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Thomas Cantinelli, Viviane Chenal-Francisque, Laure Diancourt, Lise Frezal, Alexandre Leclercq, Thierry Wirth, Marc Lecuit, Sylvain Brisse

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Epidemic clones of *Listeria monocytogenes* are widespread and ancient clonal groups

Thomas Cantinelli[1], Viviane Chenal-Francisque[1], Laure Diancourt[2], Lise Frezal[3], Alexandre Leclercq[1], Thierry Wirth[4,5], Marc Lecuit[1,6,7,*] and Sylvain Brisse[8,9*]

[1]Institut Pasteur, National Reference Center for *Listeria* and World Health Organisation Collaborating Center for *Listeria*, Biology of Infection Unit, Paris, France
[2]Institut Pasteur, Genotyping of Pathogens and Public Health, Paris, France
[3] Ecole Normale Supérieure, Department of Biology, 46 rue d'Ulm, Paris, France
[4]Laboratoire de Biologie Integrative, Ecole Pratique des Hautes Etudes, Paris, France
[5]Muséum National d'Histoire Naturelle, UMR-CNRS 7205, Département Systématique et Evolution, 16 rue Buffon, F-75231 Paris cedex 05, France
[6]Inserm U1117
[7] Université Paris Descartes, Assistance Publique-Hôpitaux de Paris, Service des Maladies Infectieuses et Tropicales, Hôpital Universitaire Necker-Enfants Malades, Institut Imagine, Paris, France.
8 Institut Pasteur, Microbial Evolutionary Genomics, Paris, France
9 CNRS, UMR3525, Paris 75015, France

* Corresponding authors:
S. Brisse. Institut Pasteur, Microbial Evolutionary Genomics, 28 rue du Dr Roux, F-75724 Paris, France. E-mail: sylvain.brisse@pasteur.fr; Phone +33 1 40 61 36 58.

M. Lecuit. Institut Pasteur, Biology of Infection Unit, 28 rue du Dr Roux, F-75724 Paris, France. E-mail: marc.lecuit@pasteur.fr; Phone: + 33 1 40 61 30 29.

Abstract

The food-borne pathogen *Listeria monocytogenes* is genetically heterogeneous. Although some clonal groups were implicated in multiple outbreaks, there is currently no consensus on how 'epidemic clones' should be defined. The objectives of this work were to compare the patterns of sequence diversity on two sets of genes that are widely used to define *L. monocytogenes* clonal groups: multilocus sequence typing (MLST) and multi-virulence locus sequence typing (MvLST). Further, we evaluated the diversity within clonal groups by pulsed-field gel electrophoresis (PFGE). Based on 125 isolates of diverse temporal, geographical and source origins, MLST and MvLST genes (i) had similar patterns of sequence polymorphism, recombination and selection, (ii) provided concordant phylogenetic clustering and (iii) had similar discriminatory power, which was not improved when combining both datasets. Inclusion of representative strains of previous outbreaks demonstrated the correspondence of 'epidemic clones' with previously recognized MLST clonal complexes. PFGE analysis demonstrated heterogeneity within major clones, most of which were isolated decades before their involvement in outbreaks. We conclude that the 'epidemic clones' denominations represent a redundant but largely incomplete nomenclature system for MLST-defined clones, which must be regarded as successful genetic groups that are widely distributed across time and space.

52 **Introduction**

53 *Listeria monocytogenes* is a foodborne pathogen that can cause listeriosis, a severe
54 invasive infection with a particularly high (20-30%) case fatality rate in persons at risk.
55 Listeriosis is currently regarded as increasing in incidence in Europe, especially in the elderly
56 (13, 23), and can occur in large outbreaks as illustrated in recent years (22, 38). To assist
57 epidemiological surveillance and outbreak investigation, different strain typing methods are
58 used, including serotyping and pulsed-field gel electrophoresis (17, 28). Population diversity
59 studies at the global scale have revealed that *L. monocytogenes* is a genetically heterogeneous
60 species (10, 46, 47, 56) and a variety of strain genotyping methods are used to characterize
61 and classify isolates into four major lineages and clonal groups thereof (7, 12, 37, 47, 50, 55,
62 56). The precise delineation of lineages and clonal groups is a prerequisite to characterize the
63 links between within-species genetic variation and important characteristics such as
64 pathogenic potential, virulence or epidemiology.

65 Given the facultative nature of genetic exchange in bacteria, reproduction is
66 predominantly clonal. In evolutionary biology terms, clones can be defined as groups of
67 isolates that descend from a common ancestor and accumulate differences among themselves
68 by a predominantly mutational process. As *L. monocytogenes* is one of the bacterial species
69 with the lowest rate of homologous recombination (11, 47), clones are expected to evolve
70 slowly and to be recognizable over large temporal and geographic scales. As a matter of fact,
71 the discovery of genetically similar isolates involved either in geographically and temporally
72 distant outbreaks or in large, single outbreaks led to the definition of *L. monocytogenes*
73 epidemic clones (EC) (6, 11, 27, 28, 46). Although initial ECs have been defined primarily
74 based on PFGE, MLEE and ribotyping (27, 28), subsequent ECs were mostly defined on the
75 basis of multi virulence locus sequence typing (MvLST) (6, 29, 38). MvLST, which is based
76 on the analysis of six to eight genes, has also been used to redefine previously described
77 epidemic clones (5, 6).

78 In many bacterial species, multilocus sequence typing (MLST) is used as a reference
79 method for clonal group definition (18, 39). An MLST scheme based on seven housekeeping
80 genes was developed for *L. monocytogenes* (47, 49). Using this approach, we recognized
81 highly prevalent clones (8, 47), which were defined using a simple and flexible operational
82 definition: clones are clonal complexes, i.e. groups that share 6 out of 7 allelic sequences with
83 at least one other member of the group. Currently, it is unknown how MLST clonal
84 complexes and epidemic clones correspond to each other.

The objectives of this study were (*i*) to determine the phylogenetic position of reference strains of previously defined epidemic clones within the MLST framework; (*ii*) to compare the patterns of diversity, recombination and selection of MLST and MvLST genes; and (*iii*) to estimate the amounts of PFGE diversity within clonal groups or epidemic clones. We demonstrate that MLST and MvLST define largely concordant clonal groups, and that the current approach to define 'epidemic clones' based on MvLST is redundant with the MLST nomenclature.

