ADSA Foundation Scholar Award— An Integrated Science-Based Approach to Dairy Food Safety: *Listeria monocytogenes* as a Model System

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ABSTRACT

Transmission of food- and milkborne pathogens often involves complex interactions among the pathogen, the environment, and one or multiple host species. A complete understanding of these interactions is critical to allow the development of science-based, effective intervention strategies for foodborne infectious diseases. This article summarizes our studies on the transmission, ecology, pathogenesis and population genetics of *Listeria monocytogenes*, which we have used as model for a food- and milkborne pathogen that infects multiple hosts and also has considerable ability to survive and multiply in nonhost environments. Application of molecular subtyping tools in conjunction with phenotypic characterization of selected strains has allowed us to define distinct *L. monocytogenes* subtypes and clonal groups that appear to differ in relevant phenotypic characteristics that may affect their abilities to be transmitted through food systems. For example, a genetic group designated as lineage I has been shown to be not only more common among human listeriosis cases than among animal cases, but lineage I strains also appear to show an increased in vitro ability to spread intracellularly from host cell to host cell. These findings are consistent with the fact that while genetically diverse strains may be classified to one bacterial species, these strains often differ from one another in important genetic and phenotypic characteristics. I thus propose that evolutionary- and molecular subtyping-based definitions of bacterial subtypes and clonal groups will provide critical insight into the microbial ecology of dairy food systems, including not only foodborne pathogens, but also organisms important for dairy fermentation and spoilage.

(Key words: dairy food safety, *Listeria monocytogenes*, ecology, population genetics)

Abbreviation key: CDC = Centers for Disease Control and Prevention, MEE = multilocus enzyme electrophoresis.

INTRODUCTION

A recent estimate by researchers at the US Centers for Disease Control and Prevention (CDC) projects that 76 million cases of gastrointestinal foodborne illnesses caused by viral, bacterial, protozoal, and parasitic pathogens occur annually in the United States. These illnesses are estimated to lead to approximately 325,000 hospitalizations and 5000 deaths per year in the United States (Mead et al., 1999). Worldwide, foodborne pathogens also play an important role as a cause of human infectious diseases (WHO, 1999). Thus, foodborne illnesses and their related medical sequelae have a significant negative impact on human health and well being. While estimates are available on the overall incidence of foodborne disease in the United States, estimates on the frequency of foodborne infections linked to consumption of dairy products are not easily available, particularly because a large proportion of foodborne diseases and infections is not tracked back to a specific food source. The incidence of milkborne illnesses in the United States has decreased considerably over the last six to seven decades, however. Whereas it is reported that 25% of food- and waterborne disease outbreaks were milkborne in 1938, the 1999 revision of the Pasteurized Milk Ordinance (Public Health Service/Food and Drug Administration, 1999) states that less than 1% of total reported foodborne disease outbreaks are currently linked to milk and milk products. Nevertheless, even in the last two decades, large foodborne disease outbreaks have been linked to dairy products, including outbreaks due to *Listeria monocytogenes* contaminated Hispanic-style cheeses and due to *Salmonella* contaminated fluid milk (Boor, 1997, 2001; Anonymous, 2001).

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larly, changes in food consumption patterns, as well as in the human population (such as an increase in the number of elderly and immunocompromised people) can alter patterns of foodborne infections. Separately, or together, changing factors that affect our food system may furthermore contribute to the emergence or re-emergence of newly recognized foodborne pathogens (Tauxe, 1997). Improved diagnostic methods also may lead to the discovery of pathogens that had been previously unrecognized as causes of foodborne infections. Continuous efforts to better understand and control transmission of foodborne pathogens in the dairy food system are thus key to assure safe dairy foods for the future.

Infections with foodborne pathogens are a consequence of the interactions of a given pathogen, the environment, and the host. While some foodborne pathogens may be predominantly associated with a single host (the human), a considerable number of foodborne pathogens have the ability to cause disease in a variety of hosts, often in farm animals as well as in humans. Highly host-adapted foodborne and waterborne pathogens (e.g., *Vibrio cholerae*, *Salmonella* Typhi) are generally more easily controlled than pathogens that have the ability to infect multiple hosts and/or have the ability to effectively survive and multiply outside a human host. Our capabilities for controlling multihost food- and milkborne pathogens are dependent on the thoroughness of our understanding of their ecology and pathogenesis in different hosts and nonhost environments. Only this basic knowledge will allow the development of science-based, effective, and economical food safety measures from farm to table. As reviewed below, my laboratory has chosen the foodborne pathogen *L. monocytogenes* as a model system to probe the pathogenesis and transmission of foodborne pathogens. We hope that our studies, together with ongoing work in many other laboratories, will ultimately provide a framework for the development and implementation of science-based food safety assurance strategies, not only for *L. monocytogenes*, but also for other pathogens.

