Optimized Multilocus Variable-Number Tandem-Repeat Analysis Assay and Its Complementarity with Pulsed-Field Gel Electrophoresis and Multilocus Sequence Typing for Listeria monocytogenes Clone Identification and Surveillance

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JCM Accepts, published online ahead of print on 10 April 2013
J. Clin. Microbiol. doi:10.1128/JCM.00606-13

An optimized MLVA assay and its complementarity with PFGE and MLST for *Listeria monocytogenes* clone identification and surveillance

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Abstract

Populations of the foodborne pathogen *Listeria monocytogenes* are genetically structured into a small number of major clonal groups, some of which have been implicated in multiple outbreaks. The goal of this study was to develop and evaluate an optimized multilocus variable number of tandem repeat (VNTR) analysis (MLVA) subtyping scheme for strain discrimination and clonal group identification. We evaluated 18 VNTR loci and combined the 11 best ones into two multiplexed PCR assays (MLVA-11). A collection of 255 isolates representing the diversity of clonal groups within phylogenetic lineages 1 and 2, including representatives of epidemic clones, were analyzed by MLVA-11, multilocus sequence typing (MLST) and pulsed field gel electrophoresis (PFGE). MLVA-11 was less discriminatory than PFGE, except for some clones, and was unable to distinguish some epidemiologically unrelated isolates. Yet it distinguished all major MLST clones and therefore constitutes a rapid method to identify epidemiologically relevant clonal groups. Given its high reproducibility and high-throughput, MLVA represents a very attractive first-line screening method to alleviate PFGE workload in outbreak investigations and listeriosis surveillance.

Introduction

Listeriosis is a foodborne infection caused by the bacterium *Listeria monocytogenes*. Invasive forms of human listeriosis include septicaemia, meningitis, and maternal-fetal infections (31). Listeriosis is associated with high hospitalization and fatality rates (almost 100% and 25-30%, respectively). Populations at risk include pregnant women, immunocompromised individuals and the elderly. *L. monocytogenes* is widely present in the environment, including soil, water, vegetation and silage, as well as in animals and animal-derived food, and can contaminate food in processing plants and retail establishments. *L. monocytogenes* is recognized as a public health issue and a serious challenge for the food industry, and this has led to the establishment of national surveillance system in several countries. *L. monocytogenes* also stands out as a model system in the fields of microbiology, cell biology and immunology and for the study of host-pathogens interactions (9, 18, 27, 50).

L. monocytogenes strain characterization based on serotyping and molecular typing methods is used for surveillance, epidemiological tracking and outbreak investigation purposes (24, 44). Genetic variants of *L. monocytogenes* have diversified into four major phylogenetic lineages, with lineages 1 and 2 each containing multiple clonal groups of public health importance (23, 36-39, 47, 48). As these groups appear to differ in virulence and epidemic potentials (21, 24), it will be interesting to better define their epidemiological, clinical and microbiological specificities. For this purposes, easy identification tools of clonal groups are needed to recognize such groups and determine their presence in a large variety of sources. Several typing methods are currently available *for L. monocytogenes* strains. Conventional serotyping (42) and its molecular proxy PCR-serogrouping (12)

discriminate major categories of strains that correlate strongly (albeit not totally) with lineages and clones (38, 39, 48), but these methods do not have the necessary discriminatory power in the context of outbreak investigations. Pulsed field gel electrophoresis (PFGE) is established as the gold standard for *L. monocytogenes* strain subtyping and is widely used for surveillance of listeriosis and outbreak investigation (16). Yet, PFGE presents several practical disadvantages, as it is time-consuming and requires stringent standardization for inter-laboratory data comparison. Multilocus sequence typing (MLST) is a well-established reference method for global epidemiology and population biology (13, 33), as it renders inter-laboratory genotype comparisons easy and unambiguous, and as sequence data can be used to infer useful population genetics information such as amounts of genetic diversity, recombination rates and strain phylogeny. MLST also provides backward compatibility with genome sequencing (22). However, MLST is neither rapid nor cheap and has limited discriminatory power within *L. monocytogenes* (11, 39). Given the current limitations of available methods for *L. monocytogenes* strain typing, a potentially useful complementary approach is multilocus variable number of tandem repeats (VNTR) analysis (MLVA). This method is largely used for epidemiological tracking of bacterial pathogens (29, 46) because it is relatively easy and cheap to implement and because it has remarkable discriminatory power in many, although not all, bacterial species. MLVA relies on the study of the variability of the number of tandem repeats at specific loci in bacterial genomes. MLVA schemes are constructed based on an open choice of several VNTR chromosomal loci, and five different MLVA schemes have been developed almost simultaneously for *L. monocytogenes* strain typing (26, 30, 34, 35, 43). Subsequent use of MLVA in outbreak investigations

and strain diversity studies has relied on these different schemes or combinations thereof (2, 5, 19, 28, 32).

The five previously proposed MLVA schemes differ in the number of VNTR loci that were included, ranging from only 3 (34) to 8 (43) and even 10 (26). These studies also differed in the number, diversity and inclusion criteria of *L. monocytogenes* isolates used to evaluate strain typeability and discrimination. As strains of both lineage 1 and lineage 2 are frequent among sporadic human infections and can cause outbreaks (6, 23, 38, 48), a MLVA scheme should ideally be applicable to all strains of both lineages. Many of the primer pairs defined so far to amplify VNTR loci did not amplify a number of strains, which limits their discriminatory power and complicates the interpretation of differences among strains. Besides, it is important to calibrate the MLVA method against PFGE on the one hand, and MLST on the other hand. Indeed, PFGE is the standard for epidemiological investigations, whereas MLST is well established as a population biology tool and provides standard operational definition of clones as clonal complexes (8, 39). Only one of the five proposed MLVA schemes was compared with PFGE data obtained following the current standard protocol of dual restriction enzyme use, *ApaI* and *AscI* (43), while comparison with MLST was performed only in one study so far (34). The added value of MLVA relative to PFGE and MLST thus clearly remains to be precisely defined.

