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# Development of a Multilocus Sequence Typing Method for Analysis of *Listeria monocytogenes* Clones

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**This study is a first step in the development of multilocus sequence typing (MLST) method for *Listeria monocytogenes*. Nine housekeeping genes were analyzed in a set of 62 strains isolated from different sources and geographic locations in Spain. These strains were previously characterized by pulsed-field gel electrophoresis (PFGE). Because of low diversity, two loci were discarded from the study. The sequence analysis of the seven remaining genes showed 29 different allelic combinations, with 22 of them represented by only one strain. The results of this sequence analysis were generally consistent with those of PFGE. Because MLST allows the easy comparison and exchange of results obtained in different laboratories, the future application of this new molecular method could be a useful tool for the listeriosis surveillance systems that will allow the identification and distribution of analysis of *L. monocytogenes* clones in the environment.**

*Listeria monocytogenes* is an opportunistic pathogen widely distributed in the environment. The ubiquity of this microorganism makes especially necessary the use of typing methods for the study of its epidemiology. Numerous molecular methods have been applied to the characterization of *L. monocytogenes* isolates, e.g., multilocus enzyme electrophoresis (MLEE) (1), pulsed-field gel electrophoresis (PFGE) (2, 29), random amplified polymorphic differences (19), and ribotyping (30), etc. By these methods the species is divided into two genetic divisions which are correlated with the flagellar antigen groups division I, composed of strains of serotypes 1/2a and 1/2c, and division II, which is composed of strains of serotypes 1/2b and 4b. Both divisions are characterized by nonoverlapping allelic variants of different genetic markers, suggesting strong linkage disequilibrium and an apparent lack of gene exchange between them (15). This is consistent with the hypothesis that the genetic structure of *L. monocytogenes* populations is basically clonal (21). Recently, an additional division has been proposed based on the variability of the sequence of several genes involved in virulence (22).

MLEE has been the most widely used molecular method to study the genetic structure and epidemiology of pathogenic bacterial species. Recently, a novel molecular typing method based on the principles of MLEE (25) has been developed, multilocus sequence typing (MLST). This technique was primarily designed and validated for *Neisseria meningitidis* (17). Afterwards, it was successful in the characterization of several other pathogenic bacteria, such as *Streptococcus pneumoniae* (5), *Streptococcus pyogenes* (8), *Staphylococcus aureus* (7), and *Campylobacter jejuni* (4).

MLST makes use of automated DNA sequencing to characterize the alleles present at different housekeeping genes. Because it is based on nucleotide sequence, it is highly discriminatory and provides unambiguous results that are directly comparable among laboratories via the internet (7). In addition, this method is particularly suited to global epidemiology studies because the accumulation of nucleotide changes in housekeeping genes is a relatively slow process and allelic profiles are stable over time (6).

Epidemiological research requires systems that make easy the comparison and exchange of results obtained in different laboratories. The most important advantage of MLST over the typing methods based on comparisons of DNA fragments is the unambiguity and electronic portability of nucleotide sequence data (17), which allow a readily comparison of results among laboratories.

In this study we describe a first step for developing the MLST method for *L. monocytogenes.* The sequence diversity of nine housekeeping genes, possible candidates to be included in a future MLST scheme, has been analyzed.

## MATERIALS AND METHODS

**Bacterial strains.** A total of 62 strains of *L. monocytogenes* were used in this study. Thirty-one were isolated from human cases of listeriosis, 12 were isolated from animal clinical sources, and 19 were isolated from food. The strains were isolated in different regions across Spain over the period from 1995 to 2001 without an evident epidemiological link among them. Serotyping was done according the method previously described (24). The three most frequent serotypes among the clinical isolates of *L. monocytogenes* were represented: 1/2a (12 strains), 1/2b (10 strains), and 4b (40 strains).

Our laboratory uses PFGE to characterize the isolates sent by hospitals and other public health laboratories. The agarose blocks are prepared by following a method previously described by Graves and Swaminathan (12). Chromosomal DNA is digested with *Apa*I (Pharmacia), and the restricted fragments are separated at 200 V with pulse times from 0.1 to 25 s over 21 h on a CHEF DR-III PFGE system (Bio-Rad, Hemel Hempstead, United Kingdom).

Isolates with different PFGE patterns were chosen for the sequence analysis of housekeeping genes. Of those PFGE patterns more frequently found, several isolates were selected.