LISTERIA MONOCYTOGENES AND LISTERIOSIS

Listeria monocytogenes is a facultative intracellular gram-positive bacterium associated with potentially serious invasive diseases in humans and in more than 40 animal species (Seeliger, 1961). Infection in these hosts can result in abortion, septicemia, meningitis, and encephalitis as well as other less severe manifestations, including diarrhea and skin infections. Among humans, immunocompromised persons, pregnant women, the elderly, and neonates are particularly at risk of listerial infections. The frequency of clinical human listeriosis

in most developed countries is estimated to range between 2 to 15 cases/million population with case mortality rates between 13 and 34% (Farber and Peterkin, 1991). The vast majority (99%) of human *Listeria* infections is thought to be foodborne (Mead et al., 1999). Thus, although foodborne listeriosis cases are less common than many other foodborne diseases (e.g., those caused by *E. coli* O157:H7, *Campylobacter jejuni*, or *Salmonella*), they represent the second most common known cause of fatal human foodborne infections, next only to *Salmonella* infections.

The Genus *Listeria*

In addition to *L. monocytogenes* the genus *Listeria* also includes *Listeria ivanovii*, *Listeria seeligeri*, *Listeria innocua* and *Listeria welshimeri*. All members of this genus are gram-positive nonspore-forming rods, closely related to the genera *Lactobacillus* and *Streptococcus*. The G + C % DNA content of *L. monocytogenes* is about 40%. Whereas *L. monocytogenes* causes both human and animal disease, *L. ivanovii* is predominantly associated with disease (specifically abortions) in sheep, and human clinical infections with this organism appear to be extremely rare (Cummins et al., 1994; Wesley, 1999). The other *Listeria* species are considered nonpathogenic. The pathogenic species *L. monocytogenes* and *L. ivanovii* are both hemolytic, as is the nonpathogenic *L. seeligeri*, while *L. innocua* and *L. welshimeri* are nonhemolytic. *Listeria monocytogenes* can be separated into 13 different serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7), all of which appear to have been associated with animal disease, although some of them are less commonly isolated from affected animals than others (Wiedmann and Evans, 2002). Generally serotypes 1/2a, 1/2b, and 4b appear to be most common among animal isolates, although serotype 4c is also occasionally found. There are also some indications that the frequency of different serotypes and molecular subtypes among human and animal clinical cases differs. Thus, certain subtypes may show at least some level of host specificity for humans and animals (Wiedmann et al., 1997).

Physiology and Ecology of *Listeria*

All *Listeria* spp. are catalase positive, oxidase negative, and esculin hydrolysis positive. The members of the genus *Listeria* are aerobic, but also grow under anaerobic conditions and can thus be classified as facultative anaerobes. *Listeria monocytogenes* has the ability to grow and survive under a variety of different conditions. For example, *L. monocytogenes* grows from close to 0 to 44°C and is thus considered psychrotolerant.

All *Listeria* spp. are heat sensitive and pasteurization effectively kills listeria. When cultured at 20 to 25°C, *Listeria* is motile due to peritrichous flagella. In media, *Listeria* grows in a pH range from 4.4 to 9.6, with optimal growth at neutral pH. *L. monocytogenes* has a high level of osmotolerance, it can grow in media containing up to 10% (wt/vol) NaCl, and it can survive at even higher salt concentrations. Perhaps due to its considerable resistance to a variety of stress conditions, *L. monocytogenes* appears to be able to multiply and/or survive for extended time periods (up to more than 2 yr) outside mammalian hosts. Consequently it has been isolated from a variety of different environments (e.g., soil, surface water, sewage, vegetative materials, different food processing environments) (Gravani, 1999), and many authors thus consider this organism "ubiquitous."

Because *L. monocytogenes* represents a foodborne pathogen, a considerable numbers of studies and surveys on the presence of this organism in many raw and ready-to-eat foods have been published. Whereas the prevalence of *L. monocytogenes* varies tremendously by study, type of food surveyed, and other factors, this organism has been isolated from many different foods, occasionally at fairly high prevalences (>5 to 10%) (Farber and Peterkin, 1991; Farber and Peterkin, 1999). A summary of raw milk surveys for the presence of *L. monocytogenes* conducted in many different countries indicates that overall <5% of raw milk samples are positive for *L. monocytogenes* (Ryser, 1999b). Most recent surveys of ready to-eat foods in developed countries show *L. monocytogenes* prevalences considerably below 5%, however, including for dairy foods (Ryser, 1999a, 1999b). The ability of *L. monocytogenes* to survive and proliferate well in foods stored at refrigeration temperatures makes this organism a particular concern for the dairy food industry, since low initial contamination levels (possibly even at <1 cfu/25 g) may increase to numbers that could present a human health hazard if products are subject to extended refrigerated storage. Consequently, humans appear to be commonly exposed to *L. monocytogenes* by food ingestion. Based on the 2001 FDA/USDA Draft *Listeria monocytogenes* risk assessment (Food and Drug Administration and US Department of Agriculture, 2001), the average US consumer is likely to occasionally (possibly once a year) consume as many as 10^6 to 10^9 cfu of *L. monocytogenes* in a single serving. In spite of this apparent occasional high exposure, only 2500 human listeriosis cases occur annually in the United States. These data indicate that even exposure to high levels of this organism may not result in human disease, even when one considers that the at-risk population for human listeriosis (immunocompromised people, pregnant women, etc.) represents only a fraction of the total US population.