The aims of our study were (*i*) to identify novel VNTR loci and evaluate them as well as all previously described loci for typeability of isolates representative of a broad range of genotypes of lineages 1 and 2, including all major clones defined by MLST; (*ii*) to evaluate the ability of MLVA to identify MLST-defined clonal groups; and

(*iii*) to evaluate and compare to PFGE, the added value of MLVA in terms of strain discrimination within clonal groups of particular interest.

Materials and Methods

Bacterial isolates. A total of 255 isolates were included in the study (**Table S1 strains**). First, 217 *L. monocytogenes* isolates were selected from the National Reference Centre for Listeria (NRC-L) and from the World Health Organization Collaborative Centre for Listeria (WHO-CC-L) collections. This included 155 isolates previously characterized by us: 58 isolates from the study of Ragon et al. (39) and 97 isolates from the study of Chenal-Francisque et al. (8). We also included 38 isolates selected to represent additional epidemiologically unrelated isolates of clones CC3, CC4, CC5 and CC8, which were represented only by few isolates in our previous studies. To assess the epidemiological concordance of MLVA, we also included 24 strains collected during the period of a large outbreak that occurred in 1992 in France linked a ready-to-eat meat product, pork tongue in jelly (20). The 217 above isolates were epidemiologically unrelated, except for some of the 24 human or food isolates corresponding to the period of the 1992 French outbreak (see results). Overall, the 217 isolates were recovered from human infection cases (n=130), food (n=47), animal (n=18), the environment (n=9), vegetation (n=1), and 12 were of undocumented origin. They were collected from 31 countries across 6 continents and were isolated between 1933 and 2010.

Second, 38 International Life Sciences Institute (ILSI) strains were included in order to place these well-documented strains (14) within the diversity based on the Institut Pasteur MLST scheme (http://www.pasteur.fr/mlst) and thereby establish correspondence of MLST clonal groups with previously defined clonal groupings. The ILSI strains (14) included 23 strains representative of past outbreaks defined as members of epidemic clones ECI, ECII, ECIII and ECIV, and 15 strains from the ILSI

diversity set (**Table S1**). These isolates were recovered from human (n=20), food (n=10), animal (n=7) and environmental (n=1) sources.

All isolates were confirmed as *L. monocytogenes* using the API *Listeria* system (bioMérieux, France) and the multiplexed PCR serogrouping method (12). Hemolytic activity was confirmed on blood agar plates.

DNA preparation and PCR amplification. Total genomic DNA was extracted using the Promega Wizard Genomic DNA purification kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. DNA samples diluted in water at 50 ng/µl were used as template for PCR amplifications.

PCR serogrouping and MLST. PCR serogrouping and MLST genotyping were carried out following the procedures previously described (12, 39).

Primer optimization and identification of novel MLVA loci. We first included all 15 VNTR loci described previously (26, 30, 34, 35, 43). The VNTR loci common to more than one of the above studies were amplified using all different PCR primer sets defined previously (**Table 1**). Second, to identify additional VNTR loci, the tandem repeat site http://minisatellites.u-psud.fr (17), which uses the software Tandem Repeat Finder (3) was used on genomes of strains EGDe and F2365, with a minimal unit length of 6 bp, a minimal copy number of 2 repeats, and 80% sequence match. Three novel repeat regions (Lis-TR357, Lis-TR495 and Lis-TR1869) were identified. To optimize primer design for these loci as well as for previously described loci, alignment of flanking regions of the VNTR loci was performed based on publicly

available genomes. This optimization effort resulted in a total of 38 distinct PCR assays (**Table 1)** corresponding to 18 distinct loci **(Figure 1)**.

MLVA assay development. A preliminary screening was performed on a genetically diversified subset of isolates of major clonal groups of phylogenetic lineages 1 and 2. Each VNTR region was separately amplified by PCR using Isis DNA polymerase (MP Biomedicals, Santa Ana, Ca.). PCR run conditions included a denaturation step at 94°C for 4 min, followed by 35 cycles including denaturation at 94°C for 30 s, elongation at a temperature depending on the primer pair (**Table 1**) for 30 s, and an extension step of 72°C for 1 min. A final extension of 72°C for 10 min was performed. The finalized protocol consisted of two multiplexed PCR mixes called M1 (containing six primer sets) and M2 (five primer sets). The Qiagen Multiplex PCR kit (Valencia, CA, USA) was used. The concentrations of the primers were adjusted to obtain even intensities for all fragments, with the forward primers being fluorescently labelled at their 5' end (**Table 2**). The same PCR cycle was used for M1 and M2 multiplex PCRs: 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 56°C for 90 s and 72°C for 90 s, with a final extension at 72°C for 10 min.

PCR amplicon sequence verification. The purified PCR products of single PCR reactions performed with unlabeled primers were sequenced to control the molecular origin of size variation. The DNA sequence for every distinct allele of all VNTR loci was determined. Nucleotide sequence data were edited and compared using BioNumerics version 6.6 (Applied Maths, Sint-Martens Latem, Belgium) and Multalin (http://multalin.toulouse.inra.fr/multalin/).

