SPECIAL GUEST EDITOR SECTION

Molecular Subtyping Methods for *Listeria monocytogenes*

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Conventional, phenotypic, and DNA-based subtyping methods allow differentiation of *Listeria monocytogenes* beyond the species and subspecies level. Bacterial subtyping methods not only improve our ability to detect and track human listeriosis outbreaks, but also provide tools to track sources of *L. monocytogenes* contamination throughout the food system. The use of subtyping methods also provides an opportunity to better understand the population genetics, epidemiology, and ecology of *L. monocytogenes*. The last 5 years have seen tremendous advancements in the development of sensitive, rapid, automated, and increasingly easy-to-use molecular subtyping methods for *L. monocytogenes*. This review highlights key aspects of different *L. monocytogenes* subtyping methods and provides examples of their application in public health, food safety, population genetics, and epidemiology. A significant focus is on the application of subtyping methods to define *L. monocytogenes* subtypes and clonal groups, which may differ in phenotypic characteristics and pathogenic potential.

Listeria monocytogenes is a foodborne pathogen capable of causing serious invasive disease, including abortion, septicemia, meningitis, and meningoencephalitis in humans and animals. Among humans, immunocompromised persons, pregnant women, the elderly, and neonates are particularly at risk for listerial infections. The frequency of clinical human listeriosis in most developed countries is estimated to range from 2 to 15 cases per million population, with case mortality rates between 13 and 34% (1). Mead et al. (2) specifically estimated that 2500 cases of clinical listeriosis occur annually in the United States, resulting in approximately 500 deaths. 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.

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The use of subtyping methods to differentiate *L. monocytogenes* strains has important applications in foodborne disease surveillance, outbreak detection, and source tracking throughout the food chain. Differentiation of bacterial and foodborne pathogens beyond the species level also provides exciting opportunities to better understand the ecology and characteristics of bacterial strains and subtypes, including differences in their ability to cause human foodborne disease.

Subtyping Methods for *L. monocytogenes*

In the context of bacterial subtyping, the terms "subtyping," "strain typing," and "fingerprinting" are often used interchangeably. All of these terms describe the process of differentiating bacterial isolates beyond the species or subspecies level. The term "fingerprinting" is somewhat misleading when used in this context, though, because bacterial subtyping differs significantly from fingerprinting humans. Importantly, asexual reproduction in bacteria allows for the common existence of virtually identical organisms. Furthermore, bacterial subtyping is used to characterize 2 or more distinct isolates with the goal of determining their relationship. For example, in outbreak investigations, the goal of subtyping bacterial isolates is to probe the likelihood that 2 or more isolates share a very recent (days to weeks and, perhaps, months) common ancestor. Fingerprinting of humans, on the other hand, is used to characterize and track a single specific individual. As bacterial subtyping methods are discussed, it is also important to clearly define other common terms, such as "isolate," "strain," and "clonal group" (Table 1).

The choice of an appropriate subtyping method depends on the intended application and the goal of subtyping *L. monocytogenes* isolates. Commonly used criteria for evaluating a subtyping method include discriminatory ability, cost, standardization and reproducibility, automation and ease of use, and applicability of a given subtyping method to different bacterial species. The discriminatory ability of a subtyping method can be characterized using Simpson's Index of Discrimination, which quantitates the probability that 2 unrelated isolates will be characterized as different subtypes (3). For an ideal subtyping method, data should be available on the frequency of isolation of different subtypes from various sample types and ecological niches. No single subtyping method will perform optimally with regard to all of these criteria. The intended application of subtyping will determine the relative importance of each criterion. For example, a food testing labora-

Table 1. Definitions of important subtyping terms

Term	Definition
Isolate	Pure culture of bacteria, presumably derived from a single organism
Strain, subtype	Isolate or group of isolates that can be distinguished from other isolates of the same species by phenotypic and/or genotypic characteristics. Strain and subtype are often used interchangeably, although the term strain is often used to describe a set of isolates with common biochemical and/or physiological characteristics, while the term subtype is often used to describe a set of isolates with common genetic or molecular characteristics
Clonal group (clones)	Genetically related isolates that are indistinguishable from each other by genetic tests or that are so similar they are presumed to have directly descended from a common ancestor

tory, which subtypes a few *L. monocytogenes* isolates and a much larger number of *Salmonella* isolates annually to determine whether a specific source is responsible for finished product contamination will have different requirements for a subtyping method than a national or international subtyping network that needs to subtype more than 1000 human and food *L. monocytogenes* isolates annually.