Cellular and Molecular Pathogenesis

The cellular processes of *L. monocytogenes* infection have been well characterized, and genetic systems for gene replacement and for monitoring gene expression have been developed. In tissue culture models, the following stages of infection have been identified: 1) internalization of *L. monocytogenes* within the host cell; 2) bacterial escape from the host vacuole; 3) multiplication of the parasite within the host cell cytoplasm and its movement through the host cytoplasm by virtue of bacterially directed nucleation of host actin filaments; 4) bacterial movement to the host cell surface and extrusion of bacterial cells in pseudopod-like structures; 5) phagocytosis of these pseudopod-like structures by neighboring cells, followed by escape of the bacterium from the resulting double-membrane vacuole, thus allowing the cycle to repeat (Portnoy et al., 1992).

Gene products essential for each step of the infection process have been identified. Six *L. monocytogenes* virulence genes (*prfA*, *plcA*, *hlyA*, *mpl*, *actA*, and *plcB*) are located together in one virulence gene cluster. This 8.8-kbp gene cluster is located at a defined position in all *L. monocytogenes* strains characterized to date, namely between the housekeeping gene *ldh* and *prs*. Additional *L. monocytogenes* virulence-associated genes (e.g., *iap*, *inlA*, *inlB*, *inlD*, *inlE*, *inlF*) are not physically linked to this virulence island (Drams et al., 1997; Portnoy et al., 1992). The specific functions for these genes in the *Listeria* infection process have been well defined. For example, internalin A (InlA), encoded by *inlA*, interacts with E-cadherin to allow *L. monocytogenes* to induce its own uptake by specific host cells (e.g., epithelial cells) thus facilitating step 1) of the infection process described above (Gaillard et al., 1991; Mengaud et al., 1996a; Mengaud et al., 1996b). ActA, the *actA* gene product, is a surface protein required for intracellular movement and cell-to-cell spread through bacterially induced actin polymerization (steps 3 and 4 of the infection process) (Brundage et al., 1993).

Listeria ivanovii and *L. seeligeri* contain virulence genes that are highly similar to those found in *L. monocytogenes*. As in *L. monocytogenes*, the virulence gene cluster in these species is also located between the two housekeeping genes *ldh* and *prs*. Whereas the organization of the *L. ivanovii* virulence gene cluster is very similar to that in *L. monocytogenes*, the *L. seeligeri* virulence gene cluster contains additional and differently organized genes (Kreft et al., 1999). Despite the presence of *L. monocytogenes* virulence gene homologues in *L. seeligeri*, this species is considered nonpathogenic and has been shown to be avirulent in the murine animal model. In tissue culture studies, *L. seeligeri* does not trigger its own uptake in nonphagocytic

mammalian host cells and it is unable to escape from the vacuole when internalized by phagocytic cells (Kreft et al., 1999). *Listeria innocua* and *L. welshimeri* completely lack the virulence gene homologues found in *L. monocytogenes*, *L. ivanovii*, and *L. seeligeri*.

PATHOGENESIS AND ECOLOGY OF FOODBORNE AND ZONOTIC INFECTIOUS DISEASES

The pathogenesis of foodborne infectious diseases can involve complex interactions between a bacterial pathogen, a variety of environments and one or multiple host species. The ability of bacterial cells to survive and compete in a variety of environments plays a key role in the pathogenesis and transmission of many foodborne diseases. In addition, selective pressures not associated with mammalian hosts may contribute significantly to the emergence and evolution of virulence characteristics related to the ability of bacteria to effectively infect mammalian hosts. Foodborne pathogens infecting both humans and animals (such as *L. monocytogenes*) provide ideal model systems for studying the ecology of infectious diseases, including adaptation of clonal groups to specific hosts and nonhost environments as well as virulence gene expression and maintenance of virulence characteristics under widely varying conditions, including those not directly associated with a host.

