Subtyping of *Listeria monocytogenes* by Multilocus Sequence Typing and Pulsed-Field Gel Electrophoresis (PFGE)

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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. Several molecular typing methods including multilocus sequence typing (MLST), pulsed -field gel electrophoresis (PFGE) and Ribotyping have been used to characterize the molecular epidemiology for *L. monocytogenes*. These methods differ in their discriminatory abilities and reproducibility. Of all these methods 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 study, MLST was performed using 45 food isolates of *L. monocytogenes* and differentiated into two main groups. The data obtained from MLST were compared with PFGE for selected strains to compare the discriminatory power of this method. From MLST groupings, four weak and four strong biofilm forming strains were run with PFGE using restriction enzymes AscI and ApaI. These techniques could be useful tools 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, Pulsed-Field Gel Electrophoresis.

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' policy 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 examined the phylogenetic relatedness of strains of *L. monocytogenes* isolated from various sources using MLST among five genetic loci within four virulence genes that include: listeriolysin O (hlyA), 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 (prfA), which activates numerous virulence genes; a surface virulence protein, internalin A (inlA), required for the penetration of *L. monocytogenes* into non-phagocytic cells; and actin A (actA), another surface virulence factor that induces polymerization of actin molecules to propel L. monocytogenes through the cytoplasm of infected cells. Another method employed in this study for subtyping was PFGE which was developed by Schwartz and Cantor (1984) and is often considered the "gold standard" of molecular typing methods. The method involves embedding organisms in agarose, lysing the organisms in situ, and digesting the chromosomal DNA with restriction endonucleases that cleave infrequently. It has been chosen by the US PulseNet, a national network of public health laboratories, to fingerprint *L. monocytogenes.*

Materials and Methods

Multi-Locus Sequence Typing. Isolates of *L. monocytogenes* were obtained from different food samples and used for MLST. Primers designed for five different genetic loci (hlyA, inlA, prfA, actA1, and actA2) 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 µl aliquot of the lysed culture solutions was then separately subjected to PCR amplification of the five 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 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.

Pulsed Field Gel Electrophoresis Analysis. The strains used for running PFGE were four strong and four weak biofilm forming ones. The weak biofilm forming strains are cw34, cw35, cw52, and sm3 whereas; the strong biofilm forming strains are cw50, cw62, cw77, and 99-38. For these strains, plugs were prepared and 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 ApaI, 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. 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 is saved in Tiff format, and then transferred to the Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium) for computer analyses. Similarity between fingerprints was determined by the Dice coefficient using a band position tolerance of 1%.

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 (actA1)		500
	Forward	AAT ACG AAC AAA GCA GAC CTA ATA G	
	Reverse	GGT CAA TTA ACC CTG CAC TTT TA	
Primer V	Actin mobility (actA2)		500
	Forward	GAT AGA GGA ACA GGA AAA CAC TCA	
	Reverse	CGT CTT CTG CAC TTT TAG CAA TT	