Materials and Methods

Strain selection

A total of 125 *Listeria monocytogenes* isolates were included (**Table S1**). First, in order to establish the position of outbreak strains or other reference strains in the international MLST scheme maintained at Institut Pasteur (www.pasteur.fr/mlst), a group of 50 strains was assembled. This reference set included (*i*) 27 reference strains from well documented outbreaks, including 19 strains of the outbreak set included in the ILSI collection (20) and 8 genome sequence reference strains corresponding to outbreak strains; (*ii*) fifteen lineage I and II strains from the diversity set of the ILSI collection; (*iii*) seven additional reference strains representing available genome sequences, and reference strain EGD. This reference set included the intensely studied laboratory reference strains EGD-e, LO28, 10304S, F2365 and Scott A, as well as strains representing 'epidemic clones' ECI, ECII, ECIII and ECIa/ECIV.

Second, with the aim of evaluating the ability of PFGE and MvLST to discriminate within and among MLST-defined clonal complexes (CC), we selected 75 isolates (the clone diversity set) representing multiple isolates of the major MLST-defined CCs, i.e. CC1, CC2, CC6, CC7, CC8 and CC9 (8, 47). These isolates were selected from our previous global MLST study of *L. monocytogenes* (8) to represent geographically and temporally diverse isolates. They originate from the Institut Pasteur *Listeria* Collection (CLIP) and from the Seeliger's *Listeria* Culture Collection (SLCC) (Rocourt and Seeliger, 1985).

Identification and serotyping

Isolates were identified as *L. monocytogenes* using API *Listeria* strips (BioMerieux, La Balme Les Grottes, France) and their serotype determined by classical serotyping and PCR serogrouping (15). Genomic DNA used as MLST or MvLST PCR template was extracted using the Promega Wizard Genomic DNA purification kit (Promega, Madison, WI, USA).

Multilocus sequence typing (MLST)

For the purposes of this study, MLST was performed as described (47). Novel alleles and profiles were incorporated into the international MLST database at www.pasteur.fr/mlst. MLST gene sequences from 16 publicly available genome sequences were extracted from the public sequence repositories.

Multi-virulence locus sequence typing (MvLST)

In this study, eight virulence-associated genes of *L. monocytogenes* were sequenced for 111 strains, while for 16 strains, the gene sequences were extracted from the available genome sequences. Six of the MvLST genes (*PrfA, inlB, inlC, clpP, dal, lisR*) were taken from S. Knabel's group MvLST scheme (59). The two remaining genes, *inlA* and *actA*, were included as they were used to complement the six previous ones in a subsequent MvLST analysis (6). Note that we sequenced the entire length of the *inlA* gene (2,400 nt), as we did in a previous study (47), whereas only 458 nt of this gene were sequenced in Chen et al. (2007). For *actA*, because we experienced difficulties in PCR amplification using previously described primers, we sequenced 450 nt from the 3' part of the gene, whereas the 582 nt template of Chen et al. (2007) corresponds to the 5' region of the *actA* gene. PCR primers (**Table 1**) were taken from Chen et al. (6) or designed in this study with EPRIMER3 (http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::eprimer3) based on the published genome sequence of *Listeria monocytogenes* strain EGD-e (NCBI RefSeq NC_003210.1). PCR amplification conditions as follows. For gene *dal*, 10 min at 95°C followed by 30 x (95°C 30 s, 57.6°C 30 s, 72°C 36 s) and 5 min at 72°C. For *prfA*, *inlB*, *inlC*, *clpP* and *lisR*, 15 min at 95°C followed by 25 x (94°C 30 s, 55°C 30 s, 72°C 1 min) and 7 min at 72°C. For *actA*, 10 min at 95°C followed by 30 x (95°C 30 s, 54,7°C 30 s, 72°C 36 s) and 5 min at 72°C. Finally for *inlA*, 5 min at 94°C followed by 35 x (94°C 30 s, 55,2°C 30 s, 72°C 90 s) and 10 min at 72°C. The PCR products were purified by ultrafiltration (Millipore, France) and were sequenced on both strands with Big Dye v.1.1 chemistry on an ABI3730XL sequencer (Applied BioSystems). As for MLST, each nucleotide was sequenced in both directions and validated by at least two independent chromatogram traces. MvLST gene sequences from published genome were extracted from GenBank entries.

Pulsed-field gel electrophoresis (PFGE)

Each isolate was typed by PFGE according to PulseNet standardized procedures with *AscI* and *ApaI* restriction enzymes (24). Data analysis was performed using BIONUMERICS version 6.5 (Applied Maths, Sint-Martens Latem, Belgium). *Apa*I and *Asc*I PFGE types were defined as differing from other types by at least two bands for each individual enzyme.

Phylogenetic reconstructions

Clonal complexes were defined based on MLST data as groups of allelic profiles sharing 6 out of 7 genes with at least one other member of the group (47). For phylogenetic

analyses, gene sequences were concatenated independently for the MLST and MvLST schemes, and neighbour-joining trees were obtained using BIONUMERICS based on the concatenated sequences using the *p*-distance (i.e., uncorrected percent of nucleotide mismatches). Nucleotide polymorphism and summary statistics were calculated using DNASP v5 (36). Minimum spanning trees were constructed using BIONUMERICS. Simpson's index and adjusted Rand coefficient were computed using the website www.comparingpartitions.info (3).

Recombination and selection analyses

We tested for recombination within phylogenetic lineages I and II for all loci independently, as well as for the concatenated MvLST and MLST loci using the LDHAT v2.2 software (40). LDHAT employs a coalescent-based method to estimate the population-scaled mutation ($\theta = 2N_e\mu$) and recombination ($\rho = 2N_e r$) rates, where N_e is the effective population size, r the rate at which recombination events separate adjacent nucleotides and μ is the mutation rate per nucleotide. The ratio r/μ were calculated as $\rho/(\theta/L)$, where L is the gene length (sequence length). This r/μ ratio ranges from 0, which indicates full clonal reproduction, to >>1, which is expected under free recombination. Significance of the evidence for recombination was tested using nonparametric, permutation-based tests implemented in LDHAT.

To detect signatures of positive selection, flat ω (d_N/d_S) ratios (as generated with DNASP) might fail to detect on-going selection on a fraction of the molecule. We therefore tested the presence of both recombination and positive selection using OMEGAMAP (57), which is able to disentangle the confounding effects of recombination and selection in a Bayesian framework. We chose a model with variable blocks of the ratio of non-synonymous to synonymous rates, ω, and the population recombination rate, ρ. A first run was performed for all loci using a block size set at 30 codons. A second run was performed using a block size set at 10 codons for the loci where a posterior probability of selection greater than 0.80 was detected in the first run. For all runs, an inverse distribution of range [0.01, 100] was used for ω and ρ. For the other parameters μ, κ and ΦInDel we used improper inverse distributions with starting values 0.1, 3.0 and 0.1, respectively. All genes were run with 100,000 iterations and 10 re-orderings, with the first 8,000 iterations discarded as burn-in period.