A comprehensive comparison of different subtyping methods for *L. monocytogenes* was performed under the auspices of the World Health Organization (WHO), and the results from this study were published in 1996 (4). In general, bacterial subtyping methods can be divided into conventional and phenotypic, and genetic or DNA-based methods. Although conventional and phenotypic methods have been used for many years to subtype *L. monocytogenes* and other foodborne pathogens, genetic subtyping methods have revolutionized this field. Key aspects of selected *L. monocytogenes* subtyping methods are summarized below; for a more comprehensive review, the reader is referred to one of the many outstanding articles or book chapters on this topic (5, 6).

Conventional and Phenotypic Subtyping Methods for L. monocytogenes

Commonly used conventional and phenotypic subtyping methods for *L. monocytogenes* include serotyping, phage typing, and multilocus enzyme electrophoresis (MEE). Although conventional subtyping methods generally still have some utility, molecular subtyping methods may replace these methods in the near future.

(**a**) *Serotyping.*—Serotyping is a classical tool for strain differentiation of many foodborne pathogens, including *Salmonella*, *L. monocytogenes* (7), *E. coli*, and others. Serotyping is based on the fact that different strains of bacteria differ in the antigens they carry on their surfaces. These surface antigens can be detected by antibodies and antisera. Serotyping has been used in epidemiological studies, but shows poor discriminatory power for many foodborne pathogens compared with other subtyping methods, particularly many molecular subtyping methods (5). For example, serotyping differentiates 13 different *L. monocytogenes* serotypes (1), whereas some molecular subtyping methods yield more than 100 different *L. monocytogenes* subtypes. Thus, serotyping provides a relatively insensitive tool for subtype differentiation and epidemiological investigations (5). More discriminatory approaches are therefore necessary for defining *L. monocytogenes* subtypes and for accurate and effective tracking of contamination sources and foodborne disease outbreaks.

(**b**) *Phage typing*.—Phage typing characterizes bacterial isolates by their susceptibility to lysis by a standard set of phages. A variety of lytic phages are available for *L. monocytogenes*. Phage typing allows very rapid, high throughput typing, but requires a standardized reference phage set in order to be comparable between laboratories. Standardization of phage typing represents a significant challenge, as this procedure suffers from significant biological and experimental variability (5). In France, phage typing has been routinely applied to subtyping of human and food isolates and has contributed to the detection of at least one human listeriosis outbreak (8).

(**c**) *Multilocus enzyme electrophoresis*.—MEE differentiates bacterial strains by variations in the electrophoretic mobility of different constitutive enzymes. Cell extracts containing soluble enzymes are separated by size in nondenaturing starch gels, and enzyme activities are determined in the gel by color-generating substrates (5). This method usually provides 100% typability, but is difficult to standardize between laboratories. MEE has been widely used for studies on the population genetics of many bacterial pathogens, including *L. monocytogenes* (9). MEE was used for epidemiological studies of human listeriosis in the early 1990s, but appears to be less discriminatory than some DNA-based subtyping methods (5).