193 **Stability of MLVA alleles**. The *Listeria monocytogenes* EGDe strain was passaged
194 on Columbia agar medium 65 times over a period of 4 months. At each passage, a
195 sweep was taken and streaked on the next Columbia agar medium tube. After every
196 10^{th} passage and the last one, DNA from a bacterial sweep was extracted and the
197 total DNA was subjected to the MLVA assay.
198
199 **Pulsed field gel electrophoresis (PFGE)**. Each isolate was typed by PFGE
200 according to PulseNet standardized procedures with *Asc*I and *Apa*I restriction
201 enzymes (16). Data analysis was performed using BioNumerics version 6.5. *Apa*I
202 and *Asc*I PFGE types were defined as differing from other types by at least 2 bands
203 for each individual enzyme.
204
205 **Data analysis**. PCR products obtained using fluorescently labelled primers (**Table 2**)
206 were subjected to capillary electrophoresis on an ABI 3730XL DNA sequencer. The
207 size of PCR fragments was determined using the Applied Biosystems GeneMapper
208 v4.0 software. The number of tandem repeats was then calculated according to the
209 PCR fragment size and the known length of the repeat unit of each VNTR locus. The
210 number of tandem repeats was rounded to the closest integer number. Following this
211 procedure, an allele number corresponding directly to the number of tandem repeats
212 was assigned to each PCR fragment. Allele strings were then imported into a
213 BioNumerics database. Because no data were available at locus Lm11 for most
214 isolates of clones CC7 and CC8, we chose to exclude this VNTR locus in subsequent
215 polymorphism calculations and for determination of the overall number of MLVA
216 types. Each unique combination of the 10 remaining allelic numbers was converted
217 into a distinct MLVA type. PFGE patterns were compared using a tolerance

parameter of 1% and an optimization parameter of 1%. UPGMA dendrogram analysis and minimum spanning tree construction were performed using BioNumerics.

Homoplasy along the branches of the minimum spanning tree (MStree) was defined as 1 - (K - 1)/M, where K is the number of alleles and M the number of changes along the MStree. Hence, if each allele was generated by a single evolutionary event, the number of changes since the ancestral state would be K - 1, and the homoplasy index would be equal to zero. In contrast, if alleles are often changing by convergent evolution or reversion to an ancestral state, M would become much greater than K and the homoplasy index would increase toward 1.

Results

MLST analysis of the 255 study isolates. To determine their diversity and whether they belong to major MLST clones, the 255 isolates were analyzed by MLST. Data for 155 isolates were derived from our previous studies (8, 39), and we performed MLST analysis of 100 additional isolates for the purposes of the present study. These 100 isolates included 38 strains from the reference ILSI collection, 24 isolates collected during the large 1992 outbreak in France (20), and 38 isolates representing clones CC3, CC4, CC5 and CC8 (see Materials and Methods). The 7 MLST genes could be amplified for the 100 isolates and their ST was determined. The 20 STs that were not previously described (ST326 or above in **Table S1**) were incorporated into the Institut Pasteur MLST database at www.pasteur.fr/mlst**.**

The 217 isolates from the NRC-L and WHO-CC-L represented 110 STs, thus representing a genetically diverse population. The 38 ILSI strains represented 18 STs, 9 of which were unique to the ILSI strains. **Figure 2** represents the joint analysis of the 255 isolates with the MLST diversity previously described using the Institut Pasteur MLST scheme (1, 8, 39). Further, the tree locating the ILSI strains relative to the other strains of this study is shown on **Suppl. Figure S1**.

Minimum spanning tree analysis of the MLST data (**Figure 2**) confirms that the population of *L. monocytogenes* is dominated by a few, numerically dominant clonal complexes (CC), which we have named “major clones” (8, 39). In the present study, most lineage I strains (172 out of 176) belonged either to CC1 (62 strains), CC2 (41 strains), CC3 (25 strains), CC4 (16 strains), CC5 (10 strains) or CC6 (18 strains). In lineage 2, clones CC7 (22 strains), CC8 (13 strains) and CC9 (23 strains) were the

most represented, although 21 strains (27%) belonged to less frequent clonal complexes or STs.

MLST analysis of the ILSI reference strains representing past outbreaks revealed that all outbreaks except three were caused by strains that belong to major clones as defined by MLST (**Figure S1**). The three exceptions were the 2000 Queso fresco North Carolina outbreak, caused by a strain that belong to ST558, which differs by three MLST genes from ST2, and the 1988 turkey franks case and the 2000 sliced turkey deli meat multistate outbreak, caused by a strain that belongs to ST11. The two latter events were traced to the same food processing facility and corresponding strains were shown to be highly similar, which led to the definition of epidemic clone (EC) III (24). ST1, the central genotype of MLST clone CC1, included strains of the 1986 Los Angeles Jalisco outbreak, the 1981 Nova Scotia coleslaw outbreak and the 1983-1987 Switzerland Vacherin Mont d'Or outbreak. These strains were previously defined as belonging to ECI (24). ST2, the central genotype of clone CC2, comprised the reference strains of the Massachusetts pasteurized milk outbreak and the 1987 UK and Ireland pâté outbreak. These isolates were assigned to ECIa, which was later renamed ECIV (7). ST3, the central genotype of CC3, included the strain from the 1994 Illinois pasteurized milk chocolate outbreak. Finally, ST6, the central ST of clone CC6, included the 1988-1989 multistate hotdog outbreak and the 2002 multistate deli turkey outbreak. Strains involved in these two later outbreaks were assigned to ECII (24). Note that the 2008 Canadian outbreak was assigned to a newly defined ECV, which belongs to MLST clone CC8 (15, 25). The ILSI strains from the diversity set were scattered across the MStree (**Figure S1**), consistent with the fact that they were selected to represent a diversity of ribotypes or PFGE types. However, two of these strains (FSL J2-064 and FSL J1-169) belong to