In a simplified model, transmission patterns of *L. monocytogenes* as well as of other foodborne pathogens may encompass all or some of the following steps and environments: 1) bacterial survival in the environment and in animal feeds (e.g., *L. monocytogenes* and pathogenic *E. coli* are common environmental contaminants); 2) bacterial survival inside nonvertebrate hosts (e.g., protozoans); 3) establishment of clinical or subclinical infections or carrier states in food animals; 4) shedding of the organism into animal products used for human consumption or secondary contamination of animal products; 5) bacterial survival and/or multiplication in non-host environments under food processing and distribution conditions; and 6) infection of human hosts, including survival of gastric passage and establishment of enteric or systemic infections (see Figure 1).

An improved understanding of the ability of bacterial pathogens, and specifically of different clonal groups of a given pathogen, to adapt to different environments and hosts and to maintain and evolve virulence characteristics in different host- and nonhost-related environments will contribute broadly to our understanding of the ecology and transmission dynamics of bacterial pathogens. For example, it is well established that different *Salmonella* serotypes (e.g., *Salmonella* Typhi and *Salmonella* Typhimurium) differ in the type and

Hypothesized *Listeria* Transmission Pathway

Figure 1. Transmission pathway model for *Listeria monocytogenes*.

severity of disease they cause in humans, even though they belong to the same bacterial species (*Salmonella enterica*) (Kingsley and Bumler, 2000). Similarly, it has been recognized that distinct subgroups of the species *E. coli* differ in their pathogenic potential. For example, Shiga-toxin producing *E. coli* (STEC), which represent a subgroup of all *E. coli*, cause a unique and severe foodborne infection (Acheson, 2000). Although *L. monocytogenes* can be isolated from a diversity of environmental sources and human and animal hosts, I thus hypothesize that clonal groups of this organism may have adapted to different hosts and nonhost environments.

POPULATIONS GENETICS, TRANSMISSION, AND ECOLOGY OF *LISTERIA MONOCYTOGENES*

Current regulations specifying a zero-tolerance for the presence of any *L. monocytogenes* subtypes in ready-

to-eat foods are based on historical taxonomic classification schemes. These classical taxonomic definitions of bacterial species do not necessarily correlate with the ability of a group of bacteria to cause human disease. Rather, as outlined above, related bacteria that differ in their abilities to cause human and/or animal disease may be grouped together into the same species. Thus, a critical need exists for the development of better scientific definitions of bacterial groups that have the ability to cause human disease. Molecular subtyping methods provide a unique opportunity to explore the population genetics and evolution of *L. monocytogenes* (Wiedmann, 2002a). Subtyping methods not only have the potential to differentiate bacterial strains, but also facilitate the definition of subtypes and clonal groups that differ in their phenotypic characteristics and/or their abilities to cause human foodborne disease (Wiedmann, 2002b).

Whereas the majority of human clinical infections occur as sporadic cases, human listeriosis can also occur in large epidemics. Most sporadic human listeriosis cases and large human foodborne listeriosis epidemics have reportedly been caused by *L. monocytogenes* serotype 4b (Farber and Peterkin, 1991; Wiedmann, 2002a). The 4b strains isolated from most epidemic outbreaks form two closely related homogenous groups (so called "epidemic clones") (Piffaretti et al., 1989; Wiedmann et al., 1997). Serotypes 1/2a and 1/2b are also responsible for significant numbers of sporadic cases of human illness, and a serotype 1/2a strain was responsible for a recent multistate human listeriosis outbreak in the US (Anonymous, 2000). Serotyping data collected by the CDC in 1986 showed that serotypes 1/2a (30%), 1/2b (32%), and 4b (34%) represented the majority of isolates from 144 human sporadic cases (Schwartz et al., 1989). Of 1363 human isolates collected in the UK, 15% were 1/2a, 10% were 1/2b, and 64% were 4b (McLauchlin, 1990). The remaining 10 currently recognized *L. monocytogenes* serotypes have been only rarely linked to human disease. This apparent association between a few specific *L. monocytogenes* strains and most cases of human listeriosis raises the intriguing challenge of identifying unique characteristics enabling these strains to be more effective than others in causing human disease. My research group has thus applied molecular subtyping methods to characterize *L. monocytogenes* isolates from different sources with the goal of defining *L. monocytogenes* subtypes and clonal groups and their associations with different hosts and nonhost environments. Phenotypic and phylogenetic analyses of selected *L. monocytogenes* strains and isolates are used to probe the functional and evolutionary basis of differences in host distribution and ecology of different clonal groups.