Genetic Subtyping Methods for L. monocytogenes

The widespread development of different DNA-based subtyping methods has dramatically improved our ability to differentiate strains and subtypes of bacterial, parasitic, and viral pathogens. Commonly used DNA-based subtyping approaches for bacterial isolates include random amplification of polymorphic DNA (RAPD), pulsed-field gel electrophoresis (PFGE), ribotyping and, increasingly, DNA-sequencing based methods. A variety of molecular subtyping methods have also been used for strain differentiation of *L. monocytogenes*. Many DNA-based methods are superior to classical methods (e.g., serotyping) in several respects. DNA-based subtyping methods often provide more sensitive strain discrimination and a higher level of standardization and reproducibility than do conventional and phenotypic methods. The most commonly used molecular methods that provide accurate and discriminatory typing results for *L. monocytogenes* include ribotyping and PFGE. Studies involving more than

100 *L. monocytogenes* isolates have shown that at least 50 distinct types can be differentiated with ribotyping (10) and at least 72 types can be differentiated by PFGE (11). Nevertheless, the use of multiple subtyping methods may further improve subtype discrimination and may thus be appropriate for certain applications and specifically for epidemiological outbreak investigations.

(**a**) *Pulsed-field gel electrophoresis*.—PFGE characterizes bacteria into subtypes (sometimes referred to as "pulsotypes") by generating DNA banding patterns after restriction digestion of the bacterial DNA. Specifically, complete bacterial DNA is purified and subsequently cut into diagnostic DNA fragments using restriction enzymes, which cut DNA where a specific short DNA sequence is present (for example, the restriction enzyme *Asc*I will cut the bacterial DNA whenever a sequence of GGCGCGCC is present). After digestion with a restriction enzyme, bacterial DNA fragments are separated electrophoretically by size, using PFGE to generate DNA banding patterns. Restriction enzymes are chosen so that they cut DNA only rarely to yield between approximately 8 and 25 large DNA bands ranging from 40 to 600 kilobases (kb). DNA banding patterns for different bacterial isolates are compared to differentiate distinct bacterial subtypes (or strains) from those that share identical (or very similar) DNA fragment patterns. Restriction enzymes commonly used for PFGE typing of *L. monocytogenes* include *Asc*I and *Apa*I. PFGE of a given isolate is often performed with different restriction enzymes in separate reactions to improve discrimination. For example, 2 isolates with identical PFGE types for *AscI* may have 2 distinct *ApaI* PFGE patterns.

The Centers for Disease Control and Prevention (CDC) and state health departments use PFGE in a national network (PulseNet) to exchange DNA subtypes for isolates of foodborne pathogens. PFGE shows a high level of sensitivity for discrimination of *L. monocytogenes* strains, and is often considered the current gold standard for discriminatory ability. It is important to realize, however, that PFGE (as well as other subtyping methods) may also sometimes detect small genetic differences (e.g., 2–3 different bands) that may not be epidemiologically significant (12). On the other hand, the detection of an identical PFGE type (or a subtype determined by another method) in 2 samples (e.g., a food sample and a sample from a clinically affected human) does not necessarily imply a causal relationship or a link between these 2 isolates. Rather, in outbreak investigations, molecular subtyping information needs to be analyzed in conjunction with epidemiological data to determine causal relationships between 2 or more isolates.

(**b**) *Ribotyping*.—Ribotyping is another DNA-based subtyping method in which bacterial DNA is initially cut into fragments by restriction enzymes. PFGE uses restriction enzymes that cut bacterial DNA into very few large pieces, whereas the initial DNA digestion for ribotyping cuts DNA into many (>300–500) smaller pieces. For example, with the restriction enzyme *Eco*RI, most DNA fragments range in size from approximately 2 to 20 kb. These DNA fragments are separated by size through agarose gel electrophoresis, and a subsequent Southern blot step uses DNA probes to specifically label and detect those DNA fragments that contain the bacterial genes encoding the ribosomal RNA (rRNA). The resulting DNA banding patterns are thus based on only those DNA fragments that contain the rRNA genes. The restriction enzyme *Eco*RI is commonly used for ribotyping of *L. monocytogenes*. Although *Eco*RI ribotyping provides robust and sensitive differentiation of *L. monocytogenes* into subtypes that appear to correlate with phenotypic and virulence characteristics, the use of different restriction enzymes (e.g., *Pvu*II) in separate reactions provides increased strain discrimination (13, 14).

