Subtyping of *Listeria monocytogenes* by Multilocus Sequence Typing

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Story in brief

Molecular characterization of *Listeria monocytogenes* is essential for understanding the distribution of this pathogen in relation to outbreaks, contaminated foods, and/or environmental sources of contamination, notably processing plants. Accurate and highly discriminatory subtyping methods are required to recognize outbreaks of infection, to match case isolates with those from potential vehicles of infection, and to discriminate sources of contamination in processing plants. Many of the sophisticated gel-based nucleic acid methods (ribotyping, PFGE) are difficult to standardize between laboratories because size estimates of large DNA fragments can differ significantly with minor differences in gel band migration, making it difficult to accurately compare results for large strain collections. Multilocus sequence typing (MLST) is currently gaining increasing interest for use as a method of phylogenetic subtyping of bacterial strains. The DNA sequence data facilitates intra- and inter-laboratory comparisons that can easily be transmitted electronically via the internet, allowing the development of global databases. In our preliminary study, MLST was performed using 26 food isolates of *L. monocytogenes* and differentiated into 9 groups. The technique could be a useful tool for listeriosis surveillance systems that will help in identify the distribution of L. monocytogenes in the food processing environment.

Key Words: *Listeria monocytogenes*, Subtyping, Fingerprinting, DNA Sequence.

Introduction

Listeria monocytogenes is a Gram-positive, intracellular foodborne pathogen. Several foodborne outbreaks have highlighted the importance of this organism to the public health. Because of the high fatality rate associated with this organism, U.S. regulatory agencies have established a 'zero tolerance' for ready-to-eat foods (RTE) (Daeschel et al., 1999). Various molecular methods have been used to differentiate *L. monocytogenes* at the subspecies (i.e., strain) level that targets nucleotide variations at endonuclease restriction sites. These methods are mainly based on identifying the microorganisms by defining unique banding patterns obtained through electrophoretic mobility of their digested or amplified DNA fragments in agarose gels. Although these methods provide better strain differentiation than serotyping or phage typing, their discriminatory abilities are not precise and sometimes cannot differentiate epidemiologically unrelated strains of *L. monocytogenes* (Mead et al., 1999). In addition, experimental protocols of these methods may differ and are difficult to standardize. As a consequence, data comparison among different laboratories is sometimes difficult (Norton, 2002). Therefore, a DNA sequence-based method known as multilocus sequence typing (MLST) was developed by Maiden et al (1998) and targets multiple genetic loci that have slowly diversified from each other among various strains within a species. The strength of this approach is that sequence data are unambiguous, can be held in a central database and can be queried through a web server. We have started to examine the phylogenetic relatedness of strains of *L. monocytogenes* isolated from various sources using MLST among five genetic loci within 4 virulence genes that include:

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 propell *L. monocytogenes* through the cytoplasm of infected cells.

Materials and Methods

Isolates of *L. monocytogenes* were obtained from different food samples and used for MLST. Primers designed for five different genetic loci (*hly*A, *inl*A, *prf*A, *act*A*1*, and *act*A2) were used for PCR (Table 1). Overnight cultures of different isolates of *L. monocytogenes* were lysed using commercial protease and lysis solutions for bacterial PCR assays (Qualicon, Wilmington, DE). A 5 ul aliquot of the lysed culture solutions were then separately subjected to PCR amplification of the 5 gene targets. The amplicons were purified of residual primers and nucleotides using a Millipore PCR purification kit (Millipore, Billerica, MA), examined by standard agarose gel (1%) electrophoresis for quantitative DNA analysis, and then sent to the OSU DNA Core facility for DNA sequencing. The sequences of the 5 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.

Table 1. PCR primers used in this study.

Primer	Target Gene	Primer Sequence (5'→ 3')	Product size (bp)
Primer I	Hemolysin (*hlyA*)		560
	Forward	TGA ACC TAC AAG ACC TTC CA	
	Reverse	CAA TTT CGT TAC CTT CAG GA	
Primer II	Internalin A (*inlA*)		575
	Forward	GCT TCA GGC GGA TAG ATT AG	
	Reverse	AAC TCG CCA ATG TGC C	
Primer III	Positive regulatory factor (*prfA*)		590
	Forward	ATT TTT AAC CAA TGG GAT CC	
	Reverse	CAT TCA TCT AAT TTA GGG GC	
Primer IV	Actin mobility (*act*A1)		500
	Forward	AAT ACG AAC AAA GCA GAC CTA ATA G	
	Reverse	GGT CAA TTA ACC CTG CAC TTT TA	
Primer V	Actin mobility (*act*A2)		500
	Forward	GAT AGA GGA ACA GGA AAA CAC TCA	
	Reverse	CGT CTT CTG CAC TTT TAG CAA TT	