Population Genetics and Evolution of Human, Food, and Animal *Listeria monocytogenes* Isolates

Initial studies by my research group and collaborators focused on probing the population genetics of *L. monocytogenes* using isolates from human and animals with clinical listeriosis as well as from contaminated food products. For more than 10 yr, our research group has developed a variety of collaborations to collect human clinical and animal *L. monocytogenes* isolates with the goal of establishing a phylogenetic framework for probing relationships among *L. monocytogenes* strains. We have developed a network for the collection of human and animal *L. monocytogenes* isolates that includes the health departments in different states (including New York State, New York City, Michigan, and Ohio) and the New York State Veterinary Diagnostic Laboratory at Cornell. Our collection already includes more than 700 human and more than 200 animal isolates and we currently obtain approximately 150 human and 20 to 30 additional animal *L. monocytogenes* isolates/year. Since 1997 we have also collected more than 700 isolates from selected food products and from environmental samples. This strain collection provides a unique resource for our research efforts to understand the population genetics and evolution of *L. monocytogenes*.

Molecular subtyping. Most *L. monocytogenes* isolates in our strain collection have been characterized by the multiple genetic methods including automated ribotyping using rRNA probes and *EcoRI* digested chromosomal DNA and allelic analysis of the virulence genes *hly*, *actA*, and *inlA* using PCR-RFLP methods (Wiedmann et al., 1997). These genes encode a hemolysin involved in host cell vacuole lysis, ActA, and internalin A, respectively. Ribotyping is a system for generating DNA fingerprints in which the rRNA operons and surrounding regions are analyzed for polymorphic restriction sites by Southern hybridization. Fingerprints from various organisms differ by molecular weight patterns and relative band intensities. Isolates in our collection have specifically been characterized by a highly standardized and fully automated ribotyping system (RiboPrinter, Qualicon Inc., Wilmington, DE) that includes computerized data analysis (Bruce, 1996; Jeffers et al., 2001). Ribotype analysis specifically identified more than 100 *L. monocytogenes* *EcoRI* ribotypes that group into 11 ribotype subsets.

Current efforts are focusing on the development of a WWW-based molecular subtype and strain database to allow rapid exchange of strain characteristics and subtype information. A prototype database is already available at www.pathogen tracker.net. We are also developing and implementing DNA sequencing-based

subtyping methods for *L. monocytogenes* (Cai et al., 2002) to allow more definitive subtype differentiation. Multilocus DNA sequencing data for *L. monocytogenes* isolates will also provide us with the ability to further and more specifically probe the evolution of *Listeria* spp. and *L. monocytogenes*.

***L. monocytogenes* phylogenetic framework.** Initial analysis of the molecular subtyping data described above allowed us to separate *L. monocytogenes* strains into three distinct genetic lineages, designated I, II, and III (Wiedmann et al., 1997). Some or all of these lineages may represent different *L. monocytogenes* subspecies. Each lineage can be separated into three to four ribotype subsets. Ribotype subsets represent groups of similar ribotypes that share a common ribotype fragment (Bruce et al., 1995). Within each ribotype subset, we found between 5 and 20 distinct genetic subtypes as defined by *Eco*RI ribotypes and virulence gene PCR-RFLP data. Specific virulence gene alleles are generally unique to a specific lineage (Wiedmann et al., 1997). Subtype characterization by a variety of additional molecular subtyping methods performed by other groups (including multilocus enzyme electrophoresis, pulse-field gel electrophoresis; amplified fragment length polymorphism, and DNA sequencing of selected *L. monocytogenes* genes) also confirmed lineages I and II as the two primary divisions of *L. monocytogenes* (Pifaretti et al., 1989; Rasmussen et al., 1991; Mazurier et al., 1992; Brosch et al., 1994; Wiedmann, 2002a). Relatively few lineage III strains have been characterized to date as strains classified into this group represent a rare subgroup of *L. monocytogenes* that appears to be primarily associated with animal hosts (Wiedmann et al., 1997). In addition to our data (Wiedmann et al., 1997), Rasmussen et al. (1995) also reported that DNA sequencing data for selected virulence genes provided evidence for the existence of a third *L. monocytogenes* lineage.

Phylogenetic analyses of partial DNA sequences of the *actA* virulence gene from 22 isolates also cluster the strains into three main groups that are identical to the three lineages defined by ribotyping and virulence gene PCR-RFLP analyses. Serotype characterization of *L. monocytogenes* strains was also consistent with our division into three lineages. Genetic lineage showed a direct correlation with serovar clusters: lineage I contained serotypes 1/2b, 3b, and 4b; lineage II contained serotypes 1/2a, 1/2c, 3a, and 3c; lineage III contained serotypes 4a and 4c (Nadon et al., 2001). Ongoing work in my laboratory now uses sequencing of multiple *L. monocytogenes* genes to further probe the phylogeny of this species. I anticipate that results from these experiments will allow us to further refine our understanding of the population structure of *L. monocy-*

togenes and particularly of the strains grouped into lineage III.