Results and Discussion

Phylogenetic positions of outbreak and reference strains based on MLST

To position reference strains, including isolates previously assigned to epidemic clones I to IV, within the diversity of *L. monocytogenes*, the sequences of internal portions of the seven multilocus sequence typing (MLST) genes (3,288 nucleotides in total) were gathered from 14 available genome sequences, 34 strains of the ILSI reference collection analyzed previously (7) and 76 isolates of clones belonging to CC1, CC2, CC6, CC7, CC8 and CC9 (7). In addition, we sequenced the 7 MLST genes from strain EGD, which is used as a reference strain (31, 41) but has not been genotyped by MLST to our knowledge. For these 125 isolates or reference strains, alignment of the sequences of six genes revealed no insertion/deletion event, whereas the *ldh* sequence of strain EGD revealed a 2-codons insertion at alignment position 423. There were 20 to 61 variable nucleotide sites per gene, corresponding to 7.5% of variable sites on average (**Table 2**). This variation allowed distinguishing 10 to 20 alleles per genes, resulting in 51 distinct sequence types (ST).

As previously described (7), clonal complex (CC) 1 included the reference strains of the 1981 Nova Scotia coleslaw outbreak, the 1983-1987 Switzerland Vacherin Mont d'Or outbreak, the 1986-1987 California Jalisco soft cheese outbreak and the 1992 French pork tongue in jelly outbreak (**Figure 1**). These strains were previously attributed to epidemic clone ECI (28). Clone CC2 comprised reference strains of the 1983 Massachusetts pasteurized milk outbreak (strain Scott A), the 1987-1988 UK and Ireland pâté outbreak, and the 1997 Italy gastroenteritis outbreak (**Figure 1**). These strains have been attributed to ECIa (27), later renamed ECIV with a more restricted definition that excluded the 1983 Massachusetts milk outbreak (6). All these reference strains including Scott A belonged to ST2, which is the central and most frequent genotype of CC2 (**Figure 1**). These results show that reference strains of ECI, ECII and ECIa/ECIV fall in MLST-defined CC1, CC6 and CC2, respectively. CC3 included the reference strain of the 1994 Illinois chocolate milk outbreak. CC4 included the 1999-2000 France pork rillettes outbreak. CC6 included the 1998-1999 USA multistate hotdog outbreak and the 2002 USA multistate delicatessen turkey outbreak, previously attributed to ECII. Finally, the 2000-2001 North Carolina Mexican-style fresh cheese outbreak reference strain belong to ST558 (**Figure 1**). The unique genotype of this outbreak strain is consistent with its characterization as ST24 (12). Note that ST5 included the isolates of the recently described ECVI clone (38).

Lineage II comprised reference strains of fewer outbreaks. ST11 included the 1988 Oklahoma turkey franks case and the two strains from the 2000 USA multistate sliced turkey deli meat outbreak, which was traced to the same food processing facility as the 1988 isolate. All three ST11 strains were previously attributed to ECIII (28). Note that whereas ECI, ECII and ECIV have been implicated in multiple outbreaks (28, 46), this was not the case to our knowledge for ECIII. CC8 comprised the 2008 Canada ready-to-eat meat products outbreak, consistent with previous report (22). Strains of this outbreak were recently proposed to represent ECV (29). Clone ECVII (involved in the 2011 US Cantaloupe outbreak) was shown to belong to CC7 (38).

The four widely used laboratory reference strains EGD, EGD-e, LO28 and 10403S belonged to two distant clonal complexes of lineage II, CC9 (EGD-e and LO28) and CC7 (10403S and EGD). These results indicate that despite their similar names, strains EGD and EGD-e appear to be phylogenetically distant (**Figure 1**), excluding direct laboratory descent between them.

The genome reference strains of serotype 1/2b FSL J2-064 and FSL N1-017 fell in major clones CC5 and CC3, respectively, thus also representing important clonal groups of *L. monocytogenes*. In contrast, the three genome reference strains FSL J1-194 (ST88), FSL J1-175 (ST87) and FSL N3-165 (ST90) represented rare STs not previously encountered, except for ST87, which was described for one food strain in Colombia (8).

To illustrate the position of the above studied strains within the diversity of *L. monocytogenes*, a minimum spanning tree (MStree) including all currently published MLST strains was constructed (**Figure 1**). Whereas outbreak strains mostly fell into the central and most frequent ST of clonal complexes, the laboratory reference strains EGD, EGD-e, LO28 and 10403S represented variant STs that were not commonly found among clinical or food isolates (**Figure 1**). Clearly, the diversity of *L. monocytogenes* is only partly represented by the laboratory reference strains that have been analysed so far in studies of host-pathogen interactions, virulence or other characteristics.

The distribution of MLST alleles showed that most CCs can be differentiated based on one or a few genes (**Table 3**). For example, allele *lhkA*-3 was uniquely observed for isolates of CC1, *abcZ*-1 was specific for CC2, and bglA-9 was shared by isolates of CC6. These CC-specific alleles allow a rapid screening of isolates to identify those belonging to CCs of interest. However, because recombination can occur, MLST based on the 7 genes sequences should be used for confirmation on selected isolates.

Diversity of MvLST genes and comparison with MLST

It has been suggested that virulence-associated genes included in MvLST studies evolve faster that MLST genes, which code for central, housekeeping functions of the cell (59). To test this hypothesis, the 125 study isolates were sequenced at eight MvLST genes used for epidemic clone definition (6, 29, 38). The genetic variation recorded at MvLST genes (**Table 2**) represented 6.62% of nucleotide sites on average, ranging from 3.06 for *inlC* to 12.6% for gene *dal*. This level of variation was therefore slightly lower than the amount observed for MLST genes (7.5%). The number of distinct alleles per gene varied from 4 alleles for gene *clpP* to 28 for gene *inlA*. When considering the eight MvLST genes, there were 364 nucleotide polymorphisms out of the 5,496 sites that were sequenced, resulting in 40 distinct MvLST types. 51 distinct STs were found based on MLST. When excluding the atypically variable gene *ldh* from the MLST data (see below), 41 sequence types were distinguished, very similar to the number of MvLST types. These results show that MvLST does not provide more discrimination than MLST even when including 8 MvLST genes, among which the entire *inlA* gene sequence. In previous work, MvLST was found to be more discriminatory than MLST (59), but this suggestion was based on different MLST genes (*prs, sigB* and *recA*) and only 14 strains.