A completely automated system for ribotyping (the RiboPrinter® Microbial Characterization system) has been developed by Qualicon, Inc. (Wilmington, DE) and is commercially available (15). This automated system provides a high level of reproducibility and standardization. Subtyping of 8 isolates can be completed in 8 h, starting from an isolated colony on an agar plate, and as many as 24 isolates can be subtyped in less than 24 h. Automated ribotyping is often considered the DNA subtyping method of choice for many large-scale studies as well as for industrial applications, as PFGE is more time-consuming and labor-intensive, and requires more personnel with a higher level of technical expertise.

(**c**) *DNA sequencing-based subtyping*.—DNA sequencing of one or more selected bacterial genes represents another genetic subtyping method. Multilocus sequence typing (MLST) refers to a molecular subtyping approach that uses DNA sequencing of multiple genes or gene fragments to differentiate bacterial subtypes and to determine the genetic relatedness of isolates. MLST often refers to sequencing of multiple housekeeping genes, but sequencing of multiple virulence genes can also be used as a subtyping method.

MLST approaches for *L. monocytogenes* are currently being developed in different laboratories. The development of MLST is aided by the fact that *L. monocytogenes* is a well characterized facultative intracellular pathogen, and a key group of virulence genes and their specific functions in the intracellular infection process have been identified and characterized. In tissue culture models of infection, the following stages of infection can be defined: (*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 movement through the 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 (16). 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. Additional virulence-associated genes (e.g., *inlA*) not linked to this virulence island have also been identified (17). These virulence genes are unique to *L. monocytogenes*, thus providing ideal targets for the development of DNA sequencing-based subtyping methods.

DNA sequences for a variety of the *L. monocytogenes* virulence genes have been determined for one or more strains; however, development of MLST methods requires the identification of highly polymorphic genes and/or gene fragments using a large, representative *L. monocytogenes* strain collection. Strains in this collection should already be characterized by other commonly used subtyping methods to allow comparison of the discriminatory ability of MLST to other typing methods. Once suitable target genes are identified, the development of an MLST/DNA sequence database for *L. monocytogenes* will be necessary to facilitate the application of MLST for this organism. Ultimately, MLST may have the potential to allow integration of polymerase chain reaction (PCR)-based detection and subtyping in a single rapid format, possibly eliminating the requirement for a culturing step in certain diagnostic applications.

Applications of Molecular Subtyping Approaches to *L. monocytogenes*

The application of conventional, phenotypic, and molecular subtyping methods has tremendously improved our understanding of the biology, ecology, and epidemiology of *L. monocytogenes* and other foodborne pathogens. The use of subtyping methods in surveillance programs has significantly contributed to the rapid detection of foodborne disease outbreaks. Some examples that highlight applications of molecular subtyping methods to *L. monocytogenes* are presented below. A significant focus is on the application of subtyping methods to define *L. monocytogenes* subtypes and clonal groups, and to probe their associations with phenotypic characteristics and pathogenic potential.

Foodborne Disease Surveillance and Tracking of Foodborne Disease Outbreaks

Changes in our food system, such as an increase in the range of distribution from local to state or national to international for foods produced by a single manufacturer, impact the epidemiology of foodborne disease outbreaks (18). Multistate foodborne disease outbreaks, which may also occur over prolonged time periods, are often difficult to detect by classical epidemiological approaches and surveillance systems. Molecular subtyping of bacterial isolates from human patients provides an opportunity to rapidly detect widespread clusters of human foodborne disease cases caused by a specific genetic type. Analysis of molecular subtyping data in conjunction with epidemiological data not only helps in detecting an outbreak, but also in detecting and eliminating the outbreak source. Surveillance of human listeriosis and detection of listeriosis clusters represent a particular challenge. Unlike diseases caused by many other foodborne pathogens, such as *E. coli* O157:H7 or *Salmonella*, foodborne listeriosis is characterized by long incubation periods (7–60 days). Furthermore, only specific segments of the population (e.g., the immuncompromised, elderly, and pregnant women) are likely to develop clinical disease after exposure to contaminated foods. Thus, listeriosis outbreaks often appear to occur over a wide geographical and temporal range and are consequently difficult to detect by classical epidemiological approaches. Even if a cluster of cases is detected, it can be extremely difficult to identify a common source because of the long incubation period of this disease. For example, *E. coli* O157:H7 outbreaks are frequently characterized by a cluster of cases that occur within a few days to weeks. Interviews of affected individuals often quickly reveal a common source, such as attendance at a public event or a specific restaurant, or specific food consumption within a few days before disease onset (e.g., hamburgers, apple cider, etc.). This is normally not the case for clusters of human listeriosis cases, because patients can rarely recall all specific foods consumed within the last 7–60 days. Consequently, application of sensitive subtyping methods may help to detect human listeriosis clusters and their sources.