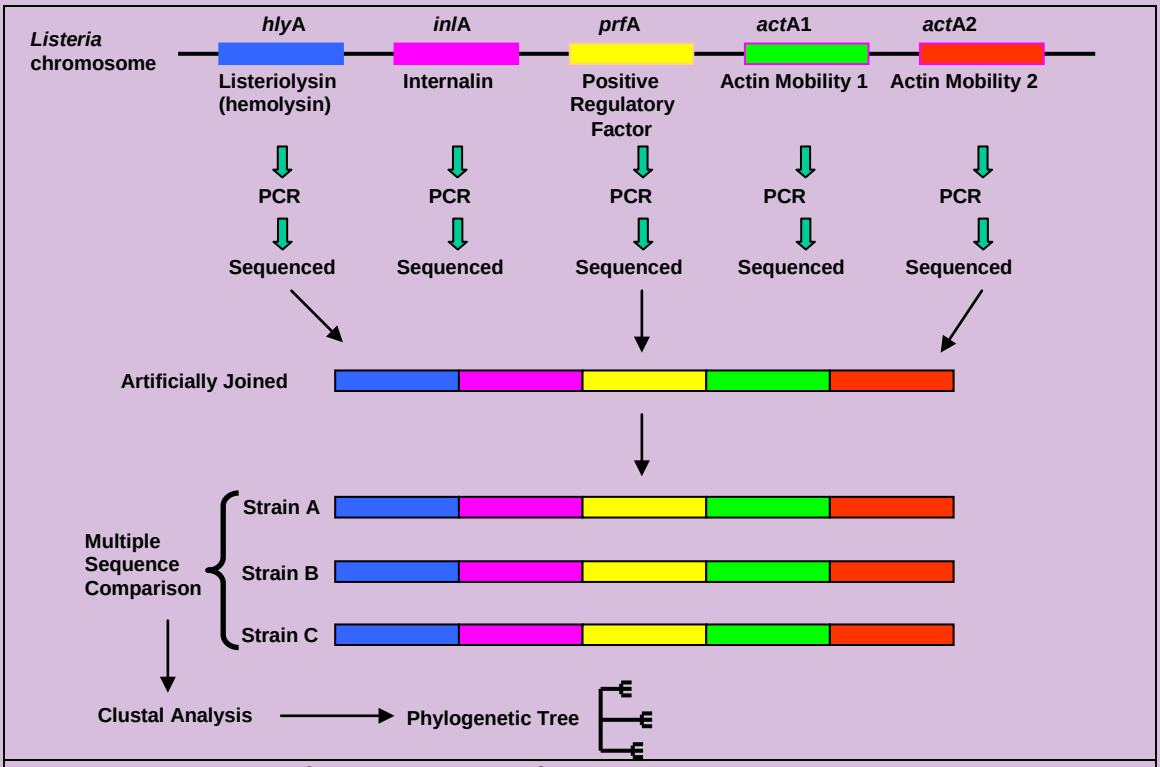

Figure 1. Sequences of separately-amplified regions pertaining to several *Listeria* virulence factors are joined into an 'artificial composite sequence' and subjected to multiple sequence alignment and clustal analysis for phylogenetic typing.

Results and Discussion

MLST involves simultaneous sequence based analysis of several genetic loci and differentiation based on their subtle genetic heterogeneity (Enright and Spratt, 1999). To date we have typed 26 food isolates strains into a phylogenetic tree of 13 groups based on genetic sequence of the *hylA*, *inlA* and *prfA* virulence genes (Fig. 2) and approximately 10 strains using these loci as well as *act*A1 and *act*A2 (data not shown). The use of a greater number of genetic loci provides additional discriminatory power. The dendrogram indicates that strains cw34, cw59, cw73, sm1, sm2, and sm3 strains are genetically related as they do not show much genetic diversity among these same genetic loci. This is interesting as the 'cw'strains were isolated from retail franks whereas the 'sm' strains were isolated from raw ground meat products. MLST can effectively distinguish strains that possess high degrees of homology within the compared gene sequences. This technique is user friendly and not as laborious as PFGE or expensive as Ribotyping, and it provides an ideal balance between sequence-based resolution and technical feasibility.

