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# Discrimination of *Listeria monocytogenes* by Sequence Typing Based on Two Housekeeping Genes and Its Comparison to PFGE Patterns

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Two housekeeping genes, of *Listeria monocytogenes dat* and *hlyA*, were analyzed in a set of 28 isolates from different sources to estimate their genetic diversities. These strains were previously characterized by pulsed-field gel electrophoresis. Complete gene sequences for *dat* (465 bp) and *hlyA* (584 bp) had sequence similarity of 99.87~100% *S* and 99.96~100% *S* among isolates, respectively. Also, we found that the numbers of sequence types (ST) were about 3-fold less than those of PFGE types (3 STs versus 11 PFGE types). There was, however, a good correlation between the PFGE patterns and phylogenetic grouping of two gene sequences among the isolates. Further studies on analyzing additional loci would increase the discriminatory power of sequence typing for *L. monocytogenes* strains.

**Key Words:** Sequence typing, *dat*, *hlyA*, *Listeria monocytogenes*

## INTRODUCTION

*Listeria* spp. are ubiquitous bacteria widely distributed in the environment, and *Listeria monocytogenes* is commonly pathogenic for human and animal among the seven species of *Listeria*. It can cause serious infections such as meningitis or septicemia in newborns, immunocompromised patients, and the elderly or lead to abortion (Lober, 1997). Meningoencephalitis is perhaps the most easily recognized form of listeriosis seen in domestic animals. The name circling disease was given to this disease in sheep, and has since been observed in cattle and sheep in many parts of the world (Timoney et al., 1988).

Numerous molecular methods have been applied to the characterization of *L. monocytogenes* isolates, e.g., multilocus enzyme electrophoresis (MLEE) (Bibb et al., 1990), pulsed-field gel electrophoresis (PFGE) (Vela et al., 2001), random amplified polymorphic differences (RAPD) (Mazurier et al., 1992) and ribotyping (Wiedmann et al., 1996). The methods differ in their discriminatory abilities and reproducibility, and some of them are limited to a few laboratories.

DNA sequence-based methods are increasingly used for subtyping and characterizing bacterial isolates. In these methods, complete or partial nucleotide sequences are determined for one or more bacterial genes or chromosomal regions, thus providing unambiguous and discrete data. Recently, a novel molecular typing method, multilocus sequence typing (MLST) has been developed. It is a nucleotide sequence-based typing method that indexes the variation present in bacterial housekeeping genes, where most of the variation is selectively neutral (Maiden et al., 1998). Internal fragments of several housekeeping genes, approximately 450~500 bp in length, are sequenced and novel alleles are assigned with arbitrary numbers sequentially to provide an allelic profile of their integers that defines the sequence type (ST) of each isolate. This technique has rapidly been applied to the study of many other bacterial species (Dingle et al., 2001; Kotetishvili et al., 2002; Enright et al., 2000).

As a preliminary data for MLST, the aim of this study was to estimate the genetic diversity of 28 *L. monocytogenes* isolates using sequence-based typing method based on two housekeeping genes, *dat* and *hlyA*, and to compare them with PFGE patterns.

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## MATERIALS AND METHODS

### 1. Bacterial strains

A total of 28 *L. monocytogenes* strains were included in this study (Table 1). Two isolates from the previous study (Kim et al., 2005) were excluded due to uncultivable condition in this experiment. They consisted of 12 isolates from pork, 9 from beef and 7 from chicken carcasses including 3 chicken products. Thirteen isolates among them were isolated from the imported meats. All isolates were biochemically identified as *L. monocytogenes* by the commercial API system (bioMerieux, France). PFGE procedure has been performed in previous study mentioned above.