In foodborne disease outbreak investigations, interpretation of molecular subtyping results needs to occur in conjunction with epidemiological data. Proper interpretation of subtyping data also requires a good understanding of bacterial physiology and genetics. Interpretation of bacterial subtyping results involves comparisons of DNA subtypes for 2 or more bacterial isolates to determine whether they are likely to share a recent common ancestor. For example, bacterial isolates from foods may be compared to isolates from infected humans to help clarify whether the bacteria present in a specific lot of food are linked to a human infection. However, human and food isolates have been exposed to many different environments (e.g., passage through the human body) and may actually differ in their genetic materials as a result of mutations and different selective pressures. Thus, human and food isolates may differ slightly in their bacterial subtypes, even if the *L. monocytogenes* present in the food was responsible for the specific human infection(s).

Over the last few years, listeriosis surveillance programs increasingly incorporated subtyping of human clinical and food *L. monocytogenes* isolates. Since 1998, these efforts have contributed significantly to the detection of at least 3 human listeriosis outbreaks in the United States that were linked to a specific food source. In 1998–1999, a multistate listeriosis outbreak was responsible for at least 100 human cases, including 21 deaths in at least 15 states. This outbreak was initially detected by an increase in the number of listeriosis cases caused by a specific molecular subtype as determined by both automated ribotyping and PFGE typing (19, 20), and was ultimately linked to the consumption of contaminated hot dogs and deli meats. In December 1999, a smaller cluster of human listeriosis cases was linked to consumption of contaminated pate. Between May and December 2000, another cluster of at least 29 human listeriosis cases in 10 states was identified by both automated ribotyping and PFGE typing. Deli turkey was identified as the likely source of this outbreak (21). Before the identification of these 3 U.S. listeriosis outbreaks, only one common-source human listeriosis outbreak had been reported in the United States between 1990 and 1998 (22): an outbreak of gastrointestinal listeriosis linked to consumption of contaminated chocolate milk in 1994 (23). The increasing use of molecular subtyping methods may be responsible for an improved de-

tection of human listeriosis outbreaks, leading to an apparent increase in the number of human listeriosis outbreaks.

In-Plant Tracking of L. monocytogenes Spread and Sources

Subtyping methods have also been valuable tools in tracking the sources and spread of *L. monocytogenes* contamination throughout the food chain (24–28). We have specifically used *L. monocytogenes* as a model system for application of molecular subtyping methods to track in-plant *Listeria* contamination patterns. In a pilot study using 3 smoked fish processing plants, we showed that specific *L. monocytogenes* subtypes persisted in the environment of a given processing plant (24). These persistent *L. monocytogenes* subtypes were the major cause of finished product contamination, although, in a single incident, the raw materials appeared to be the source of finished product contamination. These findings are consistent with other reports, which 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 (25–28). Thus, molecular subtyping methods may also provide a new approach to track in-plant sources and spread of bacterial contaminants and to provide information that can be used to control finished product contamination.

