jaradat and Bhunia, 2003; Meyer et al., 1992) and our study confirms this.

Invasion abilities of these strains varied irrespective of their adherence level indicating that adherence and invasion are not dependent upon each other. Low correlation (R^2 =0.1419) was observed between adhesion data with invasion index for strains of *L. monocytogenes* and *Listeria* spp. using a high MOI and long incubation time. Meyer et al. (1992) and Jaradat and Bhunia (2003) reported that there is no correlation between invasion and adhesion among different *L. monocytogenes* serotypes.

Virulence assays carried out with high MOI and long incubation time indicated that both *L. monocytogenes* adhere and invade but it may not reflect the correlation of abiotic adherence to virulence potential. Invasiveness by weakly adherent strains indicated that these strains were apparently equally virulent. Based on these findings we investigated the influence of different incubation time on adhesion and invasion for two strong and two weakly adherent strains at a constant MOI of 100:1. The ability of these strains to adhere and invade Caco-2 monolayer is shown in Fig 2. The results indicate that adherence level by both strong and weakly adherent strains is almost same irrespective of the incubation time and slight variation is observed in the invasion of the strains. Under these conditions both strong and weakly adherent strains showed adherence and invasion after 15, 30, 60 and 90 minutes of infection (Bigot et al., 2005) reported that strains infected at different time points showed similar adhesion and entry efficiencies in human Caco-2 and hepatocyte Hep-G2 cell line. These results indicate that factor (s) necessary for bacterial adhesion is already present on both the host cell and at least in a fraction of the bacteria. Screening of these bacteria at different incubation times did not show the differences in the adherence characteristics on abiotic surfaces (Fig. 5).

Furthermore, we monitored the invasion of strong and weakly adherent strains with different levels of MOI varying from 10:1, 1:1 and 0.1:1 and incubating the infected cell lines for 15 minutes. There was no bacterial recovery with 1or 0.1 bacterial cell used with low incubation time. Whereas, MOI of 10 bacterial cells per 1 Caco-2 infected and 15 min of incubation time resulted in invasion by strongly adherent strains and weakly adherent strains were not capable of invading Caco-2 cell line except for CW52. Results indicated that the strains that had shown high adherence on abiotic surfaces were able to invade the cell line with minimum incubation time and low MOI whereas, weaker adherent strains were unable to invade with similar conditions provided. Although adhesion level was almost same for all strains the variation in invasion may be determined in part by combined invasion and adherence capabilities of the organism (Meyer et al., 1992). Invasion efficiency for all the four strong adherent strains was about 0.7 indicating that strong adherence strains are more virulent than weak adherent strains**.** Among weakly adherent strains invasion by CW52 could indicate that it might be false virulent strain.

We have shown that all *L. monocytogenes* strains express hemolysin but only those strains which had weak adherence on abiotic surfaces were non invasive indicating that the hemolysin detection gene alone is not reliable criterion for the identification of *L .monocytogenes* pathogenicity (Hof et al., 1992). Moreover, all *L. monocytogenes* strains in this study were positive for the presence of the virulence genes tested for, but no invasion by weakly adherent strains with minimal conditions indicated that these strains were not pathogenic. The results of cell culture assays were consistent with the results of *in vivo* virulence assay carried out using A/J mice (Kushwaha and Muriana, 2009).

Invasion of strong adherent strains with low MOI and minimum incubation may show that these strains may be able to express *inl*A which encodes a protein required for invasion of intestinal epithelial cells (Kazmierczak et al., 2003; Kim et al., 2004; Kim et al., 2005; Sue et al., 2004). Although, Gaillard et al. (1987) and Kuhn et al. (1988) showed that *L. innocua* was unable to invade enterocytes and hence considered as non pathogenic species but our study showed that *L. innocua* were capable of invading and invasion level was very much similar to *L. monocytogenes* strains. Meyer et al. (1992) suggest that the avirulent nature of the species is determined not by their ability to invade, but by a combination of factors including their hemolytic nature and adherent efficiencies, as well as other as yet unidentified determinants.

Though the virulence-associated genes in *L. monocytogenes* are thermoregulated, they showed reduced expression at low temperature (Dramsi et al., 1993; Leimeisterwachter et al., 1992) however, in our study virulence assay for strong and weakly adherent strains grown at 20^{o} C showed no difference with regard to invasion for strains grown at 20^{o}C (data not shown) demonstrating that the invasion of *L. monocytogenes* is not influenced by growth temperatures which was similarly reported by Conte et al. (1994).

CONCLUSION

Our results based on the use of 12 strains of *L. monocytogenes* demonstrate the fact that those strains with strong adherence in the microplate assay were invasive in Caco-2 cells with modified conditions indicating existence of high virulence for strains isolated from raw and RTE meat. Using cell culture models we have established that both strong and weakly adherent strains of *L. monocytogenes* are equally capable of adhering and invading Caco-2 cell line and our results suggest that regulating the infection level and incubation time is a key factor in determining the virulence potential of the strains.

Therefore, understanding the adherence and invasion of these strains could help us to determine their virulence potential since strong adherence not only promote retention of such strains in food processing facilities, but enhanced virulence as well. This study demonstrates that invasion by strongly adherent strains may be useful in screening differences in infection potentials between different strains of *L. monocytogenes* when minimal conditions are used for discrimination between strains.

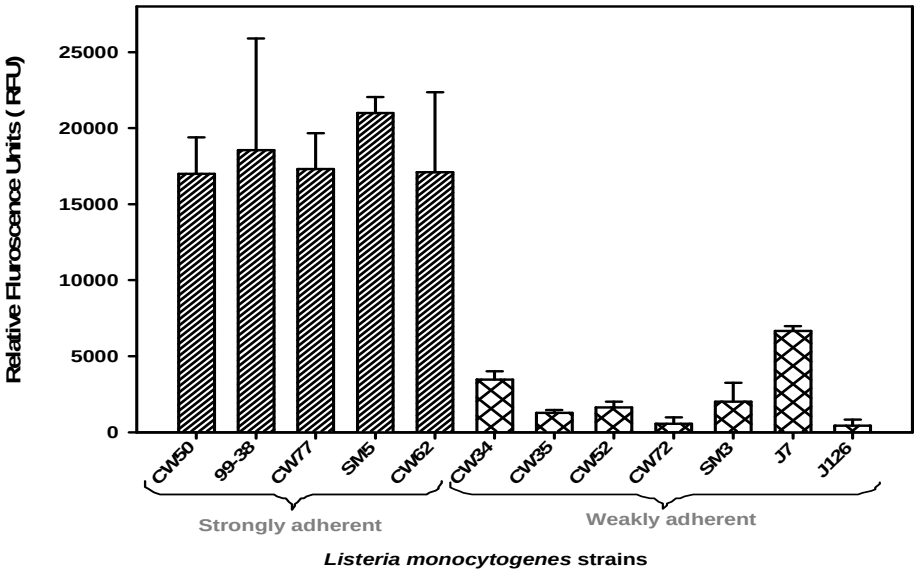

Figure 1. Relative fluorescence of *L. monocytogenes* strains isolated from raw, RTE meat and RTE meat processing plants using the microplate adherence assay. The data bars are presented as mean of triplicate replications, and the error bars represent the standard deviation of the mean

.

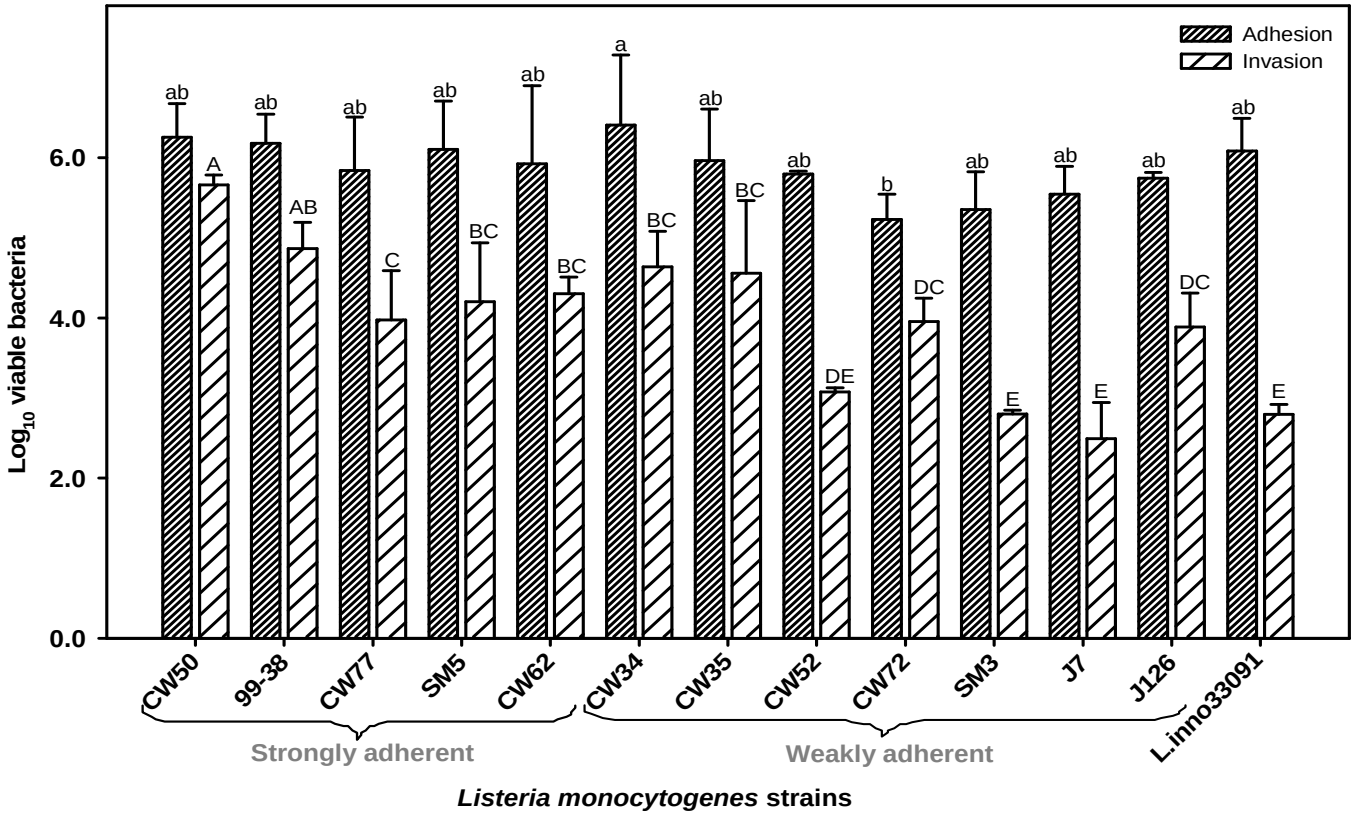

Figure 2. Adhesion and invasion assay for strong and weakly adherent strains of *L. monocytogenes* into Caco-2 cells. The cells were infected with an MOI of 100 bacteria per cell. Values represent the mean of three experiments carried out in duplicate. (Uppercase and lower case letters indicate means compared separately for significance testing).