**Amplification and nucleotide sequencing.** The following 9 housekeeping genes were chosen to be analyzed: ABC transporter (*abcZ*), D-amino acid aminotransferase (*dat*), L-lactate dehydrogenase (*ldh*), superoxide dismutase (*sod*), catalase (*cat*), succinyl diaminopimelate dessucinylase (*dapE*), phosphoglucomutase (*pgm*), beta-glucosidase (*bglA*), and histidine kinase (*lhkA*). The DNA sequences

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TABLE 1. Primers used for PCR amplification and sequencing of *L. monocytogenes* housekeeping genes

| Gene | Primer and sequence (5′–3′) | |
|---|---|---|
| | Forward | Reverse |
| *abcZ* | *abcZ*-Up, TCGCTGCTGCCACTTTTATCCA | *abcZ*-Dn, TCAAGGTCGCCGTTTAGAG |
| *bglA* | *bglA*-Up, GCCGACTTTTTATGGGGTGGAG | *bglA*-Dn, CGATTAAATACGGTGCGGACATA |
| *cat* | *cat*-Up, ATTGGCGCATTTTGATAGAGA | *cat*-Dn, AGATTGACGATTCCTGCTTTTG |
| *dapE* | *dapE*-Up, CGACTAATGGGCATGAAGAACAAG | *dapE*-Dn, ATCGAACTATGGGCATTTTTACC |
| *dat* | *dat*-Up, GAAAGAGAAGATGCCACAGTTGA | *dat*-Dn, TGCGTCCATAATACACCATCTTT |
| *ldh* | *ldh*-Up, ATTTTGATCGTATTGGGGTTTT | *ldh*-Dn, TACTGAATGGATTAGCGAAGATGA |
| *lhkA* | *lhkA*-Up, AGAATGCCAACGACGAAACC | *lhkA*-Dn, TGGGAAACATCAGCAATAAAC |
| *pgm* | *pgm*-Up, CCGATGATCAGGAAGAAGAAAT | *pgm*-Dn, CTGTCAAAATCGCCATCAAA |
| *sod* | *sod*-Up, GCGGTTGCTGGTCATCCT | *sod*-Dn, GCGTTTGTTAGCTTCATCCCAGTT |

of these candidate loci were available from GenBank. Some of these genes (*cat*, *ldh*, *sod*, and *pgm*) have been previously included in the MLEE system, and their genetic diversity in this species is known.

To identify the most polymorphic regions within each locus, several internal fragments were analyzed in 10 *L. monocytogenes* strains not related by PFGE (27). Those fragments showing the highest number of alleles were chosen for the analysis. The most variable ones were selected to be analyzed for the remaining isolates. DNAStar (Madison, Wis.) was used to design the primers for the amplification and sequencing of the selected fragments (Table 1).

Bacterial cell lysates were obtained by sonication (10 min) followed by centrifugation for 5 min. The PCR conditions were initial denaturation at 94°C for 4 min followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s (except for *pgm* and *bglA*, which had an annealing temperature of 45°C), and extension at 72°C for 2 min followed by a final extension step of 72°C for 10 min. The DNA fragments were purified by using a PCR purification kit (Qiagen) and were sequenced in each direction with Big Dye fluorescent terminators (PE Applied Biosystems) on an Applied Biosystems Prism 377 automated sequencer.

**Data analysis.** The analysis of sequences obtained for each gene fragment was performed with the DNAStar program. For each fragment, the sequences obtained from the 62 strains were compared and allele numbers were assigned to each unique sequence. Each isolate was defined by the combination of numbers corresponding to the alleles at the loci analyzed, which is an allele profile or sequence type (ST). Sequences different even at a single nucleotide site were considered distinct alleles.

A dendrogram displaying the genetic linkage distance between each ST was constructed from the matrix of pairwise differences among the allelic profiles by the unweighted pair group cluster method with arithmetic mean (Fig. 1). This data analysis was performed by using the START software (http://www.mlst.net). The index of association ($I_A$) (18) was used to test for linkage disequilibrium among alleles at the housekeeping loci. The significance of the $I_A$ was calculated by using the program at the MLST website (http://www.mlst.net).

**Nucleotide sequence accession numbers.** The nucleotide sequences of each allele at the nine loci analyzed in the present work have been assigned the following GenBank accession numbers: AY 158265 to AY 158276 (*abcZ*), AY 158286 to AY158295 (*bglA*), AY158249 to AY 158264 (*cat*), AY 158303 to AY 158316 (*dapE*), AY158277 to AY 158284 (*dat*), AY 160115 to AY 160122 (*ldh*), AY 158296 to AY 158302 (*lhkA*), AY 158322 to AY 158326 (*pgm*), and AY 158317 to AY158321 (*sod*).