### 2. PCR and nucleotide sequencing

The two gene loci selected for sequence typing were *dat* (encoding D-amino acid aminotransferase) and *hlyA* (encoding listeriolysin O). Bacterial DNA extraction for PCR was prepared by the boiling of each 2~3 colonies in 500 μl distilled water followed by centrifugation for 5 min. The PCR amplification was performed as previously described (Revazishvili et al., 2004; Salcedo et al., 2003). The PCR condition for *hlyA* gene was as follows: an initial cycle at 94℃ for 5 min; 35 cycles, each consisting of sequential incubation at 94℃ for 45 s, 55℃ for 45 s and 72℃ for 1 min; and a final incubation at 72℃ for 5 min. When the primers for *dat* gene were used, the condition was an initial denaturation at 94℃ for 4 min followed by 25 cycles of sequential incubation at 94℃ for 30 s, 52℃ for 30 s and 72℃ for 2 min, and a final incubation at 72℃ for 10 min. Amplified fragments were purified and sequenced in both directions on ABI 3100 capillary DNA sequencer (Applied Biosystems, USA). Only sequences with complete agreement between the two strands were used for further analysis.

### 3. Data analysis

Initial trimming and multiple-sequences alignment of the two housekeeping gene segments were performed with CLUSTALX program (Jeanmougin et al., 1998). Those base positions that could not be aligned unambiguously were removed. Sequencing analyses were carried out with PHYLIP program. Pairwise distances between sequences were inferred to the Jukes and Cantor one-parameter model. Trees were constructed using neighbor joining algorithm.

**Table 1.** Characteristics of 28 *L. monocytogenes* according to PFGE and sequence types

| Isolate | Origin | Source | PFGE type[a] | STs[b] |
|---|---|---|---|---|
| B1/03 | domestic | Beef | 1c | 1, 1 |
| B2/03 | domestic | Beef | 1c | 1, 1 |
| B3/03 | domestic | Beef | 1c | 1, 1 |
| B4/03 | domestic | Beef | 1c | 1, 1 |
| B5/03 | domestic | Beef | 1c | 1, 1 |
| B6/03 | domestic | Beef | 1b | 1, 1 |
| B7/03 | domestic | Beef | 1c | 1, 1 |
| B8/03 | Austria | Beef | 4b | 2b, 1 |
| B9/03 | USA | Beef | 8 | 3a, 3b |
| P1/03 | domestic | Pork | 2c | 1, 1 |
| P2/03 | domestic | Pork | 2b | 1, 1 |
| P3/03 | domestic | Pork | 2b | 1, 1 |
| P4/03 | domestic | Pork | 2c | 1, 1 |
| P5/03 | domestic | Pork | 2d | 1, 1 |
| P6/04 | Belgium | Pork | 4a | 2b, 1 |
| P7/01 | Canada | Pork | 6 | 2a, 2c |
| P8/01 | Denmark | Pork | 10 | 2a, 2a |
| P10/03 | France | Pork | 5 | 2b, 2b |
| P11/03 | France | Pork | 5 | 2b, 2b |
| P12/02 | Hungary | Pork | 11 | 3a, 3b |
| P13/01 | Netherlands | Pork | 3a | 1, 1 |
| C1/03 | domestic | Chicken | 2a | 1, 1 |
| C2/03 | domestic | Chicken | 1a | 1, 1 |
| C3/02 | Thailand | Chicken | 9 | 3b, 3a |
| C4/02 | Thailand | Chicken | 9 | 3b, 3a |
| M1/03 | domestic | Chicken fried | 2b | 1, 1 |
| M2/03 | domestic | Chicken rib | 2e | 1, 1 |
| M3/03 | domestic | Chicken wing | 7 | 3a, 3a |

[a] See reference Kim et al. (2005).
[b] Sequence types: sequence type by *dat* and *hlyA* gene in order

## RESULTS

A total of 465 nucleotide bases for *dat* gene of each isolate comprised the final alignment, and three main phylogenetic clusters were found (Fig. 1). Seventeen isolates showing all 100% sequence similarity (percent *S* value) were observed in cluster I. Cluster II consisted of six isolates, which was divided into two subgroups with isolates in each subgroup showing an identical sequence. Isolates belonging to cluster II had sequence similarity of 99.99% *S* with isolates in cluster I. An outlier group, cluster III was formed by five isolates, and were divided into two subgroups. Those five isolates had sequence similarity of 99.87% *S* with isolates in cluster I and II.