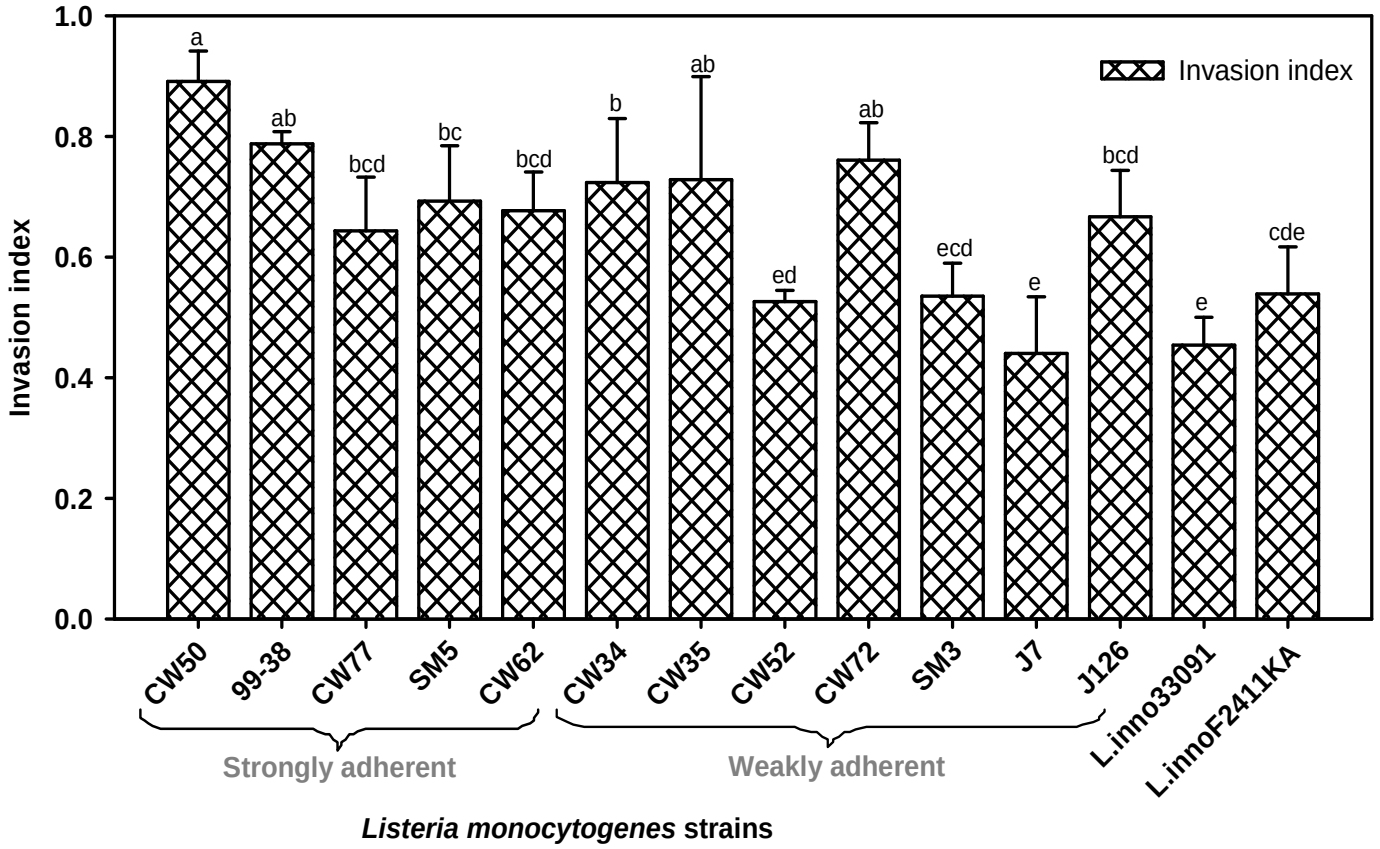

Figure 3. Invasion index (ratio of internalized cells vs. total) for strong and weakly adherent strains of *L. monocytogenes*

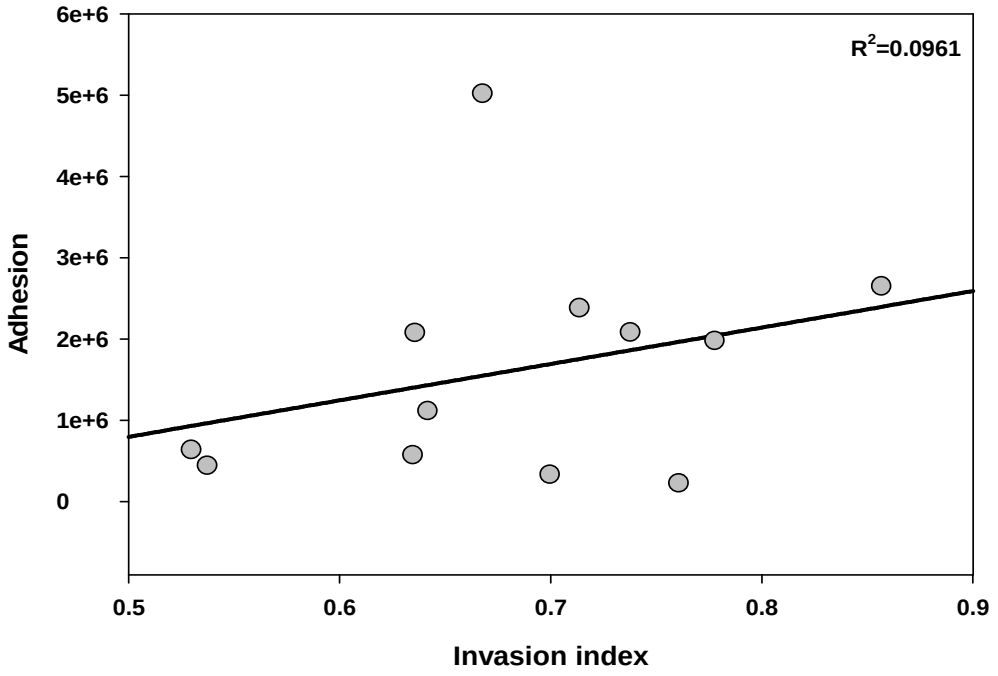

Figure 4. Correlation plot for the adhesion and invasion index for strains of *L. monocytogenes*.

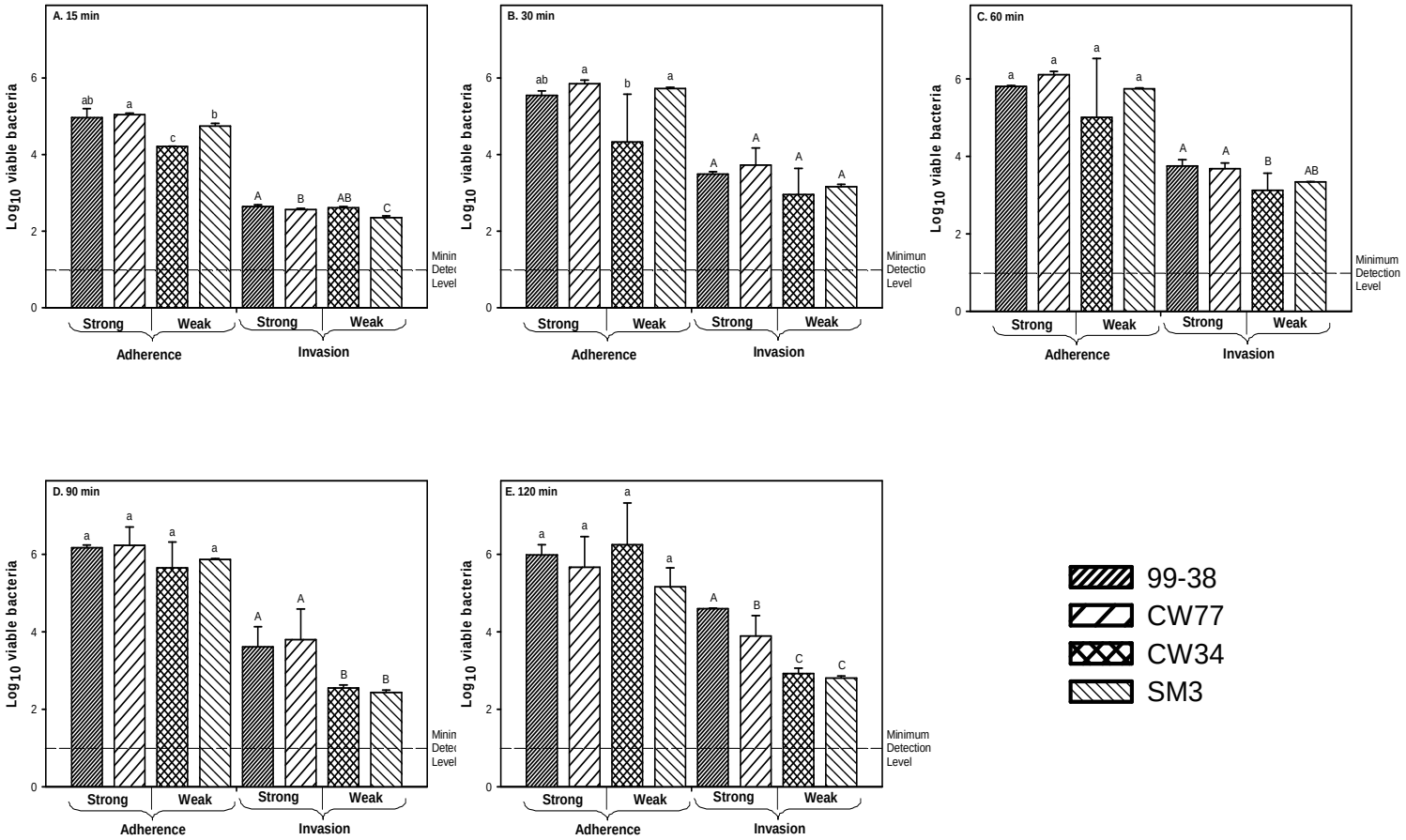

Figure 5. Adhesion and invasion assay for strong (99-38, CW77) and weakly adherent strains (CW34, SM3) using different bacterial incubation times (15, 30, 60, 90, and 120 min). (Uppercase and lower case letters indicate means compared separately for significance testing).