Similarly, the variation of MvLST types within the major MLST clones was limited (**Table 4**). No more than 4 distinct MvLST types were observed within CCs. Besides, most isolates within a CC were identical at the eight MvLST genes, as the other MvLST types were represented by only one or few isolates. Remarkably, the homogeneity of MvLST gene sequences within CCs was observed across large geographical and time scales (**Table S1**). For example, the most frequent MvLST type in CC1 was observed for isolates collected between 1963 and 2000 from 13 distinct countries in North America, Europe, Oceania and Asia. These observations show that MvLST gene sequences are stable through time and space, resulting in the same MvLST type being observed for isolates that are considered unrelated from an epidemiological point of view. From a practical standpoint, the low variation of MvLST within single STs or CCs implies that adding the analysis of virulence genes to MLST does not improve the discriminatory power significantly: Simpson's index of discrimination was 0.946 (59 types, 95% confidence interval 0.925-0.967) for MLST+MvLST combined, whereas it was 0.932 (0.908-0.956) for MLST and 0.898 (0.871-0.925) for MvLST.

InlA evolves by in-frame deletions as well as variation leading to truncated forms of internalin

Internalin, encoded by *inlA*, plays a critical role in *L. monocytogenes* invasion of cultured epithelial cells (21), and is a critical virulence determinant, mediating *L. monocytogenes* crossing of the intestinal and placental barriers (14, 35). Alignment of the MvLST gene sequences revealed that the gene *inlA* differed from the seven other genes by the presence of insertion-deletion events. First, all strains of CC6 had a deletion of 9 nucleotides corresponding to positions 2,212-2,220 in the *inlA* sequence of the other strains which result in the predicted loss of 3 amino acids in the pre-anchor region of InlA. Second, ILSI strain FSL C1-122 had a 30-nt deletion in *inlA* (positions 2,141-2,170), which results in the predicted loss of 10 amino acids (723-732) in the pre-anchor region of InlA. These two in-frame changes are not expected to impair InlA function, as the functional domain of InlA is the LRR-IR region (34), and the CC6 and FSL C1-122 InlA variants harbour an intact anchor region (32). Third, three strains of CC9 had a G to A change at position 2,054 resulting in a stop codon at amino-acid position 694. Fourth, seven other strains of CC9 including LO28 had a one nt deletion in *inlA* at position 1635, resulting in the first premature stop codon described for *inlA* (26). Fifth, three other CC9 isolates had a G to A change at position 1,380, resulting in a stop codon at position 460. Six, strain LM05-01099 had a G to T change at position 976, resulting in a stop codon at position 326. Finally, strain CLIP 11308 had a one nt deletion at position 5 in gene *inlA*, resulting in a predicted stop codon at amino-acid position 9. Functional, non-truncated InlA has been associated with the clinical origin of *L. monocytogenes* isolates (25), and premature stop codons in *inlA* leading to the secretion of a non-functional truncated protein have been associated with reduced pathogenicity (25, 26, 42, 47).

Pattern of recombination, selection and phylogenetic relationships

Homologous recombination and selection both influence the evolutionary rate and the diversity pattern of genes that they affect. To characterize the evolutionary forces that act of the diversification of clonal groups, we searched for evidence of recombination and selection in MLST and MvLST genes. Using LDHAT, evidence for recombination was detected at five out of the 15 loci (**Suppl. Table S2**). Within lineage I, recombination was only detected at the *inlB* locus. Within lineage II, recombination was detected at the three MvLST genes *actA, dal* and *inlA*. Among the MLST genes, recombination was detected only at gene *dapE* in lineages

I and II. These results confirm that recombination is infrequent in *L. monocytogenes* and indicate that MvLST genes are more affected by recombination than MLST genes.

To detect positive selection, the software OMEGAMAP (57) was used. This software separates the effects of recombination and positive selection, thus avoiding false positives for selection in the presence of recombination. Using a posterior probability threshold of 0.95, the results revealed positive selection within lineage I at MvLST genes *actA* and *inlC*, two major virulence factors, as well as at MLST loci *dapE* and *ldh* (**Suppl. Figure 1**). No evidence for positive selection was found at the other loci within lineage I, including at the recombining gene *inlB*. However, positive selection is highly likely to occur at gene *inlA*, which had a posterior probability of 0.94. No positive selection was detected within lineage II at any of the 15 genes. In this lineage, the highest posterior probability (0.84) was observed for gene *prfA*, coding for the master regulator of virulence genes. In protein ActA, the positively selected amino acids within lineage I were at codons 124 and 130 (88 and 94 of our sequenced region, **Suppl. Figure 1**), located within the actin tail formation domain (52). Overall, these results indicate contrasting impacts of recombination and selection between lineages: whilst lineage II was more affected by recombination, consistent with previous findings (11), lineage I was more affected by positive selection.

The phylogenetic analysis of the concatenated gene sequences from the MLST gene set (**Figure 2 left**) and the MvLST gene set (**Figure 2 right**) gave highly similar results. As expected, the two major lineages of *L. monocytogenes* corresponded to the deepest branches and were clearly separated based on both gene sets. Based on MvLST genes, both lineages showed unequally long branches, consistent with a higher impact of recombination on MvLST genes. Nevertheless, both gene sets grouped strains into the same shallow branches, and MLST-defined CCs were recovered as monophyletic groups based on both gene sets (**Figure 2**). We also classified isolates into MvLST clonal complexes using their 8-digit allelic profile. These groupings were highly concordant with MLST clonal complexes, as illustrated by the adjusted Rand coefficient (0.988, 95% confidence interval: 0.976-1.000; and 0.981 (0.964-1.000) when excluding gene *ldh*, see below). These results show that groupings of *L. monocytogenes* isolates obtained based on either MLST or MvLST gene sets are nearly identical.