ST5, the central genotype of major clone CC5, one strain (FSL C1-122) belongs to ST1, and two other strains (FSL C1-056 and FSL J1-094) represent the major lineage 2 clones CC7 and CC9, respectively. Therefore, 7 out of 9 major MLST clones appear to be represented in the ILSI collection, with the exceptions of CC4 and CC8.

PCR amplification assays of 18 VNTR loci on a diverse sample of isolates. *L. monocytogenes* phylogenetic lineages 1 and 2 account for almost all cases of human listeriosis. To identify a panel of PCR primer pairs able to amplify VNTR loci from a diverse sample of isolates of these lineages, we selected a panel of strains representing a diversity of sequence types (ST) and clonal complexes (CC) within lineages 1 and 2: two strains within each of CC1, CC2, CC3, CC4, CC5, CC7 and CC9, plus strain EGDe. A total of 38 primers sets corresponding to 18 distinct VNTR loci were investigated in this preliminary screening (**Table 1**; for convenience, we labelled the 18 loci with a single letter, from A to R). Most of these primer pairs have been defined in five previous studies (26, 30, 34, 35, 43). In addition, we tested three novel loci (P,Q and R; **Table 1**) identified herein. We noted that most VNTR loci were located in the first half of the chromosome of strain EGDe, and especially in the first quarter (**Figure 1**). We also redesigned primer pairs for the two previously identified loci A and B (**Table 1**), which correspond to loci Lm3 and Lm32, respectively (43). Published procedures were followed for previously described primer sets. The technical results obtained for the 38 PCR assays are summarized in Table 1. For each of the loci A, B, C, E, F, G, H, I and J, we could observe a given PCR primer pair that gave optimal amplification results with all screening isolates, with a unique amplified fragment of the expected size. One PCR primer set was thus selected for

each of these loci (**Table 1, Figure 1**): LisTR881 (locus A), Lis-TR1317 (locus B), LMV1 (locus C), LMV6 (locus E), JLR1 (locus F), JLR2 (locus G), Lm11 (locus H), LM-TR4 (locus I) and LMV9 (locus J). For loci L and R, a single primer pair defined previously gave a unique expected band for all screened isolates. In contrast, none of five PCR primer sets defined for locus D were satisfactory, as some did not amplify all the tested isolates or non-specific PCR products were observed. Besides, this locus exhibits size variation due to insertion-deletions in the sequences flanking the tandem repeat region (see below), rendering fragment size variation difficult to interpret in terms of tandem repeat numbers. Similarly, a lack of PCR amplification was observed for some screening isolates with LisTR-495 (locus Q), LM-TR5 (locus N) and LM-TR6 (locus O). The latter is consistent with the initial report of PCR failure for many strains for locus LM-TR6 (35). For locus K, the two PCR primer sets also turned out to be unsatisfactory: TR3 gave non-specific or no amplification depending on the strain, and primer pair Lm8 yielded a unique PCR fragment but this fragment was of the same size for nearly all strains, indicating a lack of discriminatory power, as reported previously (43). Likewise, LM-TR2 (locus M) and Lis-TR357 (locus P) provided low discrimination. Loci D, K, M, N, O, P and Q were thus eliminated from the assay optimization. The 11 loci that were retained (**Figure 1**, **Tables 2 and 3**) included one novel locus identified herein (Lis-TR1869), one locus (JLR4) uniquely described by Larsson (26) and 9 loci previously identified by various authors. Among the latter, two novel primer pairs were used (Lis-TR881 for locus A and Lis-TR1317 for locus B). Notably, the 11 retained loci included 7 out of 8 loci selected for inclusion in the Sperry scheme (43) but only 2 out of 6 loci proposed by Murphy et al. (35) and 1 of the three loci used by Miya et al. (34).

328 **VNTR allele sequencing and confirmation of repeat number variation**. The
329 nucleotidic sequence was determined for all VNTR loci for one representative isolate
330 of each allele. Sequence alignments confirmed that the expected target regions were
331 amplified and that the size variation of the PCR fragments as determined by capillary
332 electrophoresis was due to variation in the number of repeats. For VNTR locus LM-
333 TR3, we observed DNA sequence variability in the flanking region, as noted
334 previously (43). Independent of this observation, locus LM-TR3 was eliminated due
335 to non-specific PCR amplification. The size range of fragments obtained at the 11 loci
336 is given in **Table 3**.
337
338 **Repeatability, development of two multiplexed PCRs, and stability**. To test the
339 repeatability of these assays, we analyzed 50 isolates in duplicate, and the results
340 showed complete agreement of the deduced repeat number at all loci. In order to
341 develop a rapid and simple MLVA assay, the eleven primers sets selected from the
342 preliminary screening were distributed into two distinct groups (**Table 2**), and two
343 separate multiplexed PCR assays called M1 (6 VNTR loci) and M2 (5 VNTR loci)
344 were developed. To test for the stability of the VNTR loci during laboratory
345 subculture, strain EGDe was sub-cultured 65 times, and no repeat number variation
346 was observed during these passages at any of the 11 VNTR loci.
347
348 **Typeability of the 11 VNTR loci of lineage 1 and 2 isolates**. The two multiplexed
349 PCR tests were applied to the analysis of an expanded panel of 255
350 *L. monocytogenes* isolates. These isolates represented multiple isolates of major
351 *L. monocytogenes* MLST clones and several other sequence types (ST) of lineages 1
352 and 2 (**Figure 2**). Ten out of 11 loci showed nearly exhaustive amplification on the