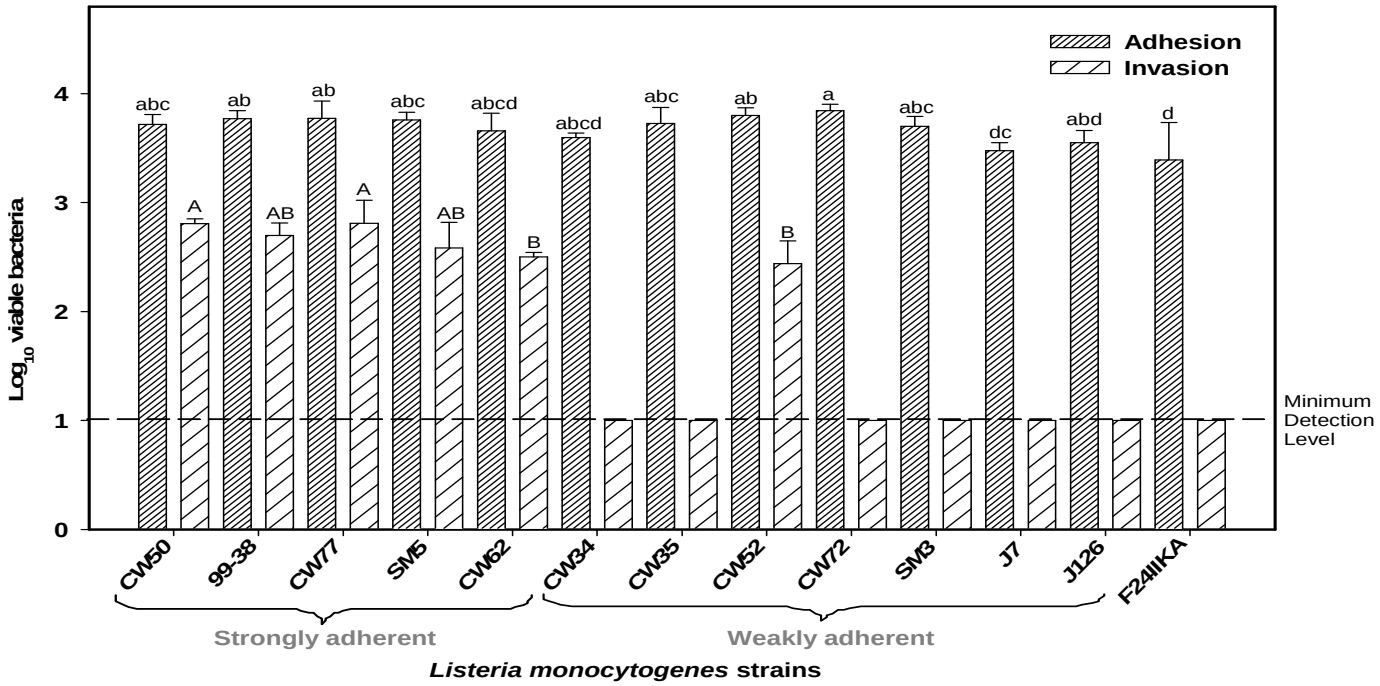

Figure 6. Adhesion and invasion assay for strong and weakly adherent strains of *L. monocytogenes* into Caco-2 cells. The cells were infected with an MOI of 10 bacteria per cell and 15 min incubation time. Values represent the mean of three experiments carried out in duplicate. (Uppercase and lower case letters indicate means compared separately for significance testing).

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CHAPTER V

COMPARISON OF VIRULENCE AMONG STRONG AND WEAKLY ADHERENT STRAINS OF *LISTERIA MONOCYTOGENES* BY ORAL INOCULATION OF A/J MICE

Kalpana Kushwaha and Peter M. Muriana[1,2] ‡

[1]Department of Animal Sciences &

[2]The Robert M. Kerr Food & Agricultural Products Center,

Oklahoma State University

Stillwater, Oklahoma 74078

INTRODUCTION

Listeria monocytogenes is an intracellular Gram-positive bacterium causing foodborne illness (listeriosis) in humans due to ingestion of contaminated ready-to-eat (RTE) foods. It is estimated that listeriosis attributes to 20-30% mortality with 2,500 illnesses and 500 deaths each year in the United States (Mead et al., 1999). The groups which are at highest risk include pregnant women and their fetuses, newborns, elderly people, and immunocompromised individuals. The United States has stringent regulations for *L. monocytogenes* in RTE foods which is less than 1 CFU per 25 gm of product (Notermans et al., 1998). Although the U.S. Food and Drug Administration (FDA, 2008) recently proposed an acceptable 'defect level' of 100cfu/gm for this organism in foods in which it is not expected to survive, there is currently a 'zero-tolerance' for *L. monocytogenes* in RTE foods. Recent epidemiological data showed that *L. monocytogenes* may be transmitted as an enteric pathogen by consuming contaminated foods such as vegetables, milk, meat and dairy products (Fleming et al., 1985; Linnan et al., 1988; Schlech et al., 1983), suggesting that natural infection can occur by the oral route (Okamoto et al., 1994).

MacDonald and Carter (1980) have proposed that the major route of invasion of *L. monocytogenes* after intragastric inoculation is through the Peyer's patches and other gut-associated lymphoid tissues. *L. monocytogenes* when ingested through contaminated food can reach the gastrointestinal tract, and translocate through intestinal barrier to

infect lymph nodes, the spleen and liver and from there will disseminate to different organs via lymphatic pathways (Marco et al., 1992). Replication of *L. monocytogenes* mainly occurs in hepatocytes and spreads cell-to-cell forming infectious foci. Development of infectious foci in liver and spleen is depended on the virulence of the strain, amount of inoculums, and sensitivity of the strain of mice (Cheers and McKenzie, 1978). In the hepatocytes >90% of bacteria are removed by neutrophils chemotactants during the first 24 hrs of infection (Conlan and North, 1991). The remaining bacteria which are not killed by neutrophilic attack are internalized by hepatocytes where they undergo intracellular replication. However, it is known that all strains of *L. monocytogenes* are not equally virulent and their virulence can be determined by their invasiveness and ability to grow *in vivo* (Barbour et al., 2001; Larsen et al., 2002; Roche et al., 2005).

Virulence of *L. monocytogenes* has been assessed by the different methods indicating that virulence varies from one strain to another (Roche et al., 2003). *In vivo* study done by infecting mice by intravenous (i.v.) intraperitoneal (i.p.) injections and oral route of inoculation are considered to be highly sensitive assays for evaluating the pathogencity of *L. monocytogenes* (Audurier et al., 1980; Hof and Hefner, 1988; Lammerding et al., 1992). In mice, virulence is evaluated either by comparing the 50% lethal dose (Conner et al., 1989; Del Corral et al., 1990) or by enumerating the viable bacterial count from spleen and liver (Hof, 1984) as described by Mackaness (1962). Enumeration of viable bacteria in the spleen and liver has provided the most consistent results in quantitative evaluation of virulence (Mackaness, 1962). Human epithelial cells

lines, such as Caco-2 and HeLa have often been used to study invasiveness and the potential virulence of *L. monocytogenes* (Gaillard et al., 1987; Pine et al., 1991).

The A/J mouse strain is among the most susceptible to infection with *Listeria monocytogenes* (Cheers and McKenzie, 1978). However, the infective dose of the organism has not yet been determined because it depends upon the host and strain variability. It has been reported that higher doses are generally required for infection via oral route than either i.v. or i.p. injections. It has been reported that there is need to use higher dose (inocula of 10^8 CFU or greater) to cause systemic infection following oral inoculation (Barbour et al., 2001; Lammerding et al., 1992) however, low challenge dose via oral route has also caused lethal infection in mice (Pine et al., 1990). In immunocompetent individuals, as many as 10^6 to 10^9 CFU may be required to cause infection whereas low doses may cause illness in immunocompromised persons (Farber and Peterkin, 1991). Infections have occurred in tissues with doses as high as 5×10^7, but were never fatal even at 6×10^9 (MacDonald and Carter, 1980).

Listeria is often present on raw meat ingredients and has been a recurring problem in meat processing facilities. It is also capable of strong adherence to equipment and/or surfaces in meat processing facilities, resulting in the formation of biofilms (Borucki and Call, 2003; Gamble and Muriana, 2007). Thus, it is essential to study the *in vivo* virulence of *L. monocytogenes* originated from raw meat sources. Very limited Information is available on *in vivo* studies concerning the virulence of *L. monocytogenes* capable of strongly adhering to abiotic surfaces that are isolated from various meats. Considering the significance of *L. monocytogenes* as a food borne pathogen, it is

important to obtain quantitative data on effect of high dose of strong and weakly adherent strains *L. monocytogenes* following oral inoculation of mice.

Therefore, the purpose of our study was to determine whether strong or weakly adherent strains of *L. monocytogenes* isolated from raw and RTE meats differ in their virulence abilities when they are inoculated intragastrically (i.g.), and these strains are likely to be persistently recurring contaminants in meat processing plants.

MATERIALS AND METHODS

Bacterial strains

L. monocytogenes strains used in this study were isolated from raw and RTE meat. Strains included in this study are CW50, 99-38, CW77, CW62, CW34, CW35, CW52 and SM3. Strains designated as “CW” were isolated from retail frankfurters which are RTE product and 99-38 and SM3 were isolated from retail ground beef (Wang and Muriana, 1994).