## RESULTS

**Diversity of the housekeeping genes analyzed.** The length of the analyzed fragments ranged between 354 bp (*ldh*) and 552 bp (*abcZ*) (Table 2).

The sequence diversity found in these fragments was quite heterogeneous. The loci *sod* and *pgm* showed the lowest sequence diversity. So after the analysis of 40 strains, only one allele was found among the strains of serotypes 4b and 1/2b. Those genes were finally discarded due to a low contribution to the discrimination degree of this method. The locus with the highest diversity was *cat* with 16 alleles.

The proportion of variable sites present in the alleles ranged from 2.9% (*sod*) to 12.4% (*dat*). Ratios of nonsynonymous (*dN*) to synonymous (*dS*) changes derived from pairwise sequence comparison were much less than 1 for all studied loci (Table 2).

**Relationship of *L. monocytogenes* isolates by sequence analysis of housekeeping loci.** A total of 29 allelic profiles or STs were identified, and 22 of these (75.8%) were represented by only one strain. The most common STs were ST6 and ST2, which grouped together 14 and 10 isolates, respectively (Table 3).

Those isolates that shared five or more loci in the allelic profile were considered members of the same clonal complex (9). The clonal complex that contained the largest number of isolates (17 isolates of serotype 4b) was the ST6 complex, which included ST6 (as the prevalent ST) and three other allelic profiles (ST7, ST8, and ST9) represented by a single isolate that differed from it at only one or two loci (Fig. 1). This lineage included 11 of the 21 serotype 4b human isolates studied.

Significant linkage disequilibrium was detected when a complete data set or STs were used in the analysis ($I_A$, 2.195 [$P < 0.01$] and $I_A$, 1.183 [$P < 0.01$], respectively). However, evidence for linkage equilibrium was found when the $I_A$ was calculated at the level of STs separately for the two genetic divisions (serotype 1/2a strains and serotype 1/2b and 4b strains). Values of $I_A$ obtained for these two divisions were 0.28 ($P > 0.001$) for division I and 0.34 ($P > 0.001$) for division II.

**Relationship between allelic profiles and serotypes.** The analysis of the housekeeping genes revealed that the sequences of serotype 1/2b and 4b isolates were more closely related to each other, or even identical, and showed significant divergence from the sequences of serotype 1/2a isolates. For all analyzed loci, alleles common to serotype 4b and 1/2b STs were found, whereas alleles present in serotype 1/2a STs were only found in this serotype (Table 3).

**Congruence between STs and PFGE fingerprints.** The similarity of PFGE profiles among isolates belonging to same ST or to closely related STs was analyzed. Nevertheless, because both markers are based on very different principles, this comparison should be carefully interpreted.

We found a high number of different cases with STs composed of strains showing a unique PFGE profile (ST3 or ST29) and also STs consisting of isolates with different PFGE pat-