255 isolates, with only three missing data points out of 2550: locus JLR1 for FSL J2-063 (ST16, serotype 1/2a), locus Lis-TR1317 for FSL C1-115 (ST370, serotype 3a), and locus LMV6 for strain LM70290 (ST251, serotype 4b). All amplified PCR products were sized and the corresponding tandem repeat number was determined, indicating nearly complete typeability using these ten loci (**Table 3**). However, we observed no Lm11 PCR product for 38 isolates. Interestingly, these isolates all belonged to MLST clonal complexes CC7 or CC8, with the only exception of ST376 (strain FSL J2-066, serotype 1/2a). Locus Lm11 could be amplified and sized for two isolates of CC7 but none of CC8. This result suggests a loss of this locus in isolates of CC8 and in the majority of isolates of CC7. Search for Lm11 locus in genomic sequences confirmed its absence in SLCC5850 (CC7) and 08-5578 and 08-5923 (CC8). Regarding strains other than CC7 and CC8, locus Lm11 had allele 5 in most strains of lineage 1 and allele 4 in most lineage 2 strains. However, strains of CC4 (lineage I) had Lm11-4, and a subset of CC1 strains had allele Lm11-6, which was specific for these strains. Likewise, only strains of ST11 (ECIII) had allele Lm11-1. This locus is therefore very useful for characterization of strains from these specific groups.

Identification of major MLST clones by MLVA. MLVA analysis using the two combined multiplexed PCR assays M1 (excluding Lm11) and M2 resolved the 255 isolates into 66 different MLVA types. In contrast, the M1 multiplexed PCR generated 44 MLVA M1 types, whereas M2 multiplexed PCR distinguished 35 MLVA M2 types. **Figure 2** illustrates the distribution of the distinct MLVA types onto the MLST diversity (note that the same color in the lineage 1 and 2 panels represent distinct MLVA types). It was remarkable that all major clones were characterized by specific

MLVA patterns. In other words, no single MLVA type was shared by isolates belonging to different MLST clones. In addition, cluster analysis based on MLVA data (**Figure 3**) was highly consistent with classification into MLST clones, as isolates of a single MLST clone clustered together in all cases, except for clone CC121.

Although less informative than when used together, the two multiplexed PCRs M1 and M2 also provided useful information when considered independently. Using M2 PCR, most major clones showed clone-specific patterns that could prove useful for their identification. For example, CC1 isolates were characterized by M2 profile 8-2-21-2-11, within only four exceptions that had profile 8-2-21-2-12 due to variation at locus LMV9 (**Figure 3**, **Table S1**). Likewise, CC2 isolates had profile 7-1-21-3-12, with a single exception (7-1-21-3-11). In particular, whereas most clones showed several patterns, clones CC3 and CC7 showed only one M2 type that was specific of either CC. Cluster analysis of M2 patterns recovered all major clones as single branches, except CC9 (data not shown). In contrast, due to higher variability of M1 multiplex PCR markers, cluster analysis of M1 data did not recover the MLST clones in general.

MLVA discrimination within clones. We observed different degrees of MLVA variation according to CC (**Table 4**). In particular, it was interesting that MLVA subdivided efficiently some of the major CCs, including their central ST (**Figure 2)**. Therefore, MLVA may represent a useful typing complement to MLST for isolates of specific clones. **Table 4** gives the number of MLVA profiles and Simpson's diversity index per major clone. MLVA was more discriminatory than MLST for CC4, CC5, CC6 and CC9. On the contrary, isolates of CC7, which represented 13 STs, were

almost not discriminated at all by MLVA with the exception of one strain at locus JLR2.

Subtyping of isolates within clones was mostly contributed by the M1 multiplex PCR, independently of the use of M2 PCR (**Table 4**). For example, CC1 isolates were subdivided into 4 M1 types, and M2 PCR did not add to their discrimination; similar results were obtained for CC6 and CC8. However, in several cases, the use of multiplex M2 did improve the discrimination of isolates within clones (**Table 4**).

MLVA analysis of representative isolates of 'epidemic clones'. The 38 isolates of the ILSI collection, including the 23 isolates representing outbreaks, were divided into 22 MLVA types. The clone CC1 reference strains representing the 1981 Nova Scotia coleslaw outbreak and the 1983-1987 Vacherin Mont d'Or Switzerland outbreak shared the same MLVA type, which differed by a single marker (Lm11) from the 1986-1987 California Jalisco soft cheese outbreak (**Figure 3, Table S1)**. Clone CC2 strains from the 1983 Massachusetts milk outbreak and the 1987-1989 UK and Ireland outbreak shared the same pattern except for variation at locus Lis-TR881 (**Figure 3, Table S1)**. Clone CC6 isolates from the two represented outbreaks could be distinguished by two loci, Lis-TR1869 and LMV6. Finally, the 4 isolates of CC11 (ECIII) from 1988/1989 and 2000 were all identical by MLVA. The CC1, CC2, CC6 and CC11 strains previously assigned to different epidemic clones (ECI to ECIV) were clearly distinguished by MLVA, as were the isolates of the ILSI diversity set (**Figure 3, Table S1**).