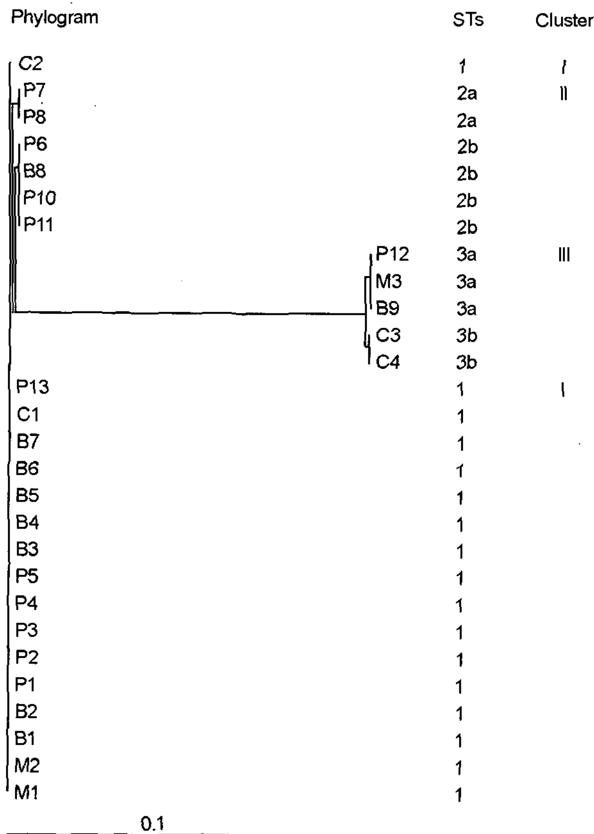


**Fig. 1.** Phylogenetic tree of 28 *L. monocytogenes* isolates based on the numbers of nucleotide differences in *dat* gene fragment. STs indicate sequence type of each isolate.

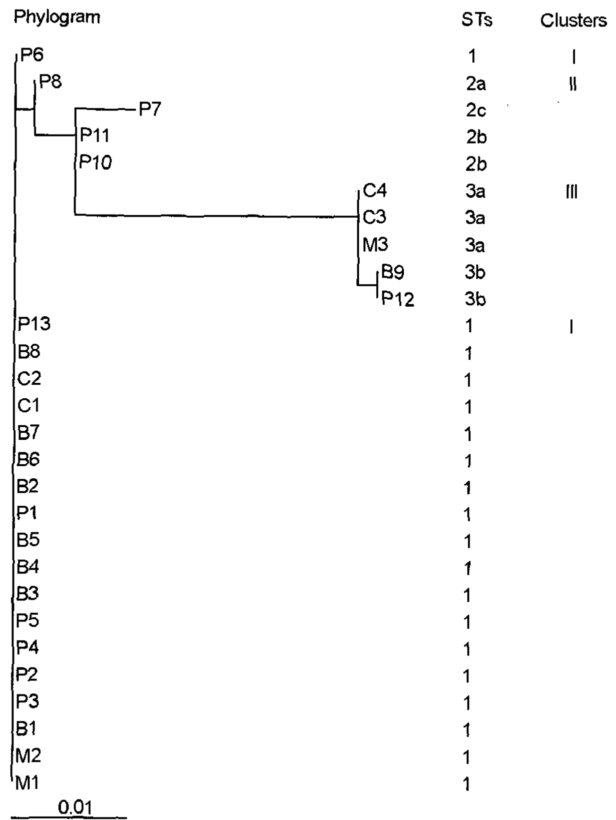


**Fig. 2.** Phylogenetic tree of 28 *L. monocytogenes* isolates based on the numbers of nucleotide differences in *hlyA* gene fragment. STs indicate sequence type of each isolate.

The results of *hlyA* gene sequence analysis based on 584 nucleotides for each isolate were shown in Fig. 2. Three main clusters were also found and showed the similar pattern of phylogenetic tree compared to that of *dat* gene. All nineteen isolates belonging to cluster I showed 100% *S* sequence similarity. They included two isolates in addition to the seventeen isolates in cluster I of *dat* gene analysis. Cluster II contained three subgroups, each consisting of one, three and five isolates, respectively. Cluster III, an outlier group, which contained the same five isolates observed in the analysis of *dat* gene, was also found. They had sequence similarity of 99.96% *S* with isolates in cluster I.