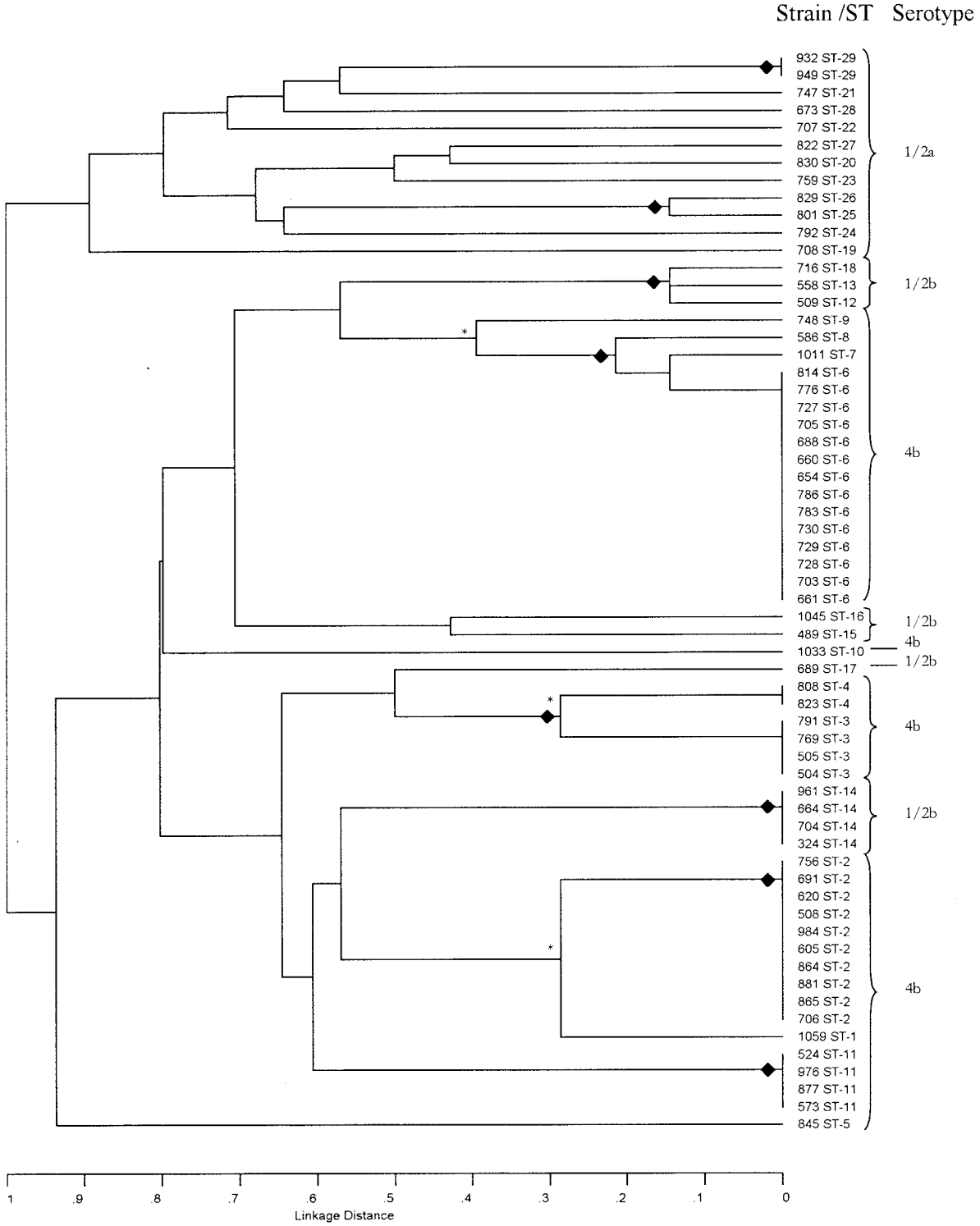


FIG. 1. Dendrogram showing cluster analysis (unweighted pair group cluster method with arithmetic mean) of the 62 *L. monocytogenes* isolates. The serotype associated with each ST is shown. *, clonal complexes that were identified; ◆, branches containing isolates that showed related PFGE patterns. Only one exception was found in ST2 (strain 706).

terns (ST2, ST4, or ST6) (Table 3). However, the consistency of the data between both methods was good in general, and the clustering of the strains was similar. Those STs defined as closely related (only one allele of difference) grouped together isolates with related or closely related PFGE pattern profiles (less than five band differences) (ST25 and ST26, or ST12, ST13, and ST18) (Fig. 1). Only one isolate of ST2 showed a higher number of fragment differences.

TABLE 2. Genetic diversity of the *L. monocytogenes* housekeeping genes analyzed

| Gene | Size of fragment (bp) | No. of alleles | No. of variable sites | % Variable nucleotide sites | *dN*/*dS* ratio |
|---|---|---|---|---|---|
| *abcZ* | 552 | 12 | 34 | 6.1 | 0.058 |
| *bglA* | 417 | 10 | 21 | 5.0 | 0.095 |
| *cat* | 501 | 16 | 39 | 7.8 | 0.153 |
| *dapE* | 480 | 14 | 40 | 8.3 | 0.225 |
| *dat* | 484 | 8 | 60 | 12.4 | 0.216 |
| *ldh* | 354 | 8 | 19 | 4.3 | 0.000 |
| *lhkA* | 488 | 7 | 17 | 3.5 | 0.117 |
| *pgm*[a] | 364 | 5 | 16 | 4.4 | 0.000 |
| *sod*[a] | 420 | 5 | 12 | 2.9 | 0.166 |

[a] Loci that were only analyzed in 40 strains. These loci were not considered for the final MLST scheme because of low sequence diversity.