Single-step model of evolution of VNTR loci and homoplasy. VNTR loci can evolve following two alternative models. In the stepwise model, the number of

repeats varies along lineages by the progressive addition or removal of single repeat units. In the saltational model, any allele has the same probability to evolve from any other allele, regardless of repeat number differences. To investigate the evolutionary mode of the 11 selected VNTR loci, we analyzed the 28 changes observed among closely related MLVA profiles, defined as profiles differing by a single allele out of 11. Out of 28 such changes, 22 (78.5%) corresponded to single-repeat differences; 3 changes involved two repeat differences, 2 changes involved three repeats, and one change (at locus LMV6) corresponded to six repeat differences. We conclude that *L. monocytogenes* VNTR markers evolve predominantly by the stepwise addition or deletion of a single repeat unit, as previously shown for other microorganisms (49).

To evaluate the degree of homoplasy, i.e. evolutionary events of convergence or reversion, we compared the number of changes observed along the minimum-spanning tree deduced from MLVA data, to the minimal number of changes, which would be obtained without homoplasy. The homoplasy ratio ranged from 0.41 for JLR1 to 0.67 for JLR2 (**Table 3**). In other words, there were approximately twice as many changes as the number required to generate all distinct alleles, indicating a substantial degree of homoplasy.

Comparison of MLVA with PFGE. PFGE analysis was performed with *Apa*I and *Asc*I enzymes for all isolates. The 255 isolates displayed 123 combined *Asc*I-*Apa*I PFGE types (**Table S1**). To estimate the respective discriminatory power of MLVA and PFGE methods using only unrelated isolates (45), we excluded 9 of the 10 isolates shown to be identical by MLVA and PFGE and that were associated to the 1992 French outbreak (see below). Based on the 246 remaining isolates, Simpson's index of discrimination was 98.5% for *ApaI+AscI* PFGE (95% confidence interval,

0.980-0.990). In contrast, Simpson's index was only 93.6% (CI, 0.921-0.951) for MLVA based on the 10 loci (excluding Lm11). When taking into account locus Lm11, 69 distinct MLVA profiles were observed and Simpson's index increased only to 94.5% (0.932-0.959), still well below PFGE discriminatory power (**Table S1**). As expected, several PFGE profiles were observed for isolates that shared the same MLVA type. However, in some cases, the reverse was observed: several MLVA types were found for isolates of the same PFGE profile. Interestingly, the relative discrimination of MLVA and PFGE appeared to depend on the MLST clone (**Table 4**). The most notable difference between MLVA and PFGE was found for CC7 isolates, with Simpson' indexes of 0.10 and 0.90, respectively. Isolates of this clone had only two distinct MLVA types, whereas they corresponded to 13 distinct PFGE types. Similarly, CC1 isolates showed only 4 MLVA types, in contrast to 28 PFGE profiles, whereas CC2 was subtyped into 5 MLVA and 19 PFGE patterns, and CC6 showed 6 MLVA and 11 PFGE patterns (**Table 4**). CC1, CC2 and CC6 include strains included in epidemic clones I, IV and II, respectively. Therefore, they correspond to clonal groups that have caused multiple outbreaks. These results show that MLVA has limited discriminatory power in epidemiologically important clones. However, interestingly, MLVA was more discriminatory than PFGE among isolates of CC4 (11 types versus 7; Simpson 0.91 versus 0.75) and CC9 (8 types versus 5; Simpson 0.78 versus 0.32). Therefore, MLVA may be useful for subtyping isolates that belong to these particular clones.

Retrospective investigation of the 1992 French outbreak based on MLVA. In order to evaluate the potential use of MLVA for outbreak investigation and to compare this method with PFGE within the context of an outbreak, we studied 24

isolates that had been collected during the period of the 1992 French outbreak of listeriosis. These isolates included 13 human clinical isolates that spanned the July-October 1992 period, as well as 11 food isolates collected during epidemiological investigations of this outbreak. The 24 isolates displayed 13 distinct combined *Apa*I/*Asc*I PFGE types and only three distinct MLVA patterns. The most frequent PFGE pattern (*Apa*I/*Asc*I-38/41) was represented by 10 isolates, among which the LM25703 strain, which was isolated from the incriminated source, pork tongue in jelly. These 10 isolates displayed MLVA type 0025, showing that MLVA did not exclude any isolate that was associated to the outbreak PFGE pattern. However, out of the 14 isolates with other PFGE types, 9 also had MLVA pattern 0025, whereas 4 had MLVA pattern 0022, differing by a single repeat unit at both JLR1 and Lis-TR1869), and one had MLVA pattern 0031, differing from MLVA pattern 0025 at 6 out of 11 loci (**Figure 3**). The 9 isolates with MLVA pattern 0025 but with a PFGE type that was distinct from *Apa*I/*Asc*I-38/41, differed from the latter pattern by up to 8 bands with *Asc*I and up to 5 bands with *Apa*I. These results showed that MLVA did not distinguish from the source isolate, several isolates that were clearly distinguished from the source isolate based on PFGE.