Mice

Female A/J mice were obtained from Jackson Laboratory (Bar Harbor, Maine, USA) at 5 to 6 weeks of age and housed under microisolator caps (six mice per group) at the Laboratory Animal Resource Center, Oklahoma State University. Mice were acclimated for at least 1 week in this facility before being used in an experiment. All animals used in this study were handled according to the guidelines approved by the Oklahoma State University Institutional Animal Care and Use Committee (IACUC).

Mouse passage

All isolates were first passaged through mice before use in mouse assays. Three days following oral inoculation of approxiamately 1 x 10^8 CFU, bacteria were isolated from the liver or spleen. Reisolated strains of *L. monocytogenes* identity were confirmed and then cultured for storage. Following this, the bacteria were harvested by centrifugation, resuspended in BHI broth containing 20% glycerol, and stored at -76°C as 1-ml aliquots.

Preparation of inoculum

For intragastric inoculation, passaged isolates from the -76^o C stocks were subcultured on to BHI broth and grown overnight at 37^{o}C. From overnight grown cultures 30µl of inoculum was inoculated with 3 ml of BHI broth, and incubated at 37°C with shaking until mid-log phase growth was reached. The optical density of the bacterial suspension was read with a spectrophotometer, and the numbers of CFU of *L. monocytogenes* were extrapolated from a standard growth curve. Exact counts were obtained subsequently from plating. To prepare the inoculums for the mice, appropriate dilutions were made in sterile phosphate-buffered saline (PBS, 0.01 M, pH 7.2) to achieve the desired bacterial concentration. The actual number of CFU in the inoculums was verified by plating on tryptic soy agar. The preparation were kept in ice until administered to the mice.

Preliminary experiment for evaluation of dose

The effect of different levels of inoculum (10^4, 10^6 and 10^8 CFU/ml) on mice was tested for one strong adherent *L. monocytogenes* strain (CW50). The concentrations of CW50 strain was prepared as described above and inoculated in mice (six mice per concentration) and the control mice were given 0.1 ml of sterile 0.01M PBS. Mice were euthanized on day 3,4, 5 and 7 day. Tissues harvested included spleen and liver. Based on the results the subssequent inoculum dose obtained with strain CW50 and days of tissue harvesting were chosen.

Inoculation of mice

Standard diet was provided and water *ad libitum* until 5 h prior to intragastric inoculation. On the day of inoculation, food and water was removed from the cage to prevent mechanical blockage of the *Listeria* inoculums by food within the stomach of mice that might lead to aspiration of the inoculums into the lungs. The cultures were grown until log phase and then centrifuged, and resuspended in sterile Phosphate Buffered Saline (PBS) to an approximate concentration of 1.0 $x10^9$ CFU/ml. Mice were inoculated with 0.1ml (approximately $1x10^8$ CFU) by i.g gavages (stainless steel ball-end feeding needle) attached to a 1ml syringe. Six mice for each concentration were inoculated and the control mice were given 0.1ml of sterile 0.01 M PBS in each experimental run.

Recovery of *L. monocytogenes* from the spleen and liver

Mice were humanely euthanized by asphyxiation with carbon dioxide after third day of i.g. inoculation for enumeration of viable bacteria in spleen and liver in the preliminary experiment. These tissues were weighed in sterile tube that contained cold sterile saline. The tissues were then homogenized, diluted in sterile saline, and plated in duplicate on tryptic soy agar to determine the bacterial counts in each organ. Dilutions of tissue homogenates plated were (10^{-1}, 10^{-2} and 10^{-3}). The plates were allowed to dry and then incubated at 37°C for 48 h. Respective colonies were checked on MOX agar for confirmation as *Listeria*.

Histopathological examination of infected mice

Histopathology for spleen and liver were done for strong and weak adherent strains. Mice were inoculated (10^{8} CFU with strong adherent strain (CW50) and a weak adherent strain (CW34)) and euthanized on day four. Necropsy was performed on each mouse and tissue samples from the spleen and liver were collected and fixed in 10% neutral buffered formalin. Following 48 hours of fixation, thin sections of the tissues were cut, paraffin-embedded on glass slides, sectioned at 5 μm and stained with hemotoxylin and eosin.

Fluorescent microplate assay for adherence

These strains were characterized for their adherence as described in Gamble and Muriana (2007). Briefly, strains were sub cultured in BHI broth held at 30°C and diluted

to 10^5-fold in fresh BHI broth, and 200 µl was transferred to designated wells of a 96-well black microwell plate with a clear lid (Nunc, Denmark). After incubation, the microplate was washed three times with Tris buffer (pH 7.4; 0.05 M) to remove loosely adhered cells. The washing was followed by the addition of 200 µl of fresh (sterile) BHI broth to each experimental well, and the plate was again wrapped in Parafilm, incubated at 30°C, and washed three times with Tris buffer (pH 7.4; 0.05 M) after another 24 h. After the final washing, 200µl of 5, 6-CFDA (Molecular Probes/Invitrogen, Carlsbad, CA) fluorescent substrate solution was added. Following incubation with the 5, 6-CFDA substrate, the plates were washed three times with Tris buffer (pH 7.4; 0.05 M) in the plate washer, and the medium was replaced with 200 µl of the same medium. The plate was then read from above or below in a Tecan GENios fluorescent-plate reader (Phenix Research Products, Hayward, CA) using a fixed signal gain of 75% with excitation at 485 nm and detection at 535 nm. Based on the level of fluorescence signals obtained with our microplate assay for the strains screened the strains were categorized as weak, medium and strong adherent strains. Strains with the high-level of fluorescence signal (>5000 RFU) were considered strong adherent strains, 1000-4000 RFU as medium adherent strains and 0-1000 RFU as weak adherent strains.

Statistical Analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) Tukey's HSD test to determine differences in mean CFU/gm for mice liver and spleen treated with strong and weakly adherent strains of *L. monocytogenes.* All statistical significance was reported for $p<0.05$ using SAS 9.1 (SAS Institute, Inc, Cary, NC, USA).

RESULTS AND DISCUSSION

We used microplate fluorescence adherence assay developed in our lab to distinguish different adherence phenotypes in strains of *L. monocytogenes* isolated from RTE meats, raw ground beef, and meat processing facilities (Gamble and Muriana, 2007). In that study, we showed that the degree of cellular attachment of the strongly adherent strains was significantly higher (10^8 CFU/well) than the weakly adherent strains (10^3 CFU/well).Select strains used in this study, isolated from raw and RTE meat were subjected to microplate fluorescence assay. Based on the relative fluorescence unit strains were categorized either weak or strong adherent. Strains CW50, 99-38, CW62 and CW77 were classified as strong adherent whereas CW34, CW35, CW52, CW72 and SM3 were categorized as weakly adherent strain as shown in Fig 1.

Our previous study using human cell line Caco-2 for the same strains revealed that those strains which had shown strong adherence on microplate fluorescence assay were more invasive in cell culture assay with low level of multiplicity of infection (1:10) and minimum incubation of time (15min) indicating that these strains were more virulent when compared to weakly adherent strains (Kushwaha and Muriana, 2009). Therefore, we further investigated the *in vivo* virulence for the same strains using intragastric inoculation of A/J mice in this study. Virulence of both strong and weakly adherent strains was tested based on the recovery of the inoculated strains from liver and spleen tissue samples from mice.

In preliminary experiments, six mice were orally inoculated with a strong adherent (CW50) strain and another set of six mice with a weakly adherent strain (CW34) using low doses (10^4 and 10^6 CFU/ml). Mice were euthanized on day 3, 4, and 5 for each inoculation dose. There was no bacterial recovery from spleen or liver for the lower doses for both strong and weakly adherent strains indicating that they failed to cause infection. Schlech et al (1993) and Conlan (1996) have indicated that there is a lower efficiency of bacterial penetration when low inoculation doses are being used due to the dose dependency of *L. monocytogenes* to establish an invasive infection.

When mice were inoculated with high doses of 10^8 CFU/ml for a strong adherent strain (CW50) and a weak adherent strain (CW34), bacterial recovery from tissue samples was observed. Higher viable bacterial counts were observed in the spleen than in the liver for strong adherent strain whereas no bacterial recovery from liver of mice was seen for the weakly adherent strain. Control mice were inoculated with 0.1 ml of PBS. Tissues were harvested on day 3^{rd}, 4^{th}, 5^{th} and 7^{th} days. Bacterial recovery for strongly adherent strains was approximately 5.0 log CFU from the liver and 4.0 log CFU from the spleen on days 3, 4 and 5 (Fig 2). In addition to liver and spleen, kidney was also harvested for these days but no bacterial recovery was observed except for the seventh day (data not shown). Limits of detection were 1.0 $\log_{10}$ in the spleen and 2.0 $\log_{10}$ in the liver. In the case of weakly adherent strains, *L. monocytogenes* were recovered from spleen which was approximately 4 logs CFU whereas no bacterial recovery made from liver of the mice. There was no significant difference observed in the mean CFU/g of spleen and liver for CW50 strain as well as spleen of CW34 strain with high dose on days 3, 4 and 5 (Fig 2). Therefore, for the evaluation of virulence of the remaining

strains, the highest dose 10^8 CFU/ml was chosen because preliminary experiments had shown maximal counts in spleen and liver. Use of relatively high numbers of *L. monocytogenes* bacteria for inoculation by gavage (10^8 CFU or greater) has been reported by (Barbour et al., 2001; Czuprynski et al., 1989; Farber and Peterkin, 1991; Marco et al., 1992a).

Maximum bacterial recovery was seen on days 3 and 4, with lower recoveries obtained on day 5 followed by day 4 and 5. By day 7 numbers of *L. monocytogenes* declined as the bacterial recovery was low from spleen and liver. There was not much difference in the bacterial recovery between day 3 and 4 and hence we decide to harvest the tissue on day 4 for the main experiment.