## DISCUSSION

The present study was conceived as a first step in the develop of MLST for *L. monocytogenes*, with the first objective being the selection of a set of housekeeping genes that were suited according to the MLST requirements. To evaluate the sequence diversity of these loci, we considered it appropriate to use a genetically diverse group of strains. With this purpose, sequence analysis of the housekeeping genes was carried out in a group of isolates previously characterized by PFGE. These strains were isolated from different sources and geographic locations of Spain during recent years. Isolates representing different PFGE patterns were included in this MLST project.

Generally, a good congruence was found among groupings obtained by sequence analysis of housekeeping genes and those obtained by using PFGE. Thus, isolates that were identical by MLST showed the same PFGE profile or patterns that differed at 1 to 5 fragments (27). We only found one exception: one isolate belonging to ST2 which showed greater differences with regard to other PFGE profiles associated with this ST. Similar patterns were also observed in those isolates that shared six of the seven alleles. The similarity was lower in those isolates that shared five of the seven loci.

Two isolates with the same allelic profile descending from a common ancestor might show PFGE profiles that are relatively different because PFGE is a highly discriminative method which is sensible to microvariation (17). It is important to take into account that a single nucleotide change that supposes a gain or loss of a restriction site can cause even three fragment differences (27), whereas MLST detects variation that accumulates slowly in housekeeping genes (26).

The validity of this molecular method is also supported by the congruence of the genetic data with previous MLEE studies that suggest that the genetic structure of *L. monocytogenes* is basically clonal (21). Thus, the value of the $I_A$ indicated a significant linkage disequilibrium among the alleles at each of the seven housekeeping loci ($P < 0.01$) (18). However, no evidence for linkage was detected when the analysis was performed separately in division I (serotype 1/2a isolates) and

TABLE 3. Properties of the 29 allelic profiles (STs)

| ST | No. of allele combinations in gene: | | | | | | | Origin(s) (no. of isolates)[a] | Serotype | PFGE pattern(s) (*Apa*I) |
|---|---|---|---|---|---|---|---|---|---|---|
| | *abcZ* | *bglA* | *cat* | *dap* | *dat* | *ldh* | *lhkA* | | | |
| 1 | 1 | 1 | 9 | 13 | 2 | 2 | 5 | H (1) | 4b | 19 |
| 2 | 1 | 1 | 11 | 11 | 2 | 2 | 5 | H (4), F (6) | 4b | 20, 21a, 21b, 22, 23, 24, 25 |
| 3 | 1 | 2 | 9 | 1 | 2 | 2 | 3 | A (4) | 4b | 26 |
| 4 | 1 | 2 | 12 | 3 | 2 | 2 | 3 | H (1), A (1) | 4b | 27, 28 |
| 5 | 1 | 3 | 7 | 2 | 4 | 6 | 2 | H (1) | 4b | 29 |
| 6 | 3 | 1 | 1 | 1 | 3 | 2 | 3 | H (10), F (1), A (3) | 4b | 30, 31, 32, 33, 34, 35 |
| 7 | 3 | 1 | 1 | 12 | 3 | 2 | 3 | F (1) | 4b | 36 |
| 8 | 3 | 1 | 11 | 1 | 3 | 2 | 3 | F (1) | 4b | 31 |
| 9 | 3 | 1 | 1 | 1 | 7 | 2 | 4 | H (1) | 4b | 37 |
| 10 | 3 | 1 | 14 | 12 | 2 | 8 | 3 | H (1) | 4b | 38 |
| 11 | 3 | 9 | 9 | 3 | 3 | 2 | 5 | H (2), F (2) | 4b | 39, 40a, 40b, 40c |
| 12 | 1 | 1 | 4 | 14 | 3 | 2 | 4 | F (1) | 1/2b | 11a |
| 13 | 10 | 1 | 4 | 14 | 3 | 2 | 4 | H (1) | 1/2b | 11b |
| 14 | 4 | 4 | 4 | 3 | 2 | 2 | 5 | H (1), F (3) | 1/2b | 12a, 12b, 13, 14 |
| 15 | 2 | 1 | 11 | 3 | 3 | 6 | 7 | H (1) | 1/2b | 15 |
| 16 | 11 | 1 | 12 | 14 | 3 | 6 | 7 | H (1) | 1/2b | 16 |
| 17 | 1 | 9 | 1 | 3 | 7 | 2 | 3 | F (1) | 1/2b | 17 |
| 18 | 12 | 1 | 4 | 14 | 3 | 2 | 4 | F (1) | 1/2b | 18 |
| 19 | 5 | 7 | 3 | 5 | 1 | 3 | 6 | H (1) | 1/2a | 1 |
| 20 | 5 | 6 | 2 | 9 | 5 | 5 | 1 | F (1) | 1/2a | 2 |
| 21 | 5 | 8 | 5 | 7 | 6 | 1 | 1 | H (1) | 1/2a | 3 |
| 22 | 6 | 5 | 6 | 4 | 1 | 1 | 1 | H (1) | 1/2a | 4 |
| 23 | 7 | 6 | 10 | 6 | 1 | 5 | 1 | H (1) | 1/2a | 5 |
| 24 | 7 | 7 | 3 | 10 | 5 | 4 | 1 | A (1) | 1/2a | 6 |
| 25 | 7 | 6 | 8 | 8 | 6 | 4 | 1 | A (1) | 1/2a | 7a |
| 26 | 7 | 6 | 8 | 8 | 6 | 7 | 1 | F (1) | 1/2a | 7b |
| 27 | 8 | 6 | 13 | 6 | 5 | 5 | 1 | H (1) | 1/2a | 8 |
| 28 | 9 | 10 | 15 | 6 | 8 | 1 | 1 | A (1) | 1/2a | 9 |
| 29 | 7 | 10 | 16 | 7 | 5 | 1 | 1 | H (1), A (1) | 1/2a | 10 |