Source-sink selection at gene *ldh* and impact on CC definitions

For most genes, all isolates of a given clonal complex shared the same allelic sequence (**Table 3**). However, gene *ldh* was a notable exception, as this gene was highly variable

within CCs, resulting in a large proportion of STs differing from the central genotype of each CC solely by gene *ldh*. We observed that almost all *ldh* nucleotide differences within CCs implied an amino-acid change in the deduced protein sequence (**Table S1**). Further, all amino-acid changes were localized in the 3'-end (codon numbers 93 to 147) of the sequenced *ldh* region, consistent with the results obtained with OMEGAMAP (**Suppl. Figure 1**). In addition, we observed the same amino-acid substitutions in two pairs of isolates from different CCs: change Y95C was observed both in CC1 (ST252) and in CC8 (ST289), whereas change S120R was found both in CC1 (ST248) and CC7 (ST85). These evolutionary parallelisms represent strong evidence for positive selection on *ldh* and contribute to the atypically high diversity of this gene within CCs. Interestingly, the 93 – 137 region within which changes were observed, includes a variable flexible loop of enzyme lactate dehydrogenase in which amino acid substitutions were associated with adaptation to cold temperatures in teleost fishes (19). In striking contrast to the observed pattern of intra-clone variation, the amino acid sequence of lactate dehydrogenase was unchanged among the central genotype of most *L. monocytogenes* clonal complexes and was even conserved between lineages I and II. This absence of diversification at the amino-acids level in the long term indicates strong purifying selection on *ldh*. Together, these results indicate that *ldh* evolves in a source-sink fashion, whereby variants are selected in the short term because they provide a selective advantage in a sink environment, but these variants are counter-selected in the source environment, which sustains the population in the long run. Strikingly, the time since initial isolation of strains with amino acid substitution in lactate dehydrogenase was significantly larger than for non-mutated isolates ($p = 0.024$, two-sided Mann-Whitney test). No substitution was observed in isolates collected after year 2000 (**Table S1**). We hypothesize that non-synonymous mutations in gene *ldh* could have been selected during long-term storage in the laboratory and would not represent naturally occurring variation.

The positive selection pattern at the 3' end of the *ldh* sequence led us to evaluate the impact of this gene on the assignment of isolates to clonal complexes. We selected the 863 isolates and reference strains previously analyzed herein and in our previous studies (2, 7, 8, 33, 47). Classification of the isolates into CCs was performed using either the 7 MLST genes (**Figure 1**) or the six genes after excluding *ldh* (MLST-6). For the 515 isolates of lineage I, there was a total correspondence between CCs obtained based on 7 or 6 genes. Similarly, in lineage III, there was no impact of exclusion of gene *ldh* on the grouping of the 25 isolates. However, when analyzing the 318 isolates of lineage II based on MLST-6, a few discrepancies were observed compared to MLST-7. First, CC8 and CC16 became merged into

a single CC, as these two CCs differed by *ldh* (alleles *ldh*-3 and *ldh*-2, respectively). Similarly, CC11 (*ldh*-2) and CC19 (*ldh*-24) became merged using MLST-6, and ST226 (*ldh*-96) became a member of CC177 (*ldh*-26). Finally, ST126 (*ldh*-65) became part of CC7 (*ldh*-2). Among the *ldh* alleles that affected CC membership, only *ldh*-65 has a non-synonymous change (H121R) that could potentially have been selected. Therefore, the positive selection on the 3' region of the *ldh* sequence potentially impacted the classification of only a single isolate (SLCC875, of ST126) out of the 863 isolates. The adjusted Rand coefficient between MLST-7 and MLST-6 was 0.992 (95% confidence interval: 0.979-1.000). We conclude that the use of gene *ldh* in MLST does not impact importantly the classification of isolates into clonal complexes.

Minimal age of clones and heterogeneity of pulsed-field gel electrophoresis (PFGE)

Although we believe it was not the intention of the author who coined the name 'epidemic clone' (28), this denomination might suggest that such groups undergo recent emergence and have diffused within epidemiological timescales. To provide a minimal age of clones, we searched the public MLST database (as of July 8th, 2013 – 2,586 isolates) for the oldest isolate of the central ST of each major clone. ST1, ST2, ST3, ST4, ST5, ST6, ST7, ST8 and ST9 were isolated as early as 1936, 1954, 1955, 1965, 1992, 1990, 1927, 1991 and 1949, respectively. These dates are several decades older than the first described outbreak of the corresponding 'epidemic clone', and it is well possible that the central STs existed much earlier.

In order to estimate the diversity within clones, 110 isolates (all except the genome references) were analyzed by PFGE using two enzymes separately. A total of 65 *Apa*I types, 48 *Asc*I types and 76 combined PFGE types were distinguished (**Suppl. Figure 2**). PFGE patterns within an MLST-defined CC were more similar among themselves than with patterns of other clones. This was illustrated by cluster analysis of combined *Apa*I and *Asc*I PFGE patterns (**Suppl. Figure 2**), which grouped isolates of each clone within a single branch. One exception was the grouping of the two CC558 strains from the 2000 Mexican-style fresh cheese outbreak, which were clustered within the CC1 branch (**Figure S2**). As ST1 and ST558 are not closely related (differing by five out of seven MLST genes), this observation might suggest an evolutionary convergence of the PFGE profiles of clones CC1 and CC558.

Although they clustered together, the PFGE patterns within a given clonal group showed a high level of heterogeneity (**Table 5**). First, the seven clonal groups represented by multiple isolates displayed 2 to 21 distinct PFGE types, when combining profiles obtained

with *Apa*I and *Asc*I enzymes. CC1 and CC2, which both comprised 25 analyzed isolates, were the most heterogeneous, with 21 and 17 distinct PFGE profiles, respectively. Furthermore, the most unrelated profiles within these CCs differed by more than 20 bands when considering both *Apa*I and *Asc*I patterns (**Table 5**). These results show that PFGE has higher discriminatory power than MLST, and that CCs exhibit high levels of PFGE diversity. As a matter of fact, isolates that differ by three or more PFGE bands are generally considered as being epidemiologically unrelated (51). Therefore, the PFGE diversity observed within *L. monocytogenes* clones confirms the observation based on date and country of origin, that most isolates that share the same or closely related MLST or MvLST type, are epidemiologically unrelated. As there is no evidence for epidemiological links between most outbreaks of *L. monocytogenes* caused by the same EC, the 'epidemic clone' denomination is potentially misleading. Instead, these results favour the view that 'epidemic clones' are evolutionary successful lineages that are widely distributed, and caused multiple independent outbreaks due to their large distribution (8, 12, 28, 46).

It is possible that 'epidemic clones' might in fact represent genetically distinct subgroups within MLST defined CCs (8), even though the MLST and MvLST genes cannot discriminate such subgroups. Comparison of PFGE patterns showed that among isolates of CC1, the strains representative of the 1981 Nova Scotia coleslaw outbreak were clearly distinct from the representatives of the two other CC1 outbreaks (1987 Vacherin Mont d'Or and 1985 Jalisco outbreaks), which were more similar among each other. Within CC2, the 1987 UK and Ireland pâté outbreak and the 1983 Massachusetts milk outbreak strains were undistinguishable. Finally within CC6, the strains from the 2002 multistate ready-to-eat meat outbreak and from the 1998 hot-dog outbreak were distinguished by a conspicuous doublet when *AscI* enzyme was used (**Suppl. Figure 2**). These results show that within a single CC, strains with distinct PFGE patterns were implicated in outbreaks. Clearly, the within-clone phylogenetic relationships need to be established more precisely, using the high-resolution of whole genome sequencing (48, 60), to determine whether they are structured into subgroups with distinctive involvements in outbreaks.