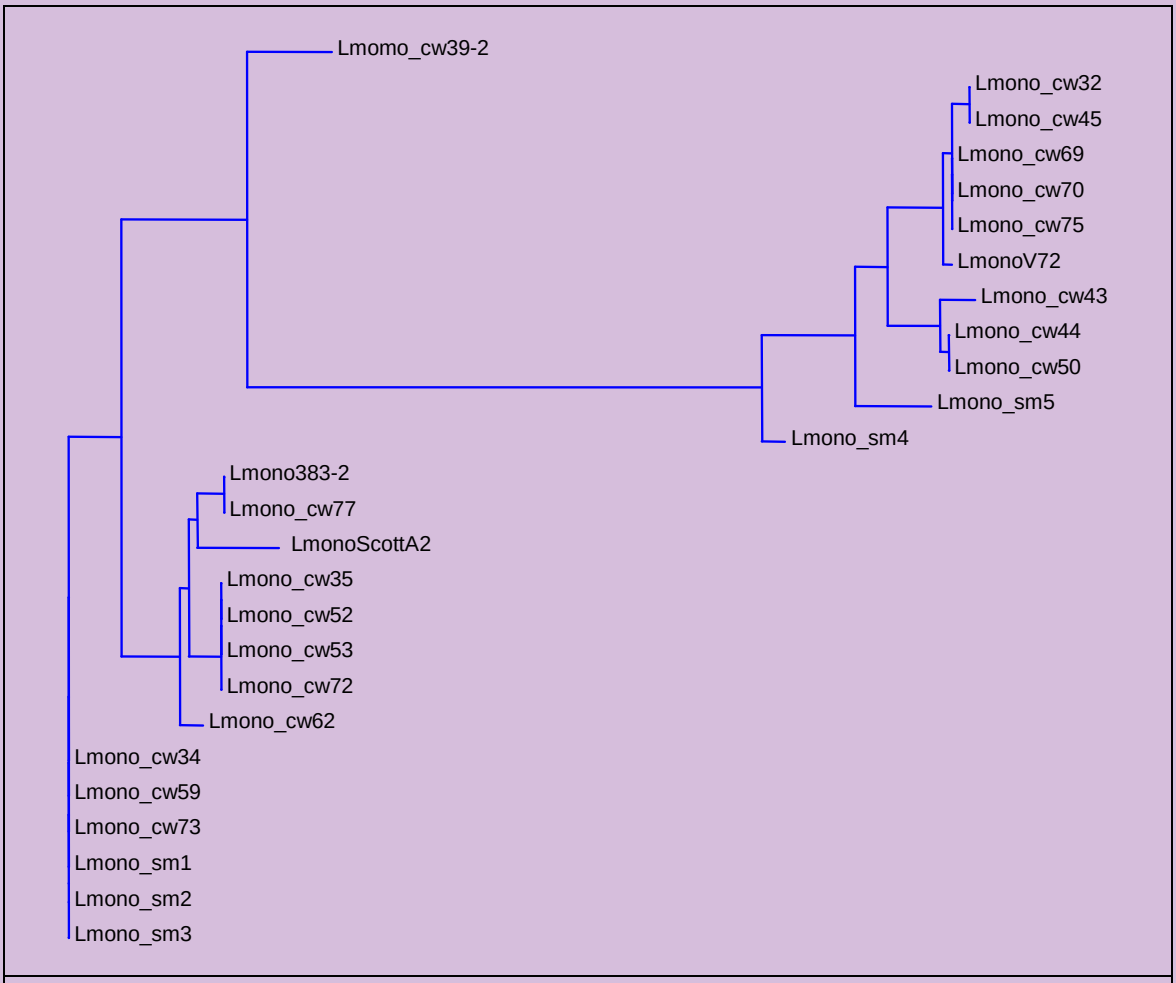

Figure 2. Dendrogram constructed by Vector NTI from a multiple sequence alignment of composite sequences using only the *hly*A, *inl*A, and *prf*A loci from 26 strains of *L. monocytogenes*. The 'CW' strains of *L. monocytogenes* were isolated from retail franks; the 'SM' strains were isolated from raw ground meat products

Maximum subtyping differentiation could be achieved by including more genetic loci. As indicated by dendrogram analysis, the inclusion of more gene loci has resulted in increased resolving power (data not shown). The resolution power of this method is unmatched by any other subtyping method as even a single nucleotide change at a targeted locus will result in a new type of subtype classification. Future work would include comparing the data obtained by MLST with pulsed-field gel electrophoresis and ribotyping to test the discriminatory power of this approach.

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