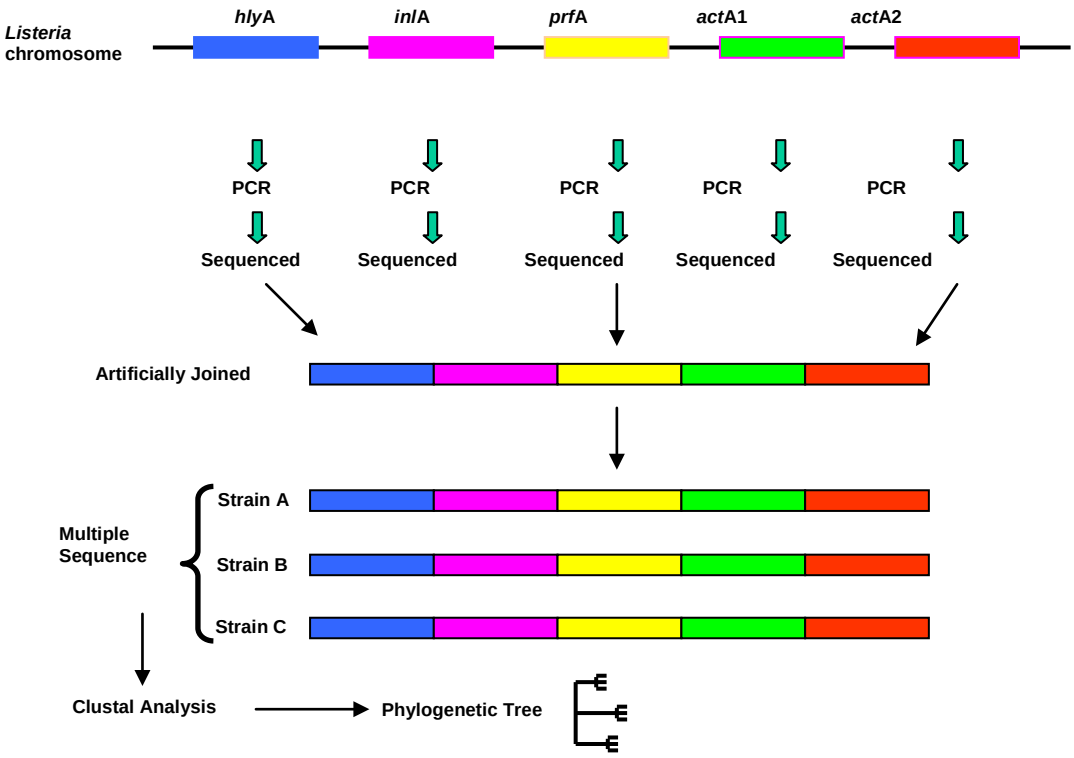

Figure 1. Sequences of separately-amplified regions pertaining to several Listeria virulence factors are joined into an 'artificial composite sequence' and subjected to multiple sequence alignment and clustal analysis for phylogenetic typing

Results and Discussion

Subtyping using MLST analyzes several genetic loci simultaneous and differentiate based on their subtle genetic heterogeneity (Enright and Spratt, 1999). In our study, 45 food isolates strains have been typed into a phylogenetic tree of two main groups based on genetic sequence of the hylA, inlA, prfA and actA1 and actA2 virulence genes (Fig. 2) 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 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. All JAG strains isolated from a food processing facility (JAG) fall in the same group, except JAG 126, which showed some divergence from these strains. *L. monocytogenes* strains isolated during year 1998 and 1999 from ground beef in a meat packaging plant also shows less divergence.

Figure 3 shows dendrogram for the four weak and four strong biofilm forming strains digested with ApaI. The isolates formed two main clusters with the second cluster having four subgroups. The strong biofilm forming strains cw50 and 99-38 belonged to one cluster whereas, in MLST, dendrogram cw50 and 99-38 are very divergent. Weak biofilm forming strains cw34, cw35, cw52 and sm3 belonged to second cluster. We observed that inclusion of virulence gene target sequences in a DNA sequence-based subtyping scheme for *L. monocytogenes* has helped to

achieve maximum subtype differentiation. As seen in the dendrogram inclusion of more gene loci has resulted in increasing the discriminatory power of MLST. Therefore, MLST can effectively distinguish strains that possess high degrees of homology within the compared gene sequences, and it provides an ideal balance between sequence-based resolution and technical feasibility.

These DNA-based methods define bacterial subtypes by using either PCR amplification and 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 biofilm forming strains of *L. monocytogenes* will help to assess the real risk posed by this pathogen found in foods.