Conclusions

Research into the pathogenicity or epidemiology differences among *L. monocytogenes* clonal groups would benefit from a widely accepted way to define clonal groups, as well as a reference system to name them. Serotyping has provided a widely shared language, but unfortunately this approach lacks discrimination and is unreliable as a classification system.

Similarly, the recognition of 'epidemic clones' as groups of similar isolates causing multiple outbreaks, has been invaluable for research on *L. monocytogenes* epidemiology and biology. However, the concept of epidemic clone was not designed to become a common nomenclature system for *L. monocytogenes*. First, it only includes clones involved in outbreaks, whereas most listeriosis cases are sporadic, thus excluding an important fraction of *L. monocytogenes* diversity. Second, there is no consensual definition of an epidemic clone. For example, authors either accept gene sequence variation within clones (12), or not (5, 6, 29, 37, 38). In particular, ECIV to ECVII were defined based on complete identity at the six MvLST genes. Strict application of this definition led, for example, to the exclusion of strain Scott A, based on a single nucleotide change in *lisR*, from ECIV (6). As shown here, this strain is otherwise identical to other strains of CC2, ECIa and ECIV at all other MvLST genes, as well as at MLST genes, suggesting that its exclusion from ECIV is too restrictive. An early definition of clone in a medical microbiology context was given by Orskov as genetically similar (but not identical) isolates from unrelated places and times that share many common features (44). The MLST-based definition of clones as CCs (18, 39) is in line with this definition, as it allows one isolate to belong to a CC even if it differs at one out of six genes from another member of the clone. The flexible definition of CCs thus accommodates random accumulation of variation within clones. In fact, MLST has initially been devised to provide a reliable way to recognize and define clonal groups (18, 39).

In this work, we have shown that reference isolates of distinct epidemic clones belong to distinct CCs. However, whereas all ECs have a corresponding group based on MLST, only a small fraction of MLST diversity is covered by the EC nomenclature (**Figure 1**). We demonstrated that MvLST genes evolve at a similar rate as MLST genes, implying that both approaches will delimit equivalent groups of isolates. Finally, we showed that CCs represent ancient, diverse and globally distributed genetic groups. This result is in line with the view of CCs in many bacterial species (1, 18, 39).

Given the above, we regard EC denominations as a redundant but partial and less flexible denomination system for MLST groups. Given the close correspondence between MLST CCs and ECs, the identification methods developed previously for specific ECs (4, 9, 16, 17, 29, 55, 58) should apply readily to the distinction of MLST clones. Unlike MvLST, MLST data are publicly available for comparison through a dedicated web site, providing a continuously growing overview of *L. monocytogenes* diversity. The MLST database (http://www.pasteur.fr/mlst) currently contains 2,586 isolates and is being used by an increasing number of laboratories (2, 7, 8, 29, 30, 38, 43, 45, 47, 49, 53, 54) for epidemiology

and population biology of this pathogen, thus providing *de facto* a common language for the denomination of clonal groups of *L. monocytogenes*.

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513 **Figure legends**

514

515 **Figure 1. Minimum spanning tree of MLST data for 863 *L. monocytogenes* isolates.**

516 Each circle represents one sequence type (ST), the size of which is related to the number of
517 isolates with this ST, as indicated (111 represents ST1, the central ST of clonal complex
518 CC1). Red sectors represent the 125 isolates or reference strains included in this work,
519 whereas white sectors represent previously published strains. Links between circles are
520 represented according to the number of allelic mismatches between STs, as indicated. Grey
521 zones surrounding groups of STs represent clonal complexes (CC). Most CC numbers are
522 indicated; those CCs that correspond to 'epidemic clones' (EC) are written in bold with
523 corresponding EC number in parentheses. Laboratory reference strains are indicated in blue.
524 STs of outbreak reference strains are indicated by colored triangles as given on the right. Top,
525 lineage II (starting with CC37); bottom, lineage I (starting with CC195).

526

527 **Figure 2. Compared phylogenies based on MLST (left) and MvLST (right) gene**
528 **sequences of the 125 study isolates.**

529 Phylogenies were obtained based on concatenated gene sequences using the neighbour joining
530 method based on uncorrected p-distance. Branches corresponding to clonal complexes are
531 colored according to the central legend. The position of reference strains is indicated.

532

533

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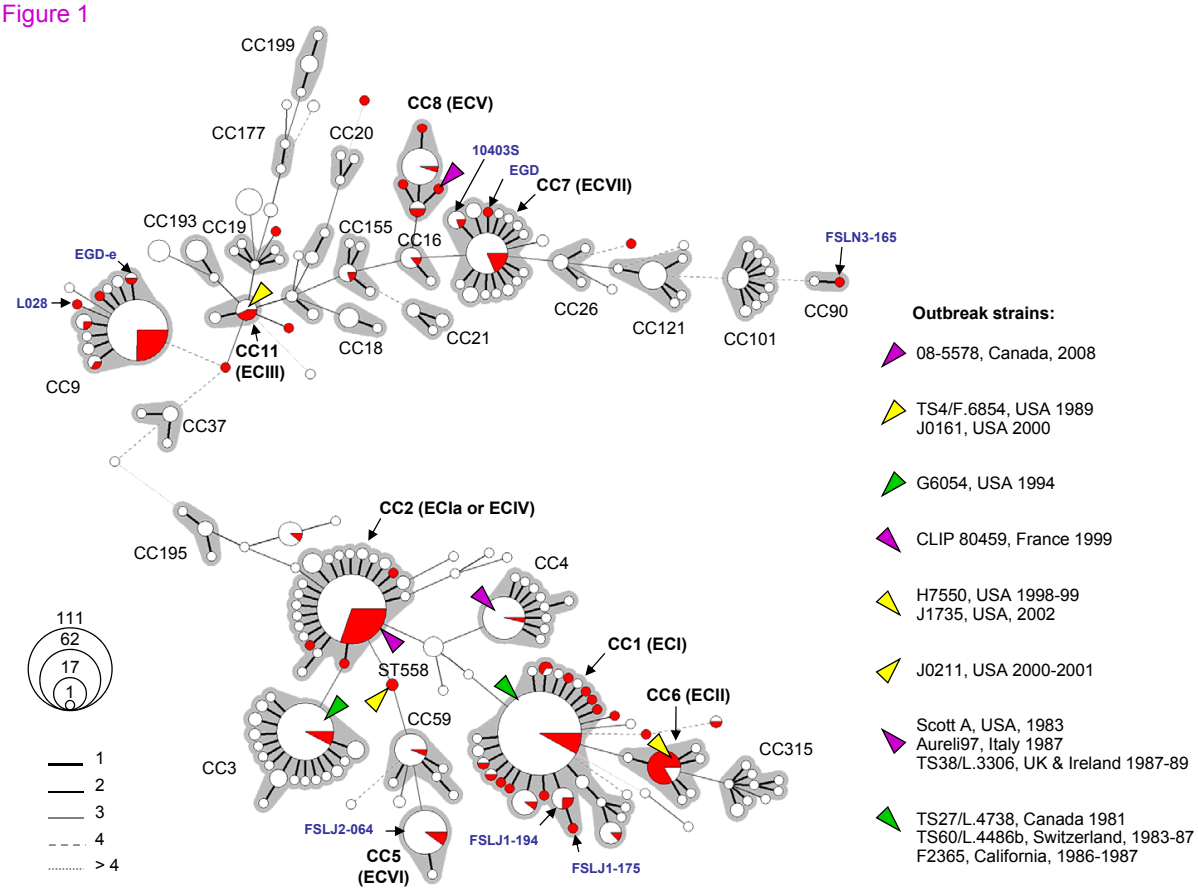
Figure 1