[a] Origin codes: H, human infections; A, animal infections; F, food products.

division II (serotype 1/2b and 4b strains). These data suggest that the recombination should be rare between strains belonging to different genetic lineages, but the evidence for clonal structure disappears when the division I and II alleles are analyzed separately. Bacteriophage might probably play an important role in the genomic plasticity of *Listeria* (28), and this factor might be involved in some of the differences found in this study. In addition, similarities in the genome sequence between serotype 4b and 1/2b isolates might explain a higher prevalence of homologous recombination between these strains. The extension of the analysis to a higher number of strains will offer information about the relative contribution of recombination and mutation to the allele variation found.

In addition, the genetic relationships among different serotypes showed by this gene allelic analysis were also consistent with those previously established by MLEE, which divides *L. monocytogenes* into the two genetic divisions already mentioned. However, an additional division has been proposed after analysis of the sequence of genes associated with virulence (22). Strains grouped in this third division consist of serotypes 4a and 4c, which were not included in our study. We think that those markers which are focused on housekeeping genes (MLEE) or random genetic characteristics (PFGE) are not able to distinguish more than two divisions, with the third division being evident when specific genes associated with virulence are analyzed. However, the future analysis by MLST of serotype 4a and 4c strains might confirm this hypothesis.

The utility of MLST and MLEE (25) for the analysis of the genetic structure of bacterial populations is mainly based on the characteristic of housekeeping genes to have a selectively neutral variability (9). Analysis of synonymous and nonsynonymous changes in the allele sequences of a locus can be used to determine if it is subject to positive selection, so a *dN*/*dS* ratio of greater than 1 implies selection for amino acid changes (4). In our genetic analysis, the seven loci had *dN*/*dS* ratios significantly lower than 1 (Table 2). Another important characteristic in relation to this fact is that the location of loci on the chromosome was distant enough to make the joint horizontal transfer of two loci unlikely. Recently, the sequencing of the *L. monocytogenes* genome has been finished and published (11), which has allowed us to know the chromosomal locations of the loci selected for this study. The analysis has revealed that all of these loci are unlinked on the chromosome, with the minimum distance being 41.3 kb.

Previous studies with the use of MLEE and other molecular methods (1, 3, 21) have revealed that despite the high diversity in natural populations of *L. monocytogenes*, only two clones have been responsible for most major outbreaks detected during the last decades in Europe and North America (10, 13, 14, 16, 23). These results suggested the hypothesis that most of the clinical cases are caused by only a few clones that either are particularly common in the environment or have an unusually high level of pathogenicity (20, 21). In this epidemiological context, MLST could be a useful tool for the surveillance systems for listeriosis that might allow the identification and analysis of the distribution of these *L. monocytogenes* clones in the environment. In fact, the isolates from ST2 and ST6 might be those strains belonging to the major clones, and the future running of a central database for the *Listeria* STs with the associated epidemiological data will allow us to confirm this and other questions.

The extension of the present analysis to a higher number of isolates could contribute to a better knowledge of the structure of the *L. monocytogenes* population. Besides, the future genetic analysis of other housekeeping loci with enough sequence diversity might improve the discrimination level of this new typing method.