Discussion

L. monocytogenes strain subtyping is widely practised in the context of human listeriosis surveillance and food safety control. Because the typing methods that are currently widely used, including serotyping, PFGE and MLST present a series of limitations, MLVA has attracted intense interest. MLVA fulfils a number of criteria recognized as important for successful implementation and interpretation of a typing method (45). In particular, the rapidity, low cost and easy implementation are practical advantages of MLVA that are largely recognized (29, 45). In the present study, we have developed an optimized MLVA scheme as a typing method for *L. monocytogenes* and compared it to two reference methods, MLST and PFGE. We have tested exhaustively the previously published VNTR primer sets and defined new ones against a genetically diverse *L. monocytogenes* set of strains. This allowed us to (i) develop a simple MLVA typing system, MLVA-11, which is based on two multiplex PCR assays combining 11 selected VNTR loci, (ii) analyse the variation at these 11 loci among a collection of 255 isolates, and (iii) compare the obtained MLVA data with PFGE and MLST data.

Previous MLVA schemes have been associated with a number of PCR failures for some VNTR loci, resulting in incomplete characterization of some isolates (28, 30, 35, 43) . Missing data lead to lower discrimination and loss of informative characters for phylogenetic placement, and render the interpretation of differences among MLVA profiles problematic. Here, we optimized the selection of VNTR loci and sequence of primers. We thus achieved nearly complete (99.9%) typeability for 10 markers, even when combining these assays into two multiplexed PCR reactions. As previously reported (43), we found null alleles at locus Lm11, but these PCR failures were

restricted to isolates of CC7 and CC8, consistent with the absence of the corresponding locus in the genome of representative strains of these two clones. However, we chose to keep this locus in the M1 multiplex assay, because it provides some discrimination within CC1, one of the epidemiologically most important clones of *L. monocytogenes*. Besides, the adjunction of the Lm11 primers in the M1 multiplex PCR mix did not affect negatively the assay for the other loci. As our sample covers a large breadth of the clonal diversity of lineages 1 and 2, including all clinically most frequent serotypes and clonal groups, we demonstrate that the MLVA-11 system has broad applicability, an important characteristic of typing systems (45).

While this work was in progress, two studies were published in which distinct MLVA schemes were compared and combined (5, 28). Our MLVA scheme includes 4 loci that were not used by Li et al., while these authors' scheme has 2 loci that we chose not to include (LM-TR3 and LM-TR6). Chen et al. combined the three VNTR loci from Murphy *et al*. (35) with the three loci from Miya *et al*. (34). However, LM4b-TR1 (34) and LM-TR3 (35) correspond to the same locus, and are therefore redundant. Besides, locus LM-TR3 was removed from our scheme as variation at this locus is difficult to interpret due to size variation in the regions flanking the repeat array (43).

Allele sequencing allowed us to confirm that size variation at the 11 selected loci is attributable to repeat number differences. We showed that MLVA loci are stable during laboratory subculture, and found complete repeatability of allele coding using capillary electrophoresis separation. Elimination of loci with non-specific amplification also facilitated the sizing of fragments and the determination of tandem repeat numbers. We also showed that the selected *L. monocytogenes* VNTR loci have a very strong tendency to evolve by the stepwise addition or removal of single

repeat units. This observation indicates that the quantitative difference in the number of repeats, rather than simple allelic mismatch, can be taken into account to estimate strain relationships.

We compared MLVA-11 with the three widely used methods serotyping, PFGE and MLST. While several MLST schemes have been published (36, 39, 41), only the Institut Pasteur scheme (39) provides a standardized nomenclature through a publicly accessible database and is used in a coordinated manner by multiple laboratories (www.pasteur.fr/mlst). Using this MLST scheme, we characterized for the first time, reference strains of several past outbreaks and show that previously defined epidemic clones appear to correspond to MLST defined major clones. Our MLVA assay successfully discriminated all MLST clonal complexes, including those that correspond to ECI, ECII, ECIII and ECIV. MLVA-11 thus appears as a very powerful method to identify these clones, as MLVA patterns are clone-specific and as cluster analysis of MLVA patterns is strongly concordant with MLST clones. This good agreement can be explained by the fact that MLVA and MLST markers have similar levels of variation and by the rarity of recombination among *L. monocytogenes* strains. MLVA could therefore be used as a rapid identification tool for epidemiologically important clonal groups. For this purpose, it will be important to elaborate a more complete MLVA-MLST dictionary, by mapping onto the MLST diversity, every novel MLVA pattern. Along the same lines, one previous study compared MLVA with MLST data and found that MLVA could be useful for recognition of three epidemic clones of serotype 4b (34). It is interesting that the two multiplex assays of the MLVA-11 system may be used for different purposes. Whereas M2 multiplex alone clustered strains according to their clone and could