We did not observe any sign of illness or death of mice following oral administration. Similar results were reported by Miller and Burns (1970) wherein no death of white Swiss mice was observed when mice drank water containing *L. monocytogenes* and evidence of infection was detected microscopically as lesions appearing on the liver and spleen. Similarly, Audurier et al., (1980) reported that there was no lethality observed in orally inoculated mice even when $7x10^{10}$ CFU of virulent bacteria were given and no significant mortality was seen by oral or i.g. inoculation of *L. monocytogenes* in mice with a dose higher than LD_{50}(Marco et al., 1992b). However, Pine et al. (1990) reported mortality of mice which were inoculated orally with approximately 50% lethal dose values that ranged from 50 to 4.4 x 10^5 CFU for different foods using clinical isolates of *L. monocytogenes*. Most studies have reported i.g. or oral inoculation does not cause mortality but causes subclinical systemic infection at high

infective log doses (Roll and Czuprynski, 1990; Roll et al., 1990; Vonkoenig et al., 1983; Zachar and Savage, 1979).

The results of bacterial recovery from CW34, CW35, SM3, CW50, 99-38, CW72, and CW62 on the fourth day of inoculation with high doses of *L. monocytogenes* shows bacterial recovery from liver for weakly adherent strains CW52 (Fig.3). The hemolysin gene of *L. monocytogenes* is important not only for intracellular survival *in vitro* but also considered to be associated with virulence after parenteral inoculation *in vivo* (Cossart et al., 1989; Gaillard et al., 1987; Kuhn et al., 1988; Portnoy et al., 1988). Even though, both weak and strong adherent strains have the known genes for virulence and may have the ability for invasion but after reaching the different organs virulent bacteria may express number of genes that are essential for their survival and replication and may have been able to resist host immune response which allowed their replication in the liver. Whereas, weakly adherent strains may have been less potential to express genes that are essential to interact with the host organs. They may be very susceptible for killing by immune response therefore, no replication was observed in the liver and hence no bacterial recovery made. This indicates that strong adherent bacteria had the ability to interact well with host tissue through expression of various genes and adherent proteins. Hence the inability of the weaker adherent strains to infect the liver may be due to its inability to attach hence reduced pathogencity due to loss or reduction of virulence factors involved in other aspects of infection which was reported by (Takeuchi et al., 2003).

Only exception was observed in CW52 which had shown weak adherence in microplate fluorescence assay and was considered as weakly adherent strain.Eventhough it was a weakly adherent strain it was able to infect the liver of mice and bacterial

recovery was 4log CFU/g from liver which was similar to CW77, a strong adherent strain. ANOVA analysis for fourth day of post inoculation showed that there is a significant difference in the mean bacterial viable counts in the spleen and liver for both strong and weakly adherent strains. Within strong adherent strains, mean bacterial cfu/g of spleen for CW62 and liver of CW50 exhibited significantly higher CFU/gm when compared with mice inoculated with weakly adherent strains that did not show any significant difference among themselves.

Evidence of infection can be detected microscopically as lesions appearing on the liver and spleen (Miller and Burns, 1970). Fixed sections of tissue from control mice and mice inoculated with strains CW50 (strong) and CW34 (weak) were examined by hematoxylin and eosin staining. Sections of the spleen tissues from mice inoculated with *L. monocytogenes* showed no histological lesions and appeared normal when compared with sections from control mice. The liver of mice inoculated with the strong adherent strain (CW50) showed conspicuous inflammatory foci whereas, the weaker adherent strain exhibited discrete, non-effacing lesions comprised primarily of macrophages with rare neutrophils with the absence of necrosis (Fig.4). The strongly adherent strains seem to establish the infection better and induce cell damage in the liver. Similar findings have been reported by Czuprynski et al. (1989), Miller and Burns (1970), Portnoy et al.(1992), and Conlan and North (1992) wherein substantial infection of mice liver and spleen tissue by hemolytic parental strains have been described. Histopathological response of spleen and liver taken from mice infected with strong and weakly adherent strain at 4 days after inoculation was confirmed by the bacteriological data (Fig.3).

In summary, the results of this study provided evidence that strong adherent strains may differ in their virulence abilities to cause infectious foci in the liver during infection. Virulence of these strains in mice demonstrated that strains that showed strong adherence are capable of invading and replicating in host tissues to the magnitude necessary to cause severe damage whereas, weakly adherent strains were eliminated from the liver with same challenge dose. This indicated that strong adherence may provide these strains with some features that make them more virulent than weakly adherent strains**.** Roche et al. (2003) also reported that even though strains may have the main known genes for virulence, but in frame mutations could decrease their virulence. Nevertheless, the results of the mouse assays were consistent with the results of *in vitro* virulence assay carried out using Caco-2 cells (Kushwaha and Muriana, 2009).

It is important to do further research (microarrays) in identifying genetic differences between strong and weakly adherent which affect the attachment of these strains to abiotic surfaces, and subsequently functional analysis. This will help us to better understand the pathogenic potential of these strains and the results we obtain when inoculated in mice.

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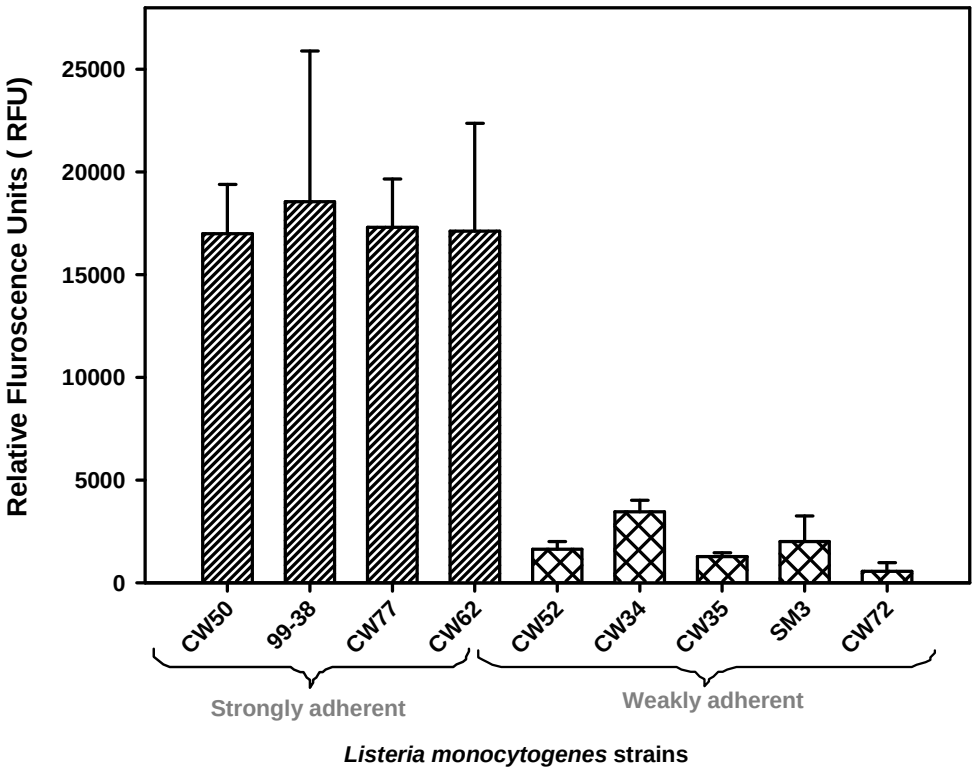


gure 1. Relative fluorescence of *Listeria monocytogenes* strains isolated from raw and RTE meat plants using the microplate adherence assay. The data bars are presented as mean of triplicate replications, and the error bars represent the standard deviation of the mean.

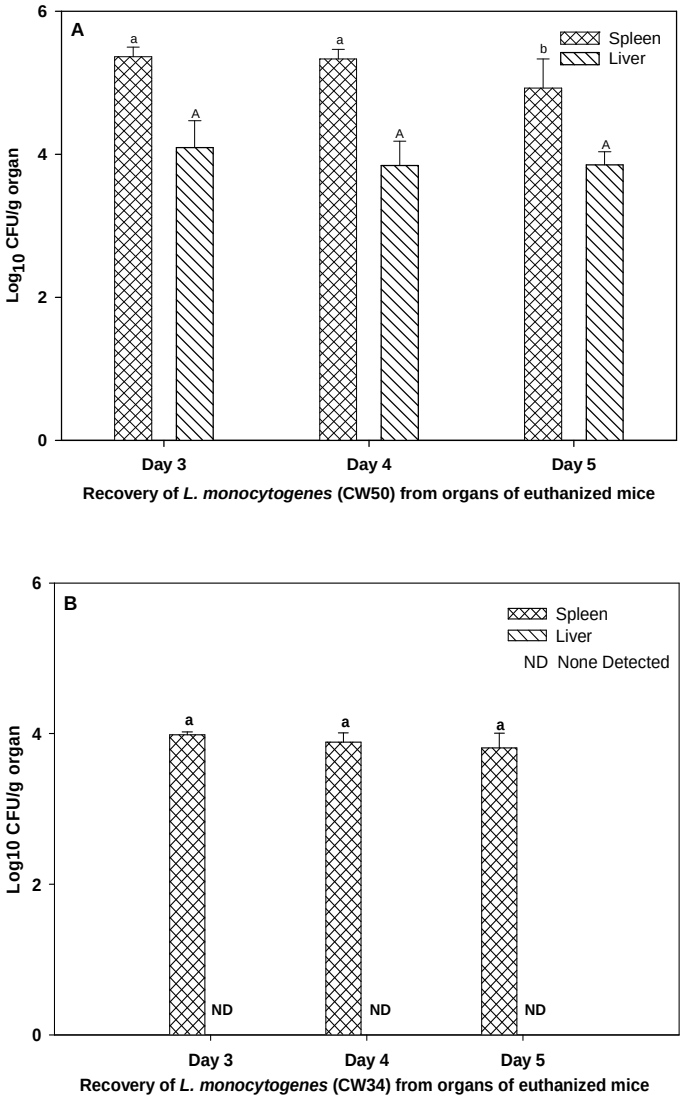


Figure 2. Recovery of *L. monocytogenes* CW50 (A) and CW 34 (B) strain from tissues of A/J mice. Groups of six mice were inoculated orally with 8 logs CFU by strong adherent strain. The mice were killed on 3day, 4 day, and 5 day post inoculation. The spleen and liver were removed aseptically and homogenized. (Uppercase and lower case letters indicate means compared separately for significance testing).