In summary, through application of molecular tools, we have been able to define distinct *L. monocytogenes* lineages and clonal groups, and also have found strong evidence suggesting a linkage disequilibrium among the genetic and phenotypic characteristics examined (Wiedmann et al., 1997). We did not find any indications for horizontal gene transfer among the genetic loci investigated. Our preliminary data thus establish a phylogenetic framework for further investigation of virulence differences and habitat specialization among *L. monocytogenes* clonal groups.

Subtype characterization of human outbreak strains. In additional studies, we used ribotype analysis to further probe the phylogenetic characterization of human epidemic *L. monocytogenes* strains (Jeffers et al., 2001). The majority of human listeriosis cases appear to be sporadic in nature and, therefore, are associated with a variety of different strains. Relatively few large epidemic listeriosis outbreaks have been reported. *Listeria monocytogenes* strains associated with these epidemic outbreaks are of particular interest as they may represent a clonal group or groups with a unique transmission potential. It is known for other bacterial pathogens that specific clonal "epidemic" groups are responsible for the majority of human cases of specific infectious diseases. Confirming previous reports, we found that two *L. monocytogenes* ribotypes were associated with the majority of human epidemic outbreaks reported before 1998. Specifically, ribotypes DUP-1038 and DUP-1042 were associated with at least six and four human epidemics, respectively (Jeffers et al., 2001). Both of these clonal groups represent serotype 4b strains and are grouped into lineage I. Recent listeriosis outbreaks in the United States (since 1998) appear to be caused by different strains. For example, the strain responsible for a multistate listeriosis outbreak in the United States in the fall of 1998 represents another lineage I ribotype (DUP-1044A) (Anonymous, 1999), and the strain associated with a multistate listeriosis outbreak in the United States in 2000 (Anonymous, 2000) is grouped into our lineage II.

Association of *L. monocytogenes* Subtypes and Clonal Groups with Different Hosts

We have used the phylogenetic framework described above to probe for possible host specificities and virulence differences among *L. monocytogenes* clonal groups using human and animal clinical isolates. Specifically, we assessed whether different clonal groups as defined by lineage, ribotype subset, and ribotype differed in their likelihood to cause human sporadic or epidemic

listeriosis cases or animal listeriosis. An initial evaluation of linkages between independent phylogenetic and virulence markers in 195 human and animal *L. monocytogenes* isolates was published in 2001 (Jeffers et al., 2001). Interestingly, the clinical histories of the *L. monocytogenes* isolates suggested differences in pathogenic potentials among the three lineages when the association between classification to lineage and the isolate source (human or animal) was evaluated. We found a statistically significant predominance of human isolates compared with animal isolates among lineage I strains as well as a significant predominance of animal isolates in comparison to human isolates among lineage II and lineage III strains (Jeffers et al., 2001). Specifically, only one human sporadic isolate and no human epidemic isolates were classified to lineage III, while 11% of animal isolates were assigned to this lineage. This suggests that strains in this lineage may have reduced virulence for humans and/or that they have limited transmission potential from animal to human hosts. Lineage I contained the majority of human outbreak and sporadic isolates, but only 42% of the animal isolates. These preliminary findings led us to hypothesize that lineage I strains or a subset of lineage I strains have a greater pathogenic potential for humans as compared to strains in the other two lineages. This hypothesis is also supported by the findings of Vines et al. (1992) who reported that perinatal listeriosis is caused more frequently by strains equivalent to those in our lineage I than by strains in lineage II. A report by McLauchlin (McLauchlin, 1990) also found that serotypes 1/2b and 4b (grouped in lineage I) represent the majority (74%) of human isolates (n = 1363), and thus further supports our findings. In conclusion, we and others have accumulated strong evidence that *L. monocytogenes* clonal groups differ in their association with human and animal hosts.

Phenotypic Characterization of *L. monocytogenes* Strains and Subtypes

Phenotypic characterization of selected isolates from our collection has been initiated to probe the functional basis of possible virulence and transmission differences among different *L. monocytogenes* clonal groups and subtypes. Initial tissue culture virulence characterization using a plaque assay in mouse L cells indicate that lineage II strains may show reduced plaquing efficiency and/or plaque size as compared to lineage I and III strains (Wiedmann et al., 1997), which may be indicative of reduced virulence for lineage II strains. These preliminary tissue culture data are consistent with data from our group and from others (detailed in the proceeding section), which indicate that lineage I strains (which

includes serotypes 1/2b and 4b) appear to be the predominant strains responsible for human listeriosis cases and outbreaks, while lineage II strains (serotypes 1/2a and 1/2c) appear to be the most common strains isolated from contaminated foods (Norton et al., 2001b). Further experiments are in progress to confirm our current working hypothesis that lineage I (or a subset of strains within this lineage) represents a subgroup with increased human virulence as compared to lineage II, while lineage II shows an increased ability for environmental survival and multiplication as compared to lineage I. Because lineage III strains represent about 10% of isolates from animals with clinical symptoms, but are considerably less frequent among human clinical isolates and among food isolates (Norton et al., 2001b), these strains may be characterized by a limited transmissibility from animals through foods (and/or environmental sources) to humans, possibly either due to limited environmental survival and growth capabilities or due to limited human virulence.