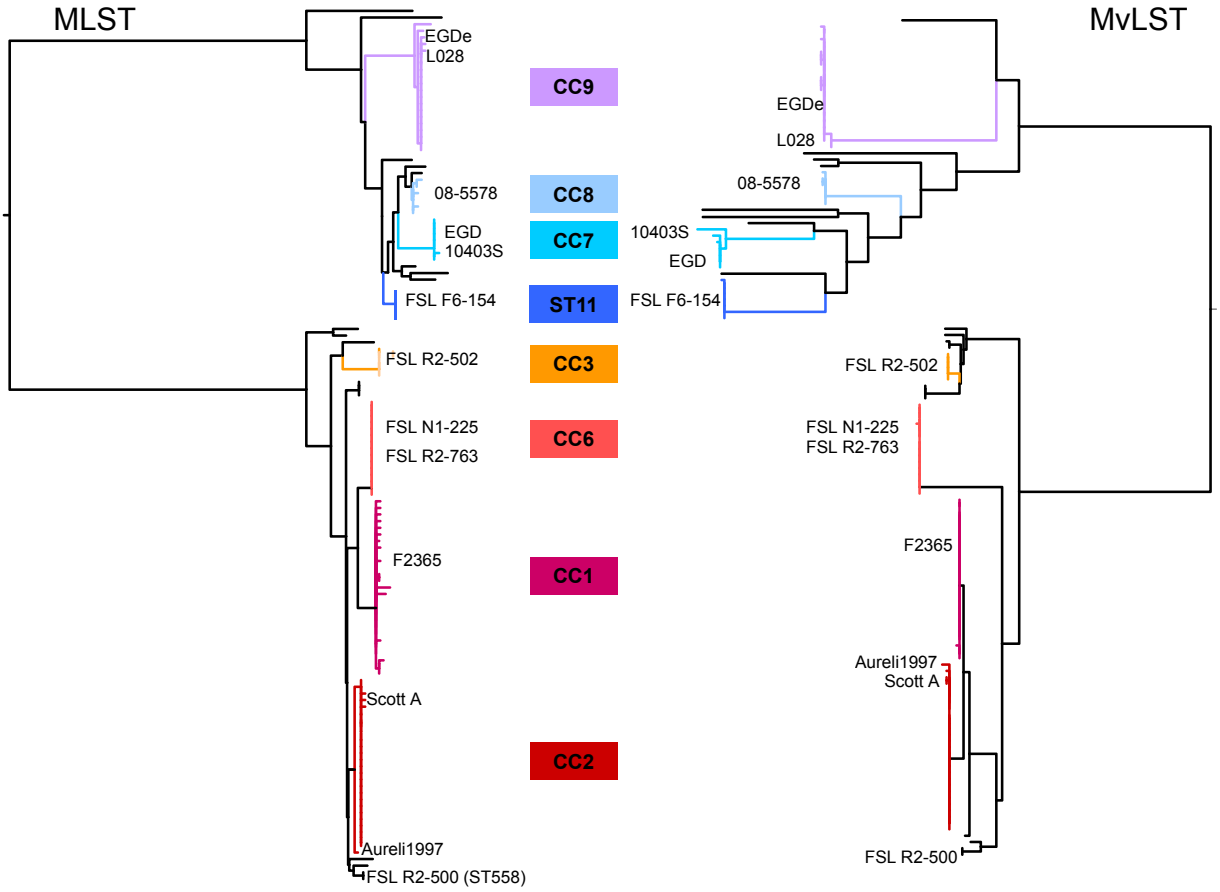


Table 1. Primers used for MvLST.

Gene	Forward primer	Reverse primer	Location (EGD-e)	Size of PCR product (bp)	Size of template (bp)	Reference
actA	CGA CAT AAT ATT TGC AGC GAC A	GAA TCT AAG TCA CTT TCA GAA GCA T	209,541 to 210,041	500	450	This study
clpP	CCA ACA GTA ATT GAA CAA ACT AGC C	GAT CTG TAT CGC GAG CAA TG	2,542,026 to 2,542,524	498	419	Zhang & al (2004)
dal	GAA GGT ATC TAC ACG CAT TTT GC	GCC AAT TAT CGT TAC TTT TGA ACC	925,391 to 925,909	518	428	This study
inlB	CAT GGG AGA GTA ACC CAA CC	GCG GTA ACC CCT TTG TCA TA	457,954 to 458,453	499	432	Zhang & al (2004)
inlC	AAC CAT CTA CAT AAC TCC CAC	CGG GAA TGC AAT TTT TCA CTA	1,860,322 to 1,860,822	500	457	Zhang & al (2004)
lisR	CGG GGT AGA AGT TTG TCG TC	ACG CAT CAC ATA CCC TGT CC	1,402,726 to 1,403,224	498	447	Zhang & al (2004)
prfA	TGC GAT GCC ACT TGA ATA TC	AAC GGG ATA AAA CCA AAA CCA	203,811 to 204,311	500	460	Zhang & al (2004)
inlA	CGGATGCAGGAGAAAATCC	CTTTCACACTATCCTCTCC	454,463 to 457,027	2,564	2,403	Ragon & al (2008)