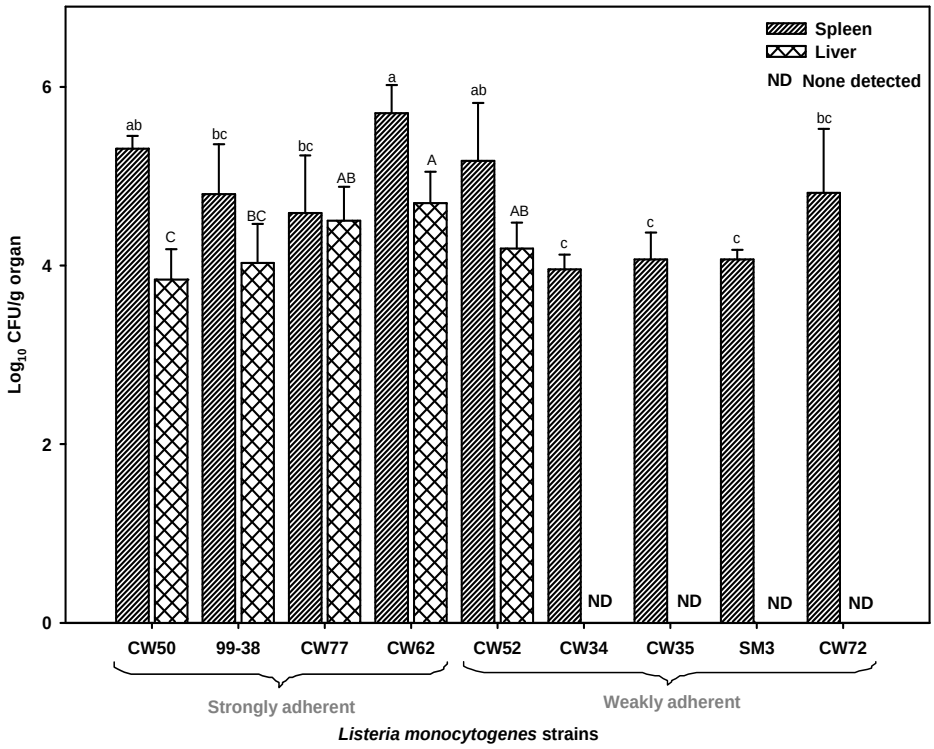
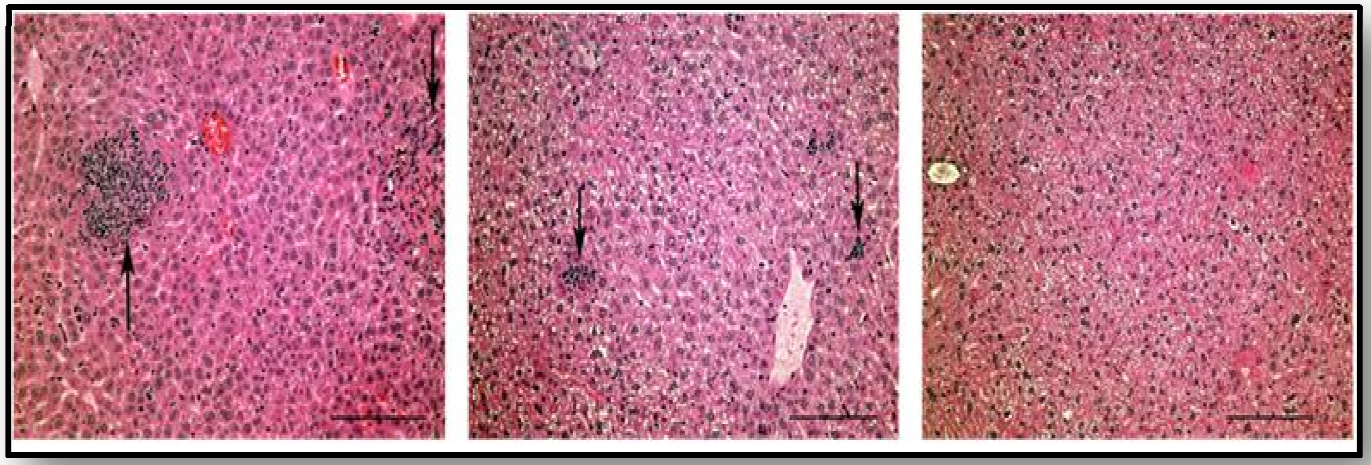


Figure 3. Bacterial recovery from spleen and liver of A/J mice on fourth day of post inoculation for strong and weakly adherent strains of *L. monocytogenes* Groups of six mice were inoculated orally with 8 logs CFU. (Uppercase and lower case letters indicate means compared separately for significance testing).



Panel A **Panel B** **Panel C**

Figure 4. Histopathology of strong (CW50) and weakly adherent (CW34) strains Tissue samples were prepared as described in materials and methods. Panel A shows conspicuous foci of inflammation dominated by neutrophils and necrosis (arrows). In comparison, the inflammatory lesions in weaker adherent strain were discrete, lacked significant (if any) necrosis, and were comprised primarily of a mononuclear infiltrate (Panel B, arrows). Control mice did not have hepatic lesions (Panel C). Haemotoxylin & Eosin stain, Bar ≈ 150 μm.

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APPENDIX

SUBTYPING OF STRONG AND WEAKLY ADHERENT STRAINS OF *LISTERIA MONOCYTOGENES* BY MLST, PFGE AND RIBOTYPING

INTRODUCTION

Listeria monocytogenes is a Gram positive, intracellular food borne pathogen causing listeriosis in humans that has a significant impact on specific risk groups, including pregnant women and their fetuses as well as immunocompromised people (Farber and Peterkin, 1991).It is estimated to cause approximately 2,500 cases of human illness and 500 deaths annually in United States (Mead et al., 1999). Because of the high fatality rate U.S regulatory agencies have established a "zero tolerance" for the species in ready-to-eat (RTE) foods (Swaminathan et al., 2001).

Molecular characterization of *L. monocytogenes* is essential for identification of specific subtypes and understanding the distribution of this pathogen in relation to outbreaks, contaminated foods, and/or environmental sources of contamination, notably processing plants These subtypes are usually characterized by various subtyping methods which needs to be accurate and highly discriminatory to help identify the potential vehicles of infection, and to discriminate sources of contamination in processing plants (Wiedmann, 2002).

Various molecular methods have been used for genotyping of *L. monocytogenes* such as multilocus sequence typing (MLST) (Maiden et al., 1998), pulsed-field gel electrophoresis (PFGE) (Brosch et al., 1996;1994) and ribotyping (RT) (Bruce et al., 1995). These DNA-based methods define bacterial subtypes by PCR amplification,

sequence analysis or restriction digestion of bacterial DNA to generate DNA fragment banding patterns.

MLST is sequence based subtyping method which uses six or more housekeeping genes and differentiates bacterial isolates by comparing the DNA sequences in these genes Data generated is unambiguous and portable through web-based database (Chan et al., 2001; Enright et al., 2001). PFGE is mainly based on identifying the microorganisms by defining unique banding patterns of their digested DNA fragments generated by gel electrophoresis apparatus (Tenover et al., 1995). PFGE is the most discriminatory subtyping because it shows high level of discrimination of *L. monocytogenes* and is often considered to be gold standard for discriminatory ability (Aarnisalo et al., 2003; Sauders et al., 2003). Ribotyping is fully automated system which allows highly reproducible subtyping of *L.monocytogenes* but is relatively costly and less discriminatory than PFGE It is based on the comparison of DNA banding pattern generated by hybridization of labeled ribosomal RNA probes with *Eco*RI digested genomic DNA of *L.monocytogenes*. In this system all process steps are automated, from cell lysis to image analysis, and provide subtyping results within 8h (Aarnisalo et al., 2003; Sauders et al., 2003).

In this study we examined the phylogenetic relatedness of strong and weakly adherent strains of *L. monocytogenes* isolated from meat, RTE meat and meat processing facilities using DNA sequencing-based subtyping method (MLST), PFGE and ribotyping.

MATERIALS AND METHODS

Bacterial strains and growth media

The *L. monocytogenes* strains used in this study included from raw and ready-to-eat (RTE) meat and meat processing facilities. The bacterial strains were cultured by transferring 100 µl of thawed frozen culture into 9 ml of brain heart infusion (BHI) (Difco, Becton-Dickenson, Franklin Lakes, NJ) broth, incubating them overnight (18 to 24 h) at 30°C, and subculturing the bacteria twice before use. Frozen culture stocks were prepared by centrifuging 9 ml of culture, resuspending the pellet in 2 ml of sterile BHI broth (containing 10% glycerol), and storing it at -76°C.

PCR for multi locus sequence typing

For MLST target genes included five genetic loci within four virulence genes for which PCR primers were designed. These were listeriolysin O (*hly*A), a bacterial pore-forming hemolysin that is essential for lysing the vacuolar membrane and allowing *L. monocytogenes* to escape into the cytoplasm of the cell; a positive regulatory factor (*prf*A), which activates numerous virulence genes; a surface virulence protein, internalin A (*inl*A), required for the penetration of *L. monocytogenes* into non-phagocytic cells; and actin A (*act*A), another surface virulence factor that induces polymerization of actin molecules to propel *L. monocytogenes* through the cytoplasm of infected cells.

Overnight cultures of different isolates of *L. monocytogenes* were lysed by using 200µl of lysis buffer with 12.5ul of protease by baxlysis program using commercial protease and lysis solutions for bacterial PCR assays (Qualicon, Wilmington, DE). A 5 µl aliquot of the lysed culture solutions was then separately subjected to PCR amplification of the five gene targets. Thermo cycling conditions included an initial hold of 4 min at 95^{o}C, followed by 40 cycles of 95^{o}C for 15 sec (denaturation), 51^{o}C for 18 sec (annealing), 72^{o}C for 40 sec (extension), with a final extension step of 72^{o} C for 4 min was followed by a hold at 4^{o}C. For primers actA1 and *act*A2, annealing temperatures 60^{o}C was used. PCR primers used in this study are summarized in table3. Purification of the PCR product was done using a Millipore PCR purification kit (Millipore, Billerica, MA), and amplification was confirmed by visualization of the PCR product of the expected size on agarose gels (1%). DNA sequencing was performed with an ABI 3730 DNA Analyzer ((Applied Biosystems, Inc.) at the Department of Biochemistry and Molecular Biology core facility, Oklahoma State University. Both forward and reverse PCR primers were used as sequencing primers. DNA sequencing chromatograms were saved as ABI files and SEQ files for analysis. The sequences obtained for the 45 isolates of *L. monocytogenes* for the five genetic loci were then artificially joined by the neighbor-joining method of the software program, Vector NTI Suite, to form an artificial composite gene. The various composite genes were then placed into a database and compared by multiple sequence alignment and clustal analysis. The different strains were then grouped to form a phylogenic tree based on the degree of divergence between the strains.