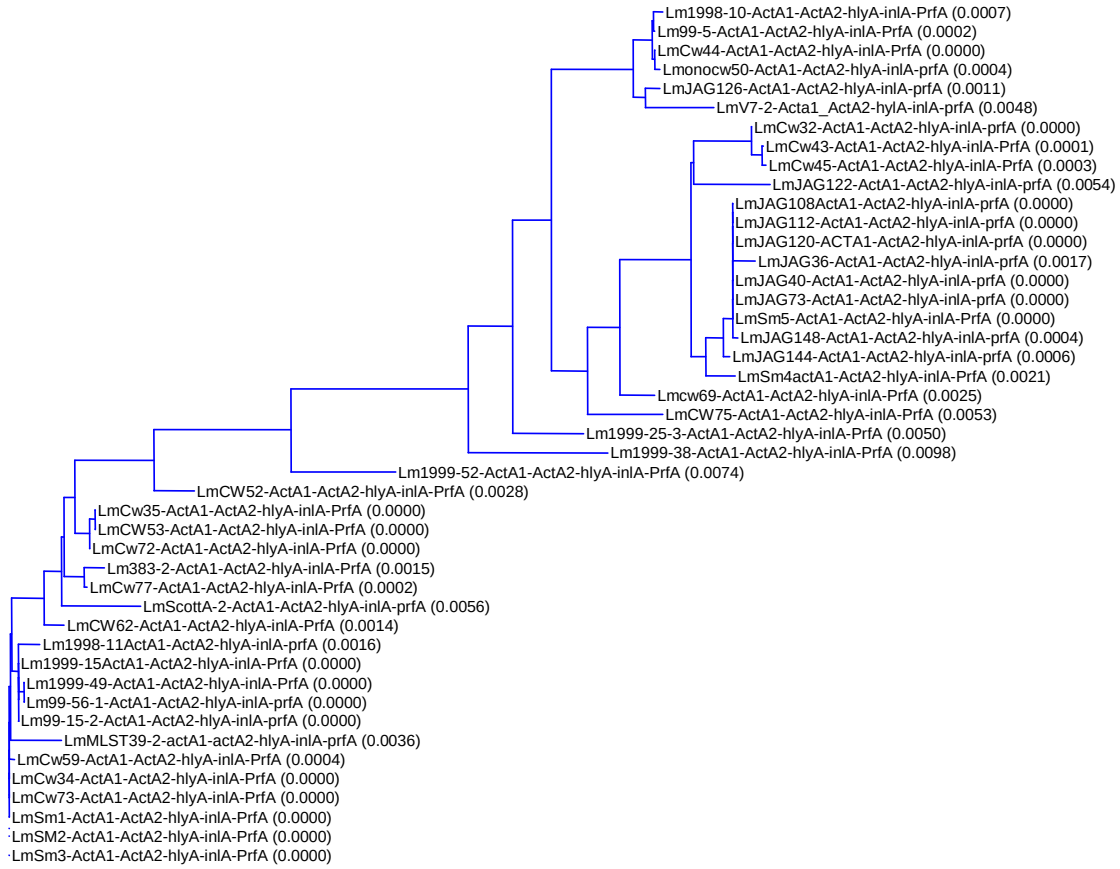

Figure 2. Dendrogram constructed by Vector NTI from a multiple sequence alignment of composite sequences using the actA1, actA2, hlyA, inlA, and prfA loci from 45 strains of *L. monocytogenes.* The 'CW' strains of *L. monocytogenes* were isolated from retail franks; the 'SM' strains were isolated from raw ground meat products.

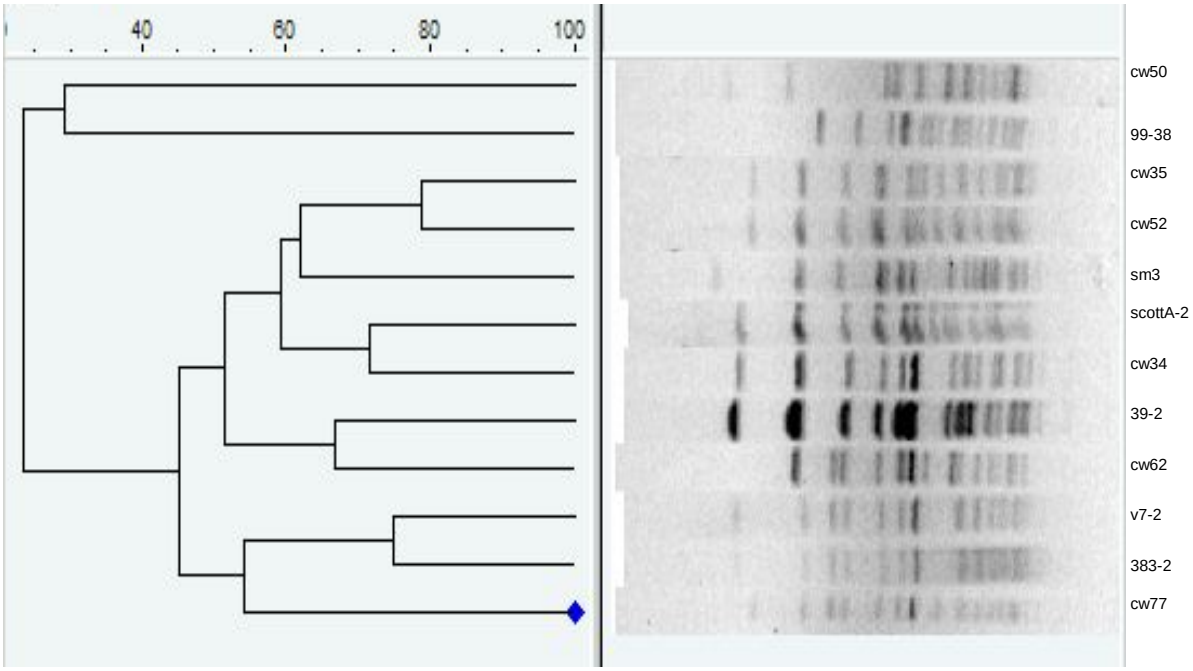

Figure 3. PFGE fingerprint patterns and dendrogram of APa I restriction digests of strong and weak biofilm forming strains

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