Phylogenetic Characterization and Differentiation of L. monocytogenes Subtypes of Varied Pathogenic Potential

Current regulations specifying a zero-tolerance for the presence of *L. monocytogenes* subtypes in ready-to-eat (RTE) foods are based on historical taxonomic classification schemes which do not necessarily correlate with the ability of a group of bacteria to cause human disease. Rather, 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 can cause human disease. Molecular subtyping methods provide a unique opportunity to explore the population genetics and evolution of *L. monocytogenes*. Subtyping methods have the potential to differentiate bacterial strains and to facilitate the definition of subtypes and clonal groups that differ in their phenotypic characteristics and/or their abilities to cause human foodborne disease. Consequently, in the future, only certain *L. monocytogenes* subtypes may be considered an adulterant when present in RTE foods.

Although most 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 (1, 29). The 4b strains isolated from most epidemic outbreaks form 2 closely related homogenous groups (so-called "epidemic clones"; 9, 30). Serotypes 1/2a and 1/2b are also responsible for significant numbers of sporadic cases of human illness. A serotype 1/2a strain was responsible for a recent multistate human listeriosis outbreak in the United States (21). 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 (31). Of 1363 human isolates collected in the United Kingdom, 15% were 1/2a, 10% were 1/2b, and 64% were 4b (32). 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 that enable these strains to be more effective than others in causing human disease. Two hypotheses could explain the apparent predominance of serotype 4b strains in human epidemic listeriosis and of 4b, 1/2a, and 1/2b strains in sporadic human cases: (*1*) Humans are more commonly exposed to these subtypes than to other *L. monocytogenes* serotypes, i.e., these strains are found in foods more frequently than other serotypes; and/or (*2*) these subtypes have a unique pathogenic potential for humans. Surveillance programs using different subtyping strategies to differentiate *L. monocytogenes* strains in conjunction with population genetics and pathogenesis studies can yield a better understanding of the transmission dynamics of *L. monocytogenes* and help us probe why specific subtypes appear to be the predominant cause of human infections. Selected findings relevant to these questions are summarized below.

Molecular subtyping methods have consistently grouped *L. monocytogenes* into 2 major lineages. MEE, PFGE, ribotyping, and amplified fragment length polymorphism (AFLP) analysis all show that *L. monocytogenes* can be separated into 2 major genetic groups (9, 11, 33, 34). Serotypes 1/2a, 3a, 1/2c, and 3c are generally found in one genetic group, while the other genetic group appears to correspond to serotypes 1/2b, 3b, 4b, 4d, and 4e. Recent allelic analyses of several virulence genes as well as ribotyping revealed a third phylogenetic lineage within *L. monocytogenes* (30, 35). Specifically, a combination of virulence gene alleles and ribotype patterns allowed separation of *L. monocytogenes* strains into 3 distinct lineages, designated I, II, and III (30). Lineages I and II correspond with the primary divisions of *L. monocytogenes* previously uncovered by MEE, PFGE, AFLP, and ribotyping, and may represent different subspecies of *L. monocytogenes*. Lineage III also represents a distinct taxonomic unit and may possibly represent a new species (30). Each lineage can be separated into 3–4 ribotype fragment subsets (characterized by common ribotype fragments), each of which contains 5–20 genetic subtypes (ribotypes) with no detectable horizontal gene transfer among them (Figure 1). These data provide a phylogenetic framework for probing virulence differences among *L. monocytogenes* subtypes.

The findings described above were used by Jeffers et al. (36) and by Norton et al. (37) to further explore an association between different *L. monocytogenes* subtypes and human and animal listeriosis infections. Jeffers et al. (36) specifically assessed whether subsets of *L. monocytogenes* as defined by lineage, ribotype fragment subset, and ribotype differed in their likelihood to cause human or animal listeriosis. The clinical histories of the *L. monocytogenes* strains suggested differ-

ences in pathogenic potentials among the 3 lineages. Lineage I contains 14 of 15 strains isolated during human epidemic outbreaks, whereas only 1% of human isolates were found in lineage III, suggesting the possibility that strains in this lineage may have reduced virulence for humans. Animal isolates were found in all 3 lineages. Jeffers et al. (36) also found a statistically significant predominance of human over animal isolates among lineage I strains and a significant predominance of animal over human isolates among lineage III strains (36). These findings led to the hypothesis that lineage I strains or a subset of lineage I strains have a greater pathogenic potential for humans than do strains for the other 2 lineages. These clonal groups may also differ in their adaptation to different ecological niches (38). Vines et al. (39) reported that perinatal listeriosis is caused more frequently by strains equivalent to those in lineage I than by strains in lineage II. Similarly, McLauchlin (32) found that serotypes 1/2b and 4b (grouped in lineage I) represent the majority of human clinical isolates.