Subtyping by Pulsed- field gel electrophoresis

PFGE was performed according to the CDC PulseNet standardized procedure for typing *L. monocytogenes* by using the CHEF-DRIII apparatus (Bio-Rad Laboratories, Hercules, Calif.). The DNA in agarose plugs were digested by incubating (at 30°C for 4h) with *Apa*I, and electrophoresis was performed in a 1% agarose gel (in 0.5X Tris-borate EDTA buffer). The agarose gel was loaded into the electrophoresis chamber containing 2000ml of 0.5X buffer. The buffer was precooled to 14°C prior to beginning gel run. The following electrophoresis conditions were used: voltage, 180V; initial switch time, 4.0s; final switch time 40s; runtime 20h. Lambda ladder (Promega markers) was loaded on the gel. *L. monocytogenes* H2446 was included as a reference which was digested with *Asc*I. After electrophoresis, the gel was stained for 30 min in 400ml of 0.5x TBE containing 10mg/ml of ethidium bromide and destained by two washes of 20-30 min each using 400 ml of deionized water and photographed with GelDoc 1000 using the Quantity one software (Bio-Rad). The image generated was saved in Tiff format, and then transferred to the Bionumerics software version 4.5 (Applied Maths, Sint-Martens-Latem, Belgium) for DNA banding patterns analysis with optimization set at 0.5% and position tolerance set at 1%. The Dice coefficient of similarity was calculated, and the unweighted pair group method with arithmetic averages (UPGMA) was used for cluster analysis. A cut-off at 80% similarity of the Dice coefficient was used to indicate identical PFGE types. This corresponds to approximately one band difference, and this degree of similarity also allows for minor technical errors that frequently occur (Salamon et al., 1998) .

Ribotying

The strong and weak adherent strains were ribotyped using the restriction enzyme *Eco*RI and the RiboPrinter Microbial Characterization System (Du Pont's Qualicon Inc., Wilmington, DE) as described by (Bruce, 1996). The generated ribotypes were imported into Bionumerics (Applied Maths, St. Martens-Latem, Belgium), and a dendrogram was generated based on Unweighted Pair Group Method with Arithmetic mean (UPGMA) and Pearson correlation coefficients.

Discrimination index

The discrimination power of ribotyping method was determined by calculating the discrimination index (DI) using the formula of (Hunter and Gaston, 1988).

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j(n_j - 1)$$

Where, **N** is the total number of strains in the sample population,

s is the total number of types described, and

nj is the number of strains belonging to the jth type.

RESULTS AND DISCUSSION

In order to understand the adherent phenotypes of *L. monocytogenes* we subtyped 45 isolates obtained from raw meat, RTE meat and meat processing facilities using MLST approach by sequencing a trimmed 500bp fragment of the *L. monocytogenes* virulence genes *hly*A, *inl*A, *Prf*A and *act*A. Subtyping using MLST analyzes several genetic loci simultaneous and differentiate based on their subtle genetic heterogeneity (Enright and Spratt, 1999).The phylogenetic tree for MLST analysis on the basis of nucleotide differences in the four gene fragments (Fig.1.) showed two major clusters. Cluster I contained all weakly adherent strains as well as two strong adherent strains (CW 50 and 99-38) while cluster II consisted of all the strains including two strong adherent strains. The dendrogram indicates that strains CW34, CW59, CW73, SM1, SM2, and SM3 were genetically related as they do not show much genetic diversity among the same genetic loci. This 'CW' strains were isolated from retail franks whereas the 'SM' strains were isolated from raw ground meat products. All JAG strains are clustered in one group which was isolated from a meat processing plants. *L. monocytogenes* strains isolated during year 1998 and 1999 from ground beef in a meat packaging plant also shows very less divergence. Cai et al. (2002) showed that inclusion of *act*A virulence gene allowed discrimination of 15 *L.monocytogenes* isolates used into 13 sequence types.

Dendrogram based on PFGE of the four weak and four strongly adherent strains digested with *Apa*I (Fig.2) formed two main clusters with a second cluster consisting of

weakly adherent strains. The strong adherent strains CW50 and 99-38 belonged to one cluster. The PFGE dendrogram was constructed by the UPGMA on the basis of the banding patterns of *Apa*I-digested genomic DNA fragments. Although PFGE has been the 'gold standard' of epidemiological DNA fingerprint analysis, the difficulty in exact band matching between different gels lends to placement of same gel patterns in different groupings. *L.monocytogenes* isolates that had shown higher and low adherence were characterized by automated *Eco*RI ribotyping using the RiboPrinter microbial characterization system (DuPont Qualicon, Wilmington De) as described previously (Bruce et al., 1996). Ribotype patterns were analyzed using the riboprinter software, which normalize fragment pattern data for band intensity and relative band position compared to those of the molecular weight marker. Sixteen strains including weak and strongly adherent ones isolated from RTE meat as well as raw ground beef were divided into two main groups. *Eco*RI ribotyping differentiated the isolates into 11 distinct ribotypes (Fig.3) and the discrimination index was 0.950. Dendrogram patterns generated from ribotype data produced three clusters. The threshold regarding the measure of similarity was fixed at 0.86%. Cluster I consisted of 8 isolates: six from RTE meat and two from raw ground beef. Cluster II had one isolate from raw ground beef and cluster III had five for raw ground beef and two from RTE meat. The results of the cluster analysis showed that there is co-typing of strains isolated from raw ground beef and RTE. The pattern of ribotype-8 was clearly different from the other *L. monocytogenes* patterns.

Our results showed that use of combined multi-method genotyping approach for subtyping of *L. monocytogenes* strains grouped 99-38 (raw beef) and CW 50 (RTE meat) in one group though these strains were isolated from different sources by MLST, PFGE

and ribotyping. Typing of additional strains by both PFGE and MLST demonstrated several instances whereby strains from raw sources co-typed with strains from RTE sources, suggesting that outbreak strains that are only compared to RTE-derived isolates (i.e., USDA-FSIS regulatory samplings) may not identify raw sources for *L. monocytogenes* should they occur because their database contains only RTE-sourced isolates.*Eco*RI ribotyping targets conserved genetic characteristics and indicates similar banding pattern for the both the strains whereas PFGE doesn't show similar fingerprint pattern but assign both strains in the same group because PFGE may not be able to detect less than or equal to 3 bands. All "CW" strains were grouped in one cluster by all three typing methods indicating less genetic diversity. This is overcome by sequence-based typing for which sequence analysis and comparison is much more 'user-friendly' and is only limited by the quality of the sequence information. It is likely that sequence-based typing will replace DNA fragment/band based typing in the future. The observed inclusion of virulence gene target sequences in a DNA sequence-based subtyping scheme for *L. monocytogenes* helped us to achieve maximum subtype differentiation. MLST can effectively distinguish strains with high degrees of homology within the compared gene sequences. MLST detects all genetic variations within the amplified gene fragment whereas PFGE only examines the variations that are in the cleavage sites for a particular restriction enzyme. Since this technique is user friendly and not laborious like PFGE, or expensive like ribotyping, it provides an ideal balance between sequence-based resolution and technical feasibility.

CONCLUSION

These DNA-based methods define bacterial subtypes by using either PCR amplification or sequence analysis or restriction digestion of bacterial DNA to generate DNA fragment banding patterns. This study demonstrated several instances whereby strains from raw sources co-typed with strains from RTE sources, suggesting that outbreak strains that are only compared to RTE-derived isolates (i.e., USDA-FSIS regulatory samplings) may not identify raw sources for *L. monocytogenes* should they occur because their database contains only RTE-sourced isolates. Use of these methodologies will help in making decision concerning which method has the superior discriminatory ability. Typing pathogenic bacteria from meat and RTE meat sources involved in food processing may help establish strains that are persistent and may have harborage sites within the processing facility.

Table 1. Virulence genes and PCR primers used in this study

Primer	Target Gene	Primer Sequence (5'→ 3')	Product size (bp)
Primer I	Hemolysin (*hly*A)		560
	Forward	TGAACCTACAAGACCTTCCA	
	Reverse	CAATTTCGTTACCTTCAGGA	
Primer II	Internalin A (*inl*A)		575
	Forward	GCTTCAGGCGGATAGATTAG	
	Reverse	AACTCGCCAATGTGCC	
Primer III	Positive regulatory factor (*prf*A)		590
	Forward	ATTTTTAACCAATGGGATCC	
	Reverse	CATTCATCTAATTTAGGGGC	
Primer IV	Actin mobility (*act*A1)		500
	Forward	AATACGAACAAAGCAGACCTAATAG	
	Reverse	GGTCAATTAACCCTGCACTTTTA	
Primer V	Actin mobility (*act*A2)		500
	Forward	GATAGAGGAACAGGAAAACACTCA	
	Reverse	CGTCTTCTGCACTTTTAGCAATT	