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## REFERENCES

1. **Bibb, W. F., B. G. Gellin, R. Weaver, B. Schwartz, B. D. Plikaytis, M. W. Reeves, R. W. Pinner, and C. V. Broome.** 1990. Analysis of clinical and food-borne isolates of *Listeria monocytogenes* in the United States by multilocus enzyme electrophoresis and application of the method to epidemiologic investigations. Appl. Environ. Microbiol. **56:**2133–2141.
2. **Brosch, R., J. Chen, and J. B. Luchansky.** 1994. Pulsed-field fingerprinting of listeriae: identification of genomic divisions for *Listeria monocytogenes* and their correlation with serovar. Appl. Environ. Microbiol. **60:**2584–2592.
3. **Buchrieser, C., R. Brosch, B. Catimel, and J. Rocourt.** 1993. Pulsed-field gel electrophoresis applied for comparing *Listeria monocytogenes* strains involved in outbreaks. Can. J. Microbiol. **39:**395–401.
4. **Dingle, K. E., F. M. Colles, D. R. A. Wareing, R. Ure, A. J. Fox, F. E. Bolton, H. J. Bootsma, R. J. L. Willems, R. Urwin, and M. C. J. Maiden.** 2001. Multilocus sequence typing system for *Campylobacter jejuni.* J. Clin. Microbiol. **39:**14–23.
5. **Enright, M. C., and B. G. Spratt.** 1998. A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. Microbiology **144:**3049–3060.
6. **Enright, M. C., and B. G. Spratt.** 1999. Multilocus sequence typing. Trends Microbiol. **7:**482–487.
7. **Enright, M. C., N. P. J. Day, and B. G. Spratt.** 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. J. Clin. Microbiol. **38:**1008–1015.
8. **Enright, M. C., B. G. Spratt, and D. E. Bessen.** 2001. Multilocus sequence typing of *Streptococcus pyogenes* and the relationships between *emm* type and clone. Infect. Immun. **69:**2416–2427.
9. **Feil, E. J., M. C. J. Maiden, M. Achtman, and B. G. Spratt.** 1999. The relative contributions of recombination and mutation to the divergence of clones of *Neisseria meningitidis.* Mol. Biol. Evol. **16:**1496–1502.
10. **Fleming, D. W., S. L. Cochi, K. L. McDonald, J. Brondum, P. S. Hayes, B. D. Plikaytis, M. B. Holmes, A. Audurier, C. V. Broome, and A. L. Reingold.** 1985. Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. N. Engl. J. Med. **312:**404–407.
11. **Glaser, P., L. Frangeul, C. Buchrieser, C. Rusniok, A. Amend, F. Baquero, P. Berche, H. Bloecker, P. Brandt, T. Chakraborty, A. Charbit, F. Chetouani, E. Couve, A. de Daruvar, P. Dehoux, E. Domann, G. Dominguez-Bernal, E. Duchaud, L. Durant, O. Dussurget, K. D. Entian, H. Fsihi, F. G. Portillo, P. Garrido, L. Gautier, W. Goebel, N. Gomez-Lopez, T. Hain, J. Hauf, D. Jackson, L. M. Jones, U. Kaerst, J. Kreft, M. Kuhn, F. Kunst, G. Kurapkat, E. Madueño, A. Maitournam, J. M. Vicente, E. Ng, H. Nedjari, G. Nordsiek, S. Novella, B. de Pablos, J. C. Perez-Diaz, R. Purcell, B. Remmel, M. Rose, T. Schlueter, N. Simoes, A. Tierrez, J. A. Vazquez-Boland, H. Voss, J. Wehland, and P. Cossart.** 2001. Comparative genomics of Listeria species. Science **294:**849–852.
12. **Graves, L. M., and B. Swaminathan.** 2001. PulseNet standarized protocol for subtyping *Listeria monocytogenes* by macrorestriction and pulsed-field gel electrophoresis. Int. J. Food Microbiol. **65:**55–62.
13. **Ho, J. L., K. N. Shands, G. Friedland, P. Eckind, and D. W. Fraser.** 1986. An outbreak of type 4b *Listeria monocytogenes* infection involving patients from eight Boston hospitals. Arch. Intern. Med. **146:**520–524.
14. **Jacquet, C., B. Catimel, R. Brosch, C. Buchrieser, P. Dehaumont, V. Goulet, V. Lepoutre, P. Veit, and J. Rocourt.** 1995. Investigations related to the epidemic strain involved in the French listeriosis outbreak in 1992. Appl. Environ. Microbiol. **61:**2242–2246.
15. **Lan, Z., F. Fiedler, and S. Kathariou.** 2000. A sheep in wolf's clothing: *Listeria innocua* strains with teichoic acid-associated surface antigens and genes characteristic of *Listeria monocytogenes* serogroup 4. J. Bacteriol. **182:**6161–6168.
16. **Linnan, M. J., L. Mascola, X. D. Lou, V. Goulet, S. May, C. Salminen, D. W. Hird, M. L. Yonekura, P. Hayes, R. Weaver, A. Audurier, B. D. Plikaytis,**