In terms of origin of country from each isolate, all seventeen isolates from domestic carcasses and meat products revealed 100% *S* sequence similarity for dat gene. Eleven isolates from imported carcasses were clustered separately except one isolate, which was originated from Netherlands pork. Similar result was obtained with *hlyA* gene sequence. Two additional isolates, however, one from Austria and the other from Belgium, had the same sequences with the domestic isolates. These sequence analysis for two genes could not differentiated the isolates in terms of different sources, e.g., beef, pork, chicken and meat product.

## DISCUSSION

While the commonly used DNA-based subtyping methods, such as RAPD, PFGE and MLEE, have proven effective for differentiating *L. monocytogenes*, their discriminatory abilities are not optimal and sometimes cannot differentiate epidemiologically unrelated strains (Gravesen et al., 2000). In addition, they generally do not provide information amenable to the inference of primary genetic characteristics for evolutionary analyses. To overcome the ambiguities of fragment-based typing methods, DNA sequencing-based methods are being developed and increasingly used for subtyping and characterizing bacterial isolates.

We selected the two housekeeping genes, *dat* and *hlyA*, to determine the suitability of DNA sequencing analysis for

differentiating *L. monocytogenes* isolates based on the previous studies by Salcedo et al. (2003) and Revazishvili et al. (2004). They found that the two loci showed the quite high sequence diversities among several housekeeping and virulent genes of *L. monocytogenes* strains. Sequence analysis of 28 *L. monocytogenes* isolates in this study found that their sequence similarities of two genes among strains were very high (>99.87% *S*) and that those isolates were grouped into three clusters each. Also, our observations that the number of STs was ca. 3-fold greater than the number of PFGE types (3 STs versus 11 PFGE types) by previous study (Kim et al., 2005) and that several strains within the same ST were differentiated by PFGE suggest that discriminatory power of PFGE is greater than that of ST using two genes. Revazishvili et al. (2004) reported that MLST using four housekeeping genes had greater discriminatory ability for subtyping *L. monocytogenes* than that of PFGE. It could be concluded that single or two genes sequence analysis were not appropriate for retaining diverse phylogenetic information though they showed the very high sequence diversity among strains. Including additional loci in the analysis is likely to further increase the discriminatory power of sequence typing for *L. monocytogenes* strains.

However, there were good congruence between the PFGE patterns and phylogenetic grouping of two gene sequences. For example, all isolates in PFGE types 1 to 3 were clustered into ST 1 using *dat* gene. And, isolates in PFGE types 4 to 6 and 7 to 10 belonged to ST 2 and ST 3, respectively. This meant that the clustering of 28 isolates by sequence analysis of *dat* gene coincided at 65% similarity with PFGE. Eleven PFGE types of 30 *L. monocytogenes* isolates were identified at 75% Similarity in the dendrogram (Kim et al., 2005). Similar results were also found in the analysis of *hlyA* gene sequence. These results supported the finding of MLST (Salcedo et al., 2003) and multi-virulence-locus-sequence typing (MVLST) by Zhang et al. (2004) that the discriminatory power of phylogenetic analysis using these methods was comparable or higher than that of PFGE.

We also found the close relationship between isolates from origin of country. All isolates from the domestic sources had the same sequences with two genes. Zhang et al. (2004) suggested based on allelic analyses of several virulence genes that clinical (presumably highly pathogenic) *L. monocytogenes* isolates were of clonal origin and that they were grouped in distinct lineages. Sequencing of additional loci including those from *Listeria* virulence gene may identify the critical nucleotide substitutions responsible for clustering of *L. monocytogenes* isolates that have high potential for the origin of isolates. In conclusion, there was a correlation between the PFGE patterns and phylogenetic grouping of two gene sequences though the sequence analysis using two genes only seemed to be less discriminatory for *L. monocytogenes* subtyping. Also, sequence typing of *L. monocytogenes* isolates using two genes with high sequence diversity appears to be available for epidemiological studies and characterizing *L. monocytogenes*.

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