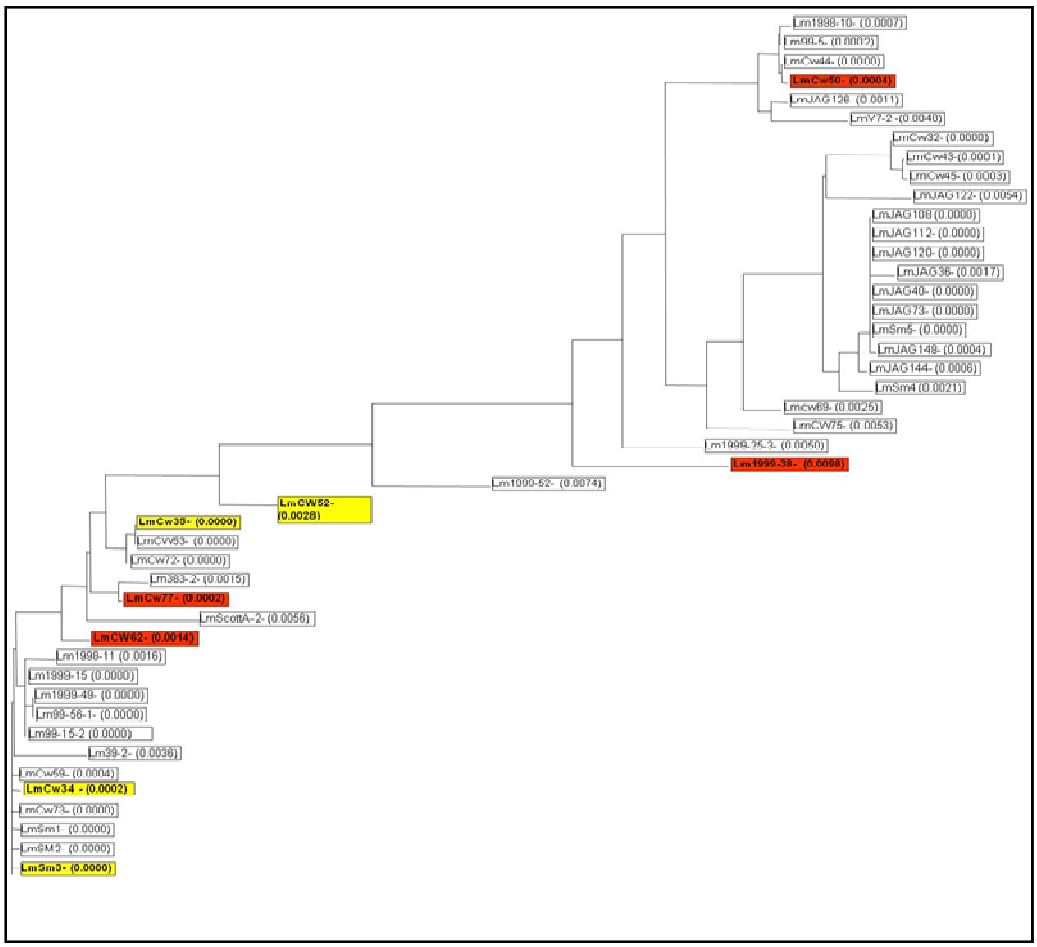

Figure.1.Multilocus sequence typing (MLST) of composite sequences of various strains of *L. monocytogenes.* (Strong adherent strains are highlighted in red whereas weakly adherent strains are highlighted in yellow).

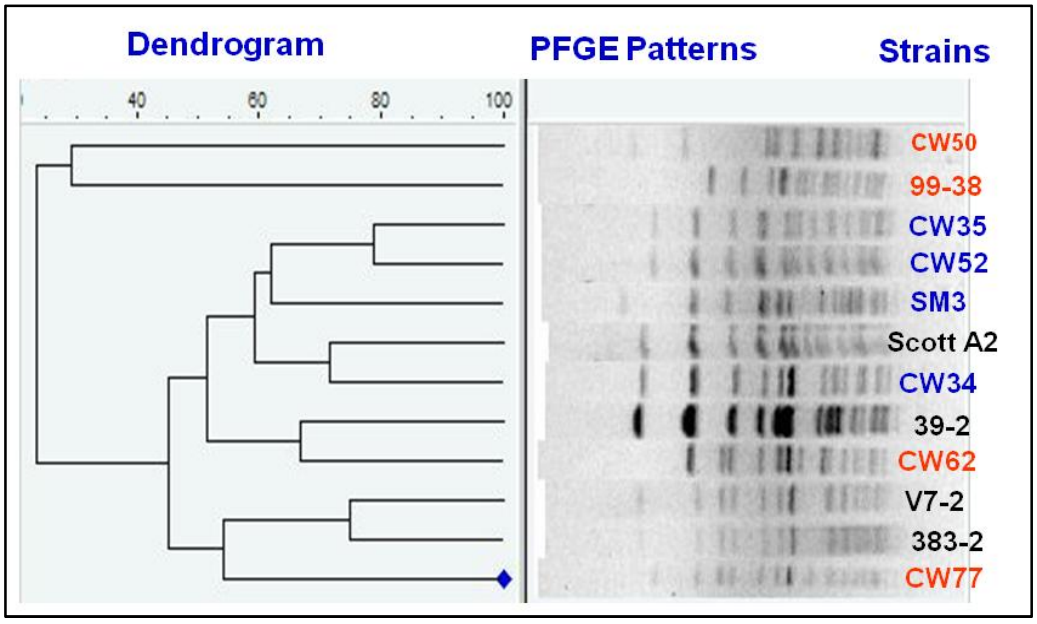

Figure 2. PFGE –based dendrogram for strong and weakly adherent strains of *L. monocytogenes* strains Similarity analysis was performed using the Dice coefficient and clustering was performed by UPGMA (position tolerance 1%).

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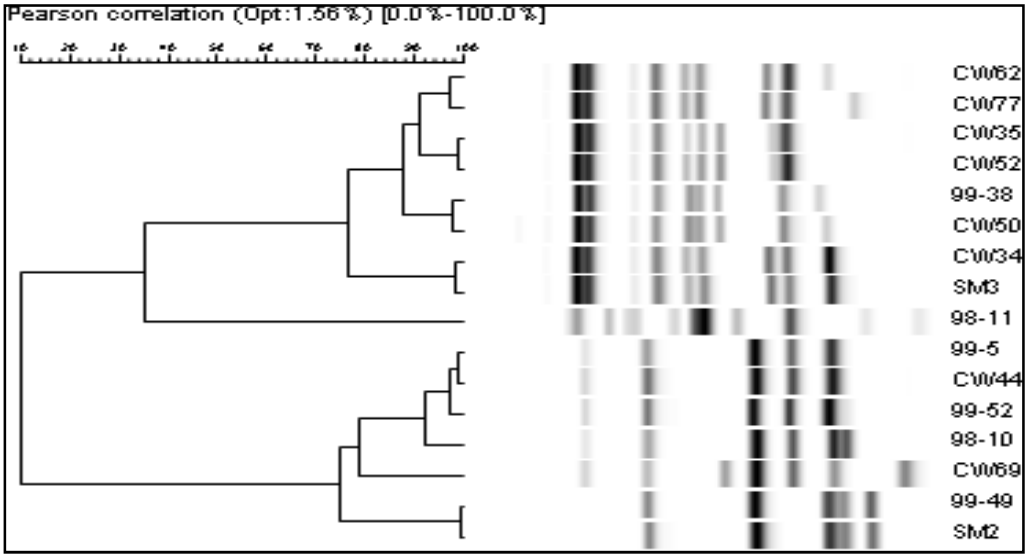

Figure 3. Riboprint patterns obtained with enzymes *Eco*RI restriction enzyme for 16 *L .monocytogenes* isolates

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VITA

KALPANA KALPANA-KUSHWAHA

Candidate for the Degree of Doctor of Philosophy

Dissertation: CHARACTERIZATION OF VIRULENCE IN ADHERENT PHENOTYPES OF *LISTERIA MONOCYTOGENES*

Major Field: Food Science

Biographical:

Education:

Bachelor of Science Degree in Agricultural from University of Agricultural Sciences, Bangalore, India in March of 1995.

Masters of Science in Agricultural Microbiology from University of agricultural Sciences, Bangalore, India in July 1997.

Completed the requirements for the Doctorate of Philosophy in Food Science/Food Microbiology at Oklahoma State University in December of 2009.

Experience:
Employed by Oklahoma State University: Robert M. Kerr Food and Agricultural Products Research Center (FAPC) as a graduate research assistant

Professional Memberships:
Institute of Food Technologist (IFT)
International Association of Food Protection (IAFP)

Name: Kalpana Kalpana-Kushwaha Date of Degree: December, 2009

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: CHARACTERIZATION OF VIRULENCE IN ADHERENT PHENOTYPES OF *LISTERIA MONOCYTOGENES*

Pages in Study: 145 Candidate for the Doctor of Philosophy

Major Field: Food Science

Scope and Method of Study:

Listeria monocytogenes is a food borne pathogen and a recurring problem in ready-to-eat (RTE) meat, raw meat and meat processing facilities. We examined 246 strains of *Listeria* isolated for over 1 year from 3 RTE meat processing facilities for their adherence phenotype using microplate adherence assay, used PCR to identity those that were *L. monocytogenes* and further typed using pulsed-field gel electrophoresis. The adherence and virulence capacity of the select strains isolated from raw and RTE meat was tested *in vitro* using human cell line Caco-2 and *in vivo* by oral inoculation into A/J mice. Further, spleen and liver tissue samples were subjected to necropsy to confirm the virulence. Subtyping was done using MLST, PFGE and RT methods to see if molecular typing methods could segregate the adherent phenotypes into different groups.

Findings and Conclusions:

Strains of *Listeria* isolated from three RTE meat processing plants were distinguished as strong and weakly adherent based on assay. The data showed that Plant C had almost 4x the prevalence of *Listeria* isolates when compared to Plants A or B indicating the significance of adherence. The use of PFGE fingerprint analysis was informative in suggesting that similar strains were isolated repeatedly within the same facility on the same day or on different dates as recurring isolates. Cell culture virulence assay carried out for four strong and four weakly adherent strains isolated from raw and RTE meat showed higher invasion for strains capable of strong adherence to abiotic surfaces with low incubation time and low multiplicity of infection. *In vivo* study showed bacterial recovery in spleen and liver by strongly adherent strains whereas weakly adherent strains were completely eliminated from liver. This was further confirmed by significant histological lesions in the liver samples caused by strongly adherent strains. Adherence properties of *L. monocytogenes* may allow persistence and recurrence in plant environments, potentially increasing the chance of eventual product contamination. The presence of generic *Listeria* spp. is significant as it represents a failure of sanitation hurdles to eliminate these organisms from the processing environment, and even more so if they are *L. monocytogenes,* as they are human pathogens. Therefore, elimination of strongly adherent strains of *L. monocytogenes* from food processing environment deserves special attention as their strong adherence not only promotes retention but may show enhanced invasion and replication in host tissues causing greater virulence.

Advisors' Approval: _____