**S. L. Fannin, A. Kleks, and C. V. Broome.** 1988. Epidemic listeriosis associated with Mexican-style cheese. N. Engl. J. Med. **319:**823–828.
17. **Maiden, M. C. J., J. A. Bygraves, and B. G. Spratt.** 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc. Natl. Acad. Sci. USA **95:** 3140–3145.
18. **Maynard Smith, J., N. H. Smith, M. O'Rourke, and B. G. Spratt.** 1993. How clonal are bacteria? Proc. Natl. Acad. Sci. USA **90:**4384–4388.
19. **Mazurier, S. I., and K. Wernars.** 1992. Typing of *Listeria* strains by random amplification of polymorphic DNA. Res. Microbiol. **143:**499–505.
20. **McLauchlin, J.** 1990. Distribution of serovars of *Listeria monocytogenes* isolated from different categories of patients with listeriosis. Eur. J. Clin. Microbiol. Infect. Dis. **9:**210–213.
21. **Piffaretti, J. C., H. Kressebuch, M. Aeschbacher, J. Bille, E. Bannerman, J. M. Musser, R. K. Selander, and J. Rocourt.** 1989. Genetic characterization of clones of the bacterium *Listeria monocytogenes* causing epidemic disease. Proc. Natl. Acad. Sci. USA **86:**3818–3822.
22. **Rasmussen, O. F., P. Skouboe, L. Dons, L. Rossen, and J. E. Olsen.** 1995. *Listeria monocytogenes* exists in at least three evolutionary lines: evidence from flagellin, invasive associated protein and listeriolysin O genes. Microbiology 141:2053–2061.
23. **Schlech, W. F., III, P. M. Lavigne, R. A. Bortolussi, A. C. Allen, E. V. Haldane, A. J. Wort, A. W. Hightower, S. E. Johnson, S. H. King, E. S. Nicholls, and C. V. Broome.** 1983. Epidemic listeriosis-evidence for transmission by food. N. Engl. J. Med. **308:**203–206.
24. **Seeliger, H. P. R., and K. Höhne.** 1979. Serotyping of *Listeria monocytogenes* and related species. Methods Microbiol. **13:**31–49.
25. **Selander, R. K., D. A. Caugant, H. Ochman, J. M. Musser, M. N. Gilmour, and T. S. Whittam.** 1986. Methods of multilocus enzyme electrophoresis for bacterial populations genetics and systematics. Appl. Environ. Microbiol. **51:**873–884.
26. **Spratt, B. G.** 1999. Multilocus sequence typing: molecular typing of bacterial pathogens in an era of rapid DNA sequencing and the internet. Curr. Opin. Microbiol. **2:**312–316.
27. **Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field electrophoresis: criteria for bacterial typing. J. Clin. Microbiol. **33:**2233–2239.
28. **Vázquez-Boland, J. A., G. Domínguez-Bernal, B. González-Zorn, J. Kreft, and W. Goebel.** 2001. Pathogenicity islands and virulence evolution in *Listeria.* Microbes Infect. **3:**571–584.
29. **Vela, A. I., J. F. Fernández-Garayzabal, J. A. Vazquez, M. V. Latre, M. M. Blanco, M. A. Moreno, L. De La Fuente, J. Marco, C. Franco, A. Cepeda, A. A. Rodríguez Moure, G. Suárez, and L. Domínguez.** 2001. Molecular typing by pulsed-field gel electrophoresis of Spanish animal and human *Listeria monocytogenes* isolates. Appl. Environ. Microbiol. **67:**5840–5843.
30. **Wiedmann, M., J. L. Bruce, R. Knorr, M. Bodis, E. M. Cole, C. I. McDowell, P. L. McDonough, and C. A. Batt.** 1996. Ribotype diversity of *Listeria monocytogenes* strains associated with outbreaks of listeriosis in ruminants. J. Clin. Microbiol. **34:**1086–1090.