Table 2. Polymorphism of the MLST and MvLST genes, based on the 125 study isolates

	Length	No. alleles	No. Polymorphic sites	% Polymorphic sites	Ks	Ka	Ka/Ks	π (%)
MvLST genes								
actA	450	14	44	9.78	0.07	0.02	0.35	3.21
clpP	419	4	14	3.34	0.06	0.001	0.02	1.44
dal	428	14	54	12.62	0.15	0.002	0.01	3.88
inlA	2403	28	140	5.83	0.06	0.06	1.01	1.76
inlB	432	15	40	9.26	0.03	0.04	1.38	3.38
inlC	457	10	14	3.06	0.03	0.01	0.25	1.18
lisR	447	5	38	8.50	0.02	0	0	0.51
prfA	460	9	20	4.35	0.06	0.00008	0.001	1.37
Concatenate	5,496	40	364	6.62	0.06	0.03	0.53	1.96
MLST genes								
abcZ	537	14	37	6.89	0.08	0.001	0.01	1.96
bglA	399	15	21	5.26	0.04	0.0006	0.01	0.91
cat	486	17	26	5.35	0.08	0.002	0.03	1.93
dapE	462	19	40	8.66	0.11	0.01	0.08	3.27
dat	471	10	61	12.95	0.20	0.01	0.07	5.72
ldh	453	20	39	8.61	0.06	0.0008	0.01	1.37
lhkA	480	10	20	4.17	0.06	0.0026	0.04	1.55
Concatenated	3,294	51	244	7.41	0.09	0.004	0.05	2.41

Ks: No. of synonymous changes per synonymous site. Ka: No. of non-synonymous changes per non-synonymous site.

π: nucleotide diversity.

Table 3. Distribution of MLST alleles within clones, observed based on the 125 study isolates.

Lineage	**CC**	**No. isolates**	**No. of distinct alleles (alleles found)**							**No. ST**	**No. ST without *ldh***
			abcZ	*bglA*	*cat*	*dapE*	*dat*	*ldh*	*lhkA*		
I	CC1	25	1 (3)	2 (1;56)	3 (1;20;60)	4 (1;34;42;44)	4 (3;14;18;42)	9 (1;11;12;61;90;101;103;104;105)	1 (3)	16	10
	CC2	25	1 (1)	2 (1;38)	1 (11)	2 (3;11)	1 (2)	1 (1)	2 (5;8)	4	4
	CC3	5	1 (3)	1 (4)	1 (4)	1 (4)	1 (2)	1 (1)	1 (5)	1	1
	CC6	15	1 (3)	1 (9)	1 (9)	1 (3)	1 (3)	1 (1)	1 (5)	1	1
	Others	10	5 (1;2;11;12;34)	4 (1;2;12;39)	3 (4;11;12)	6 (1;3;14;15;16;38)	2 (2;3)	3 (1;5;39)	5 (2;3;4;5;7)	7	7
II	CC7	6	1 (5)	1 (8)	1 (5)	1 (7)	1 (6)	1 (2;22;38)	1 (1)	3	1
	CC8	6	5 (5;57)	1 (6)	1 (2)	2 (9;29)	1 (5)	3 (3;89;121)	1 (1)	5	3
	CC9	19	2 (6;33)	1 (5)	1 (6)	2 (4;20)	1 (1)	3 (4;57;62)	1 (1)	5	3
	Others	14	5 (5;6;7;21;53)	6 (5;6;10;13;27;52)	9 (2;3;8;10;16;17;24;26;39)	6 (4;6;7;8;21;67)	5 (1;5;6;13;39)	4 (2;3;24;32)	4 (1;6;14;41)	10	10

Table 4. MvLST allelic diversity within clones, observed based on the 125 study isolates

Lineage	CC	No. strains	No. of distinct alleles (allele number)								No. MVLST types
			actA	*clpP*	*dal*	*inlA*	*inlB*	*inlC*	*lisR*	*prfA*	
I	CC1	25	2 (14;15)	1 (4)	1 (5)	1 (28)	1 (3)	2 (1;4)	1 (1)	1 (3)	3
	CC2	25	2 (10;13)	1 (4)	1 (5)	1 (28)	1 (4)	1 (1)	3 (1;2;5)	1 (2)	4
	CC3	5	1 (10)	1 (4)	1 (5)	1 (22)	1 (5)	1 (4)	1 (1)	1 (2)	1
	CC6	15	2 (10;12)	1 (4)	1 (5)	1 (29)	1 (3)	1 (4)	1 (3)	2 (2;5)	3
	Others	10	4 (9;10;11;14)	1 (4)	1 (4;5;6;7)	6 (21;23;24;25;26;27)	4 (1;2;6;7)	3 (2;3;4)	1 (3)	4 (1;2;4;6)	7
II	CC7	6	2 (6;8)	1 (2)	2 (11;13)	2 (9;10)	2 (10;13)	2 (10;11)	1 (4)	2 (7;9)	4
	CC8	6	1 (4)	1 (1)	1 (8)	2 (14;15)	2 (9;12)	1 (10)	1 (4)	1 (8)	2
	CC9	19	1 (1)	1 (2)	2 (1;2)	6 (1;2;3;4;5;6)	1 (8)	1 (5)	1 (4)	1 (8)	3
	Others	14	7 (1;2;3;4;5;6;7)	3 (1;2;3)	7 (1;3;8;9;10;12;14)	11 (4;7;8;11;12;13;16;17;18;19;20)	6 (8;9;10;11;14;15)	6 (5;6;7;8;9;10)	2 (4;6)	1 (8)	10

Table 5. Pulsed field gel electrophoresis diversity per clone

Lineage	Clone	No. Isolates	No. of *AscI* profiles (Max. No. of different bands)	No. of *ApaI* profiles (Max. No. of different bands)	No. of *Asc*I + *Apa*I profiles
I	CC1	25	11 (13)	12 (9)	21
	CC2	25	7 (7)	13 (15)	17
	CC3	3	1 (0)	2 (6)	2
	CC6	15	2 (5)	5 (10)	6
	Others	6	5 (10)	5 (12)	5
II	CC7	5	4 (5)	4 (7)	5
	CC8	4	3 (8)	2 (3)	4
	CC9	20	6 (9)	4 (16)	7
	Others	11	9 (12)	8 (13)	9