Ecology of *L. monocytogenes*

Survival of *L. monocytogenes* outside human and animal hosts plays an important role in the transmission of this organism in the dairy food system. While it is conceivable that *L. monocytogenes* can enter the dairy food chain at many and possibly even at nearly any point, agricultural environments and food processing plant environments may be of particular importance as sources for the introduction of *L. monocytogenes* into the dairy food system.

Listeriosis infections in farm animals and particularly in cattle are often linked to consumption of contaminated silage. In addition, animal listeriosis cases sometimes occur in animals that are not fed silage and environmental sources have been speculated to be responsible for at least some of these cases. The agricultural environment thus may serve not only as an important source for contamination of silage, but may also be a direct source of animal infection in some cases.

The role of contaminated raw animal-based agricultural products (e.g., milk, meat) as a direct source of *L. monocytogenes* contamination of ready-to-eat food products is likely to be minimal since commercially applied heat treatments generally kill *L. monocytogenes* effectively enough to provide an appropriate margin of safety. While, infected animal and agricultural environments appear to rarely be a direct cause of human infections, there have been cases in which a direct link has been observed. Specifically, animal sources can play an important role in animal-derived food products that are not processed before consumption (e.g., raw milk). In addition, manure from infected or shedding animals

may represent a source of food contamination. For example, an outbreak involving 42 human cases in Nova Scotia in 1981 was linked to the consumption of coleslaw. This coleslaw was produced from cabbage harvested from fields fertilized with untreated sheep manure that had been obtained from a farm with a history of ovine listeriosis (Schlech et al., 1983).

Contaminated raw meat and milk as well as agricultural environments and/or animal fecal materials may also serve as an indirect source of food contamination by contributing to contamination of the food processing plant environment. Increasing evidence indicates that the processing plant environment may be one of the most important sources of food product contamination with *L. monocytogenes*. We thus have focused research efforts on developing a better understanding of the ecology of *L. monocytogenes* in agricultural environments and in food and dairy processing plants to better understand the contributions of these environments to processed food product contamination with *L. monocytogenes*. Further, it is possible that these environments serve as reservoir for this organism. Application of molecular subtyping methods that have been developed over the last 10 to 15 yr provides an opportunity to study of the distribution of *L. monocytogenes* in different environments and to track the sources and spread of specific *L. monocytogenes* subtypes throughout the food chain, allowing insight into the ecology of different *L. monocytogenes* clonal groups (Wiedmann, 2002a).

Listeria monocytogenes in the agricultural environment. To better understand the ecology of *L. monocytogenes*, we have initially applied molecular subtyping methods to track the spread and distribution of different *L. monocytogenes* subtypes on farms with listeriosis outbreaks. These studies have found a considerable diversity of *L. monocytogenes* subtypes in feed (primarily silage) samples as well as in other environmental samples collected on outbreak farms. Interestingly, in some outbreaks multiple distinct *L. monocytogenes* subtypes were shown to be responsible for the animal cases (Wiedmann et al., 1996), while in others a single subtype was responsible for all cases observed (Wiedmann et al., 1997). These findings might indicate that listeriosis epizootics can differ with regard to the causative strains and their spread. In some epizootics, a single, possibly highly virulent, strain may be responsible for infection of all involved animals, whereas in others distinctive strains (possibly with lower virulence and/or transmission potential) may be responsible for individual infections (Wiedmann et al., 1997). In many of the listeriosis outbreaks in farm animals investigated by our group, a considerable proportion, and sometimes all, of the *L. monocytogenes* subtypes found on a given farm were only isolated from environmental samples

and not from infected animals. These findings could indicate that some environmental isolates may have a limited ability to cause disease and thus further supports that *L. monocytogenes* strains may differ in their virulence characteristics.

Ongoing research in my laboratory focuses on developing a broader understanding of the ecology of *L. monocytogenes* in farm environments (including farms without listeriosis cases) as well as in pristine and urban environments. Initial findings indicate that while *L. monocytogenes* is indeed found in many environments, including urban and pristine environments, it is considerably more frequently isolated in farm environments (Sauders et al., 2002). Further subtype and phenotypic analyses and virulence characterization of the *L. monocytogenes* isolates from these environments will provide a better understanding of the ecology of this foodborne pathogen outside human and animal hosts.