Wiedmann et al. (30) and Jeffers et al. (36) also proposed that their findings indicate that strains in lineage III may be characterized by reduced virulence for humans, and that this lineage might show host specificity towards animals (30). Lineage III represents a unique subset of *L. monocytogenes* strains, which are characterized by phenotypic and genetic features atypical for this species. Strains within lineage III have a unique 16S rRNA signature sequence that differs by at least 2 nucleotides from lineages I and II and from *L. innocua* strains (30). Interestingly, lineage III strains predominantly

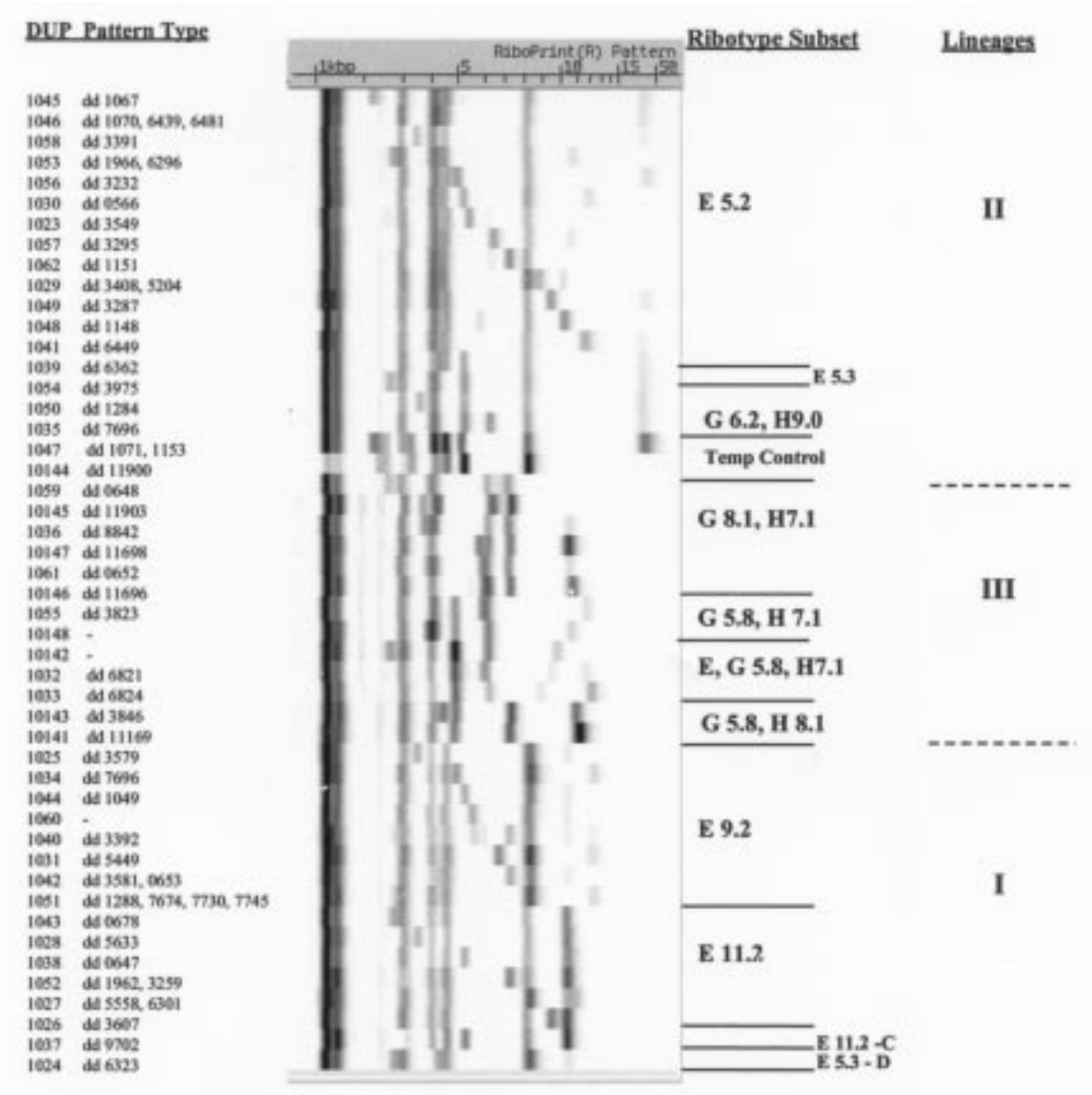

Figure 1. Ribotypes, ribotype subsets, and lineages for *L. monocytogenes*. This figure displays most previously described *L. monocytogenes Eco*RI ribotypes (10, 24, 30, 36, 37). Ribotyping was performed on the Qualicon RiboPrinter Microbial Characterization system. Numbers on left margin indicate RiboPrinter pattern type designations (DUP-1045, etc.) and previously reported manual ribotype designations (dd 1067, etc.; 10, 30). Ribotype subsets (10) and lineages (30) for the different ribotypes are indicated on right side.

represent serotypes 4a and 4c, which are generally not found among isolates of other lineages (40). One previous study found a low DNA–DNA homology of serotype 4a and 4c strains to a serotype 1/2a type strain, which groups into lineage II (72–76 and 70–71%, respectively; 41). These data further support that lineage III strains represent a distinct subset of *L. monocytogenes*, which may warrant reclassification as separate species or subspecies. At least 2 studies indicate that only 0–2% of human cases are caused by lineage III (36, 37) or serotype 4a and 4c strains (42). Jeffers et al. (36) specifically reported that only 1% of human isolates, but 10.5% of animal isolates tested, a statistically significant difference, were grouped in lineage III.