Listeria monocytogenes in food and dairy processing environments. Because *L. monocytogenes* is effectively inactivated by commercial heat treatments (pasteurization) used in the dairy industry, the food processing environment seems to represent a major source of finished product contamination. Whereas *L. monocytogenes* has been reported to be regularly isolated from food and dairy processing environments (Cox, 1989; Pritchard et al., 1995; Sutherland and Porritt, 1996; Gravani, 1999), a better knowledge of the spread and survival of *L. monocytogenes* in food processing environments and the contributions of different environmental sources to finished product contamination is necessary to understand the transmission of *L. monocytogenes*. Whereas many food processors only test for the presence of *Listeria* spp. in the processing environment, detection and isolation of *L. monocytogenes* followed by molecular subtype characterization provides an opportunity to study the molecular ecology of *L. monocytogenes* in food processing plants. A thorough understanding of the molecular ecology of foodborne pathogens is necessary for the design of rational and science-based methods and approaches for the control of *L. monocytogenes* and other foodborne pathogens.

In a pilot study using three smoked fish processing plants, we showed that specific *L. monocytogenes* subtypes persisted in the environment of a given processing plant (Norton et al., 2001a). In addition to these persistent, plant-specific, subtypes, we also observed a considerable diversity of transient *L. monocytogenes* subtypes. Interestingly, the persistent *L. monocytogenes* subtypes were the major cause of finished product contamination. Through a collaborative research project on the ecology of *L. monocytogenes* in Mexican-style cheese processing plants, we also showed a similar pattern of strain distribution in dairy processing plants, including persistence

of plant-specific subtypes over time. In one of the three plants studied, the persistent *L. monocytogenes* subtype was also responsible for finished product contamination (Kuaye et al., 2001). These findings are consistent with other reports that used bacterial subtyping methods to show the persistence of specific *L. monocytogenes* subtypes in a variety of food processing environments including those for smoked fish, poultry, meat and dairy foods (Lawrence and Gilmour, 1995; Rorvik et al., 1995; Nesbakken et al., 1996; Autio et al., 1999). Whereas some studies found that persistent strains may have an increased capability to adhere to inorganic surfaces (Lunden et al., 2000), others were not able to discern an association between strain persistence and a strain's ability to form biofilms (Djordjevic et al., 2002). Further research will be necessary to determine whether specific phenotypic and genetic strain characteristics are responsible for persistence in a processing plant environment.

While further confirmation of the association between persistent stains and finished product contamination in other dairy and food processing plants will be important, our results indicate that control and elimination of persistent *L. monocytogenes* subtypes in processing plants may allow for considerable reduction of finished product contamination. Transient *L. monocytogenes* strains on the other hand may only be minor contributors to finished product contamination. Molecular subtyping thus provides an important tool to track in-plant sources and spread of bacterial contaminants and to better control finished product contamination by foodborne pathogens (Wiedmann, 2002a, Wiedmann, 2002b).

CONCLUSIONS

Transmission of food and milkborne pathogens often involves complex interactions among the pathogen, the environment and one or multiple host species. A complete understanding of these interactions is important to develop science-based and effective intervention strategies to reduce the incidence of foodborne infectious diseases. Within this context, it is important to understand that bacteria grouped into a single species often represent a diverse group of organisms. To illustrate, bacteria sharing as little as 70% DNA-DNA homology can be grouped into a single species. In our studies using *L. monocytogenes* as a model system, we have found considerable evidence for the existence of distinct *L. monocytogenes* subtypes and clonal groups that appear not only to represent distinct phylogenetic groups, but also to differ in phenotypic characteristics and transmission dynamics. Our findings are consistent with current knowledge of other foodborne pathogens

(e.g., *Salmonella*, *E. coli*), where strains and clonal groups within a given species can differ considerably in their transmission characteristics and in their ability to cause foodborne disease. I thus propose that rational and science-based approaches to food safety should be based on specific definitions of bacterial subtypes and clonal groups that have the ability to cause human disease, rather than on broad bacterial species definitions, which may not relate to the ability of a group of bacteria to cause human disease. Understanding bacterial diversity beyond the species level thus represents an important task for all food and dairy microbiologists. Strain differences among microbial organisms grouped into a single bacterial species are not only important for our understanding of bacterial pathogens, but these differences also play important roles for spoilage organisms and starter cultures. While it may be difficult to imagine dairy microbiology without the species and taxonomic units we are familiar with, a move towards evolutionary- and molecular subtyping-based definitions of bacterial and clonal groups will improve our understanding of microbes associated with the dairy food system. These ideas can be summarized best with a quote from Norman Pace, a renowned microbiologist: "I think the concept of species is a fallacy in the microbial world" (Stencel, 2000).

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