Lineage III can be further divided into 2 groups: lineages IIIA and IIIB. All isolates grouped in lineage IIIB are rhamnose-negative, although *L. monocytogenes* is generally rhamnose-positive. Although these strains appear closely related to *L. innocua*, the presence of the *L. monocytogenes* virulence genes *actA*, *hlyA*, and *inlA* strongly suggests that they should not be classified as *L. innocua* (10, 30). No human isolates in the Cornell *Listeria* strain collection were characterized as lineage IIIB, but a strain responsible for an outbreak of listeriosis in goats was classified in this lineage (43).

Conclusions

The last 5 years have seen tremendous advancements in the development of DNA-based subtyping methods for *L. monocytogenes*. Although PFGE and ribotyping are now commonly used to subtype *L. monocytogenes*, DNA sequencing-based methods and, specifically, MLST are being developed and are likely to become the typing methods of choice for many applications. Phenotypic and molecular subtyping methods for *L. monocytogenes* have improved our ability to detect human listeriosis outbreaks and provide tools to track sources of *L. monocytogenes* contamination throughout the food system. Subtyping methods thus provide an opportunity to improve our ability to control and prevent foodborne listeriosis cases and outbreaks. With the use of subtyping methods, we can develop a better understanding of the population genetics, epidemiology, and ecology of *L. monocytogenes*. A variety of subtyping studies have shown that *L. monocytogenes* can be separated into 3 lineages that may differ in their pathogenic potential. Lineage I, which is defined by unique molecular characteristics, causes the vast majority of human listeriosis outbreaks and approximately 70% of human sporadic listeriosis cases. Lineage III strains, on the other hand, very rarely cause human disease, but are more commonly involved in animal listeriosis cases and outbreaks. Similar observations of host specificity among different subtypes have been reported for other foodborne pathogens. Further research in these areas will allow more rational and science-based food safety regulations and directives based on specific definitions of human health hazards, rather than on bacterial species definitions, which may not relate to the ability of a group of bacteria to cause human disease.

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