569 therefore represent a useful rapid clone identification method by itself, in turn, M1
570 PCR was more useful to discriminate among isolates inside clones.
571 One advantage of molecular typing methods, in contrast to serotyping, is that
572 genetic markers may be used to estimate phylogenetic relationships among strains.
573 MLVA is generally regarded as an unreliable phylogenetic method, due to the high
574 frequency of homoplasies. Such events can either correspond to reversion to
575 ancestral states or to convergent evolution leading to a same allele by independent
576 changes in different lineages (4, 40). We calculated that approximately half of the
577 changes in our dataset were evolutionary reversions or convergences. Therefore,
578 MLVA data comprise a substantial degree of phylogenetic noise and must be
579 interpreted with caution. As most VNTR loci are located within genes putatively
580 encoding surface exposed proteins (**Table 2**), it is possible that the number of
581 repeats is subjected to selective pressures. In this context, it is remarkable that
582 cluster analysis of the MLVA-11 profiles did recover the two main subdivisions
583 corresponding to the two major phylogenetic lineages 1 and 2, and classified strains
584 according to their MLST clone (**Figure 3**). This result indicates that despite
585 homoplasy, MLVA variation in *L. monocytogenes* does convey useful phylogenetic
586 information. Several MLVA alleles are largely conserved within either lineage 1 and
587 2: for example, Lis-TR1317 has predominantly allele 4 in lineage 1 and allele 3 in
588 lineage 2, while locus JLR4 has mostly alleles 8 and 4 in lineages 1 and 2,
589 respectively. This remarkable stability within lineages indicates that some VNTR
590 markers diversify very slowly in *L. monocytogenes*. Another requirement for MLVA to
591 convey phylogenetic information is that these loci must undergo restricted amounts of
592 genetic recombination among *L. monocytogenes* strains, which is consistent with low
593 recombination rates estimated based on MLST and full genome sequence analyses

(10, 39). It is also important to use a relatively high number of VNTR markers, as the use of each multiplex individually did not recover phylogenetic placements as accurately (data not shown).

Listeriosis is a global public health issue that led to the implementation of surveillance systems in several countries in the EU, the USA and Canada (16). In this context, the high discriminatory power of PFGE made this method the *de facto* gold standard typing method. Our study indicates that MLVA has lower discriminatory power than PFGE based on enzymes *Apa*I and *Asc*I, which is the current standard. Two previous evaluations of MLVA have concluded that MLVA had better discrimination than PFGE (30, 34). However, PFGE in these studies was performed with a single enzyme, either *Apa*I or *Asc*I, and were thus based on a less discriminatory implementation of PFGE. Sperry et al. (43) compared MLVA with PFGE data for 123 isolates and demonstrated a lower discrimination of MLVA as compared to *Apa*I and *Asc*I PFGE, a conclusion with which our findings fully agree. Our scheme includes 7 of the 8 loci included in the scheme of Sperry et al. and 4 additional loci. A surprisingly high discrimination was achieved by Li et al. (28) based on 9 MLVA loci, possibly due to inclusion of solely unrelated strains, many of which from food and the environment. From the above, we conclude that MLVA cannot be viewed as a replacement for PFGE when discrimination is a key requirement.

Interestingly, we showed that the discrimination of MLVA relative to PFGE is highly dependent on the clone. In particular, MLVA should be a useful addition to PFGE for the discrimination of strains that belong to CC4 or CC9. It is conceivable that MLVA loci evolve faster in some clonal lines, even though the reasons behind this heterogeneity are currently unknown. Alternatively, mobile elements including insertion sequences and phages may be less dynamic in some clones.

The high number of distinct MLST sequence types (STs) per clone and the Simpson index of discrimination of the MLST method (**Table 4**) should not be taken as evidence that MLST has higher discrimination power than MLVA. Indeed, strains with distinct STs were included purposefully in the study, based on our previous MLST analyses (8, 39), in order to test the ability of MLVA for identification of all variants within clones. This selection has inflated MLST's Simpson index artificially. However, as multiple isolates with the same ST or closely related ones were not discriminated by MLVA, it indicates that MLVA does not subtype MLST clones efficiently, with the exceptions of CC4 and CC9.

Concordance of typing results with epidemiological information is a desirable characteristic of a typing method (45). In our study, we used mostly independent isolates collected over wide temporal and geographical scales. Nevertheless, we found several groups of epidemiologically unrelated isolates that shared the same MLVA pattern, and sometimes also the same PFGE pattern (**Table S1**; PFGE16/54 and MLVA0010 (n=9), PFGE27/11 and MLVA0055 (n=6), PFGE53/14 and MLVA0048 (n=7), PFGE42/41 and MLVA0022 (n=5). These results show that identity of MLVA and PFGE profiles does not necessarily imply a direct epidemiological link. This is especially true for MLVA, and in our retrospective study from the French pork jelly outbreak, some isolates defined as unrelated by PFGE were not distinguished by MLVA.

MLVA has strong potential for inter-laboratory standardization, as this method is highly reproducible and as data scoring into integer numerals provides unambiguous results. This is largely regarded as an advantage over PFGE, which can imply partly subjective decisions during band scoring. Standardization would provide benefits to international surveillance and population biology. However, MLVA

644 standardization requires calibration of fragment sizing apparatuses and inter-
645 laboratory reproducibility needs to be carefully evaluated. To this purpose, we
646 identified a set of 12 isolates, which display distinct alleles at each of the 11 loci, and
647 together represent 80% of all distinct alleles found in this study (**Table S1**). This
648 MLVA reference set of strains is available upon request and should constitute a
649 useful resource for inter-laboratory calibrations.
650
651 **Conclusions**
652 MLVA was implemented in this study in the form of two multiplexed PCRs combining
653 a total of 11 VNTR markers. The variation disclosed at these loci proved highly
654 consistent with MLST data and was phylogenetically informative. These results show
655 that MLVA could be used as a rapid identification method for MLST defined clonal
656 groups, including those corresponding to the so-called epidemic clones ECI to ECIV.
657 Because it has lower discriminatory power, MLVA cannot replace PFGE for outbreak
658 investigations. However, given its simplicity, low cost, high throughput and rapid time
659 to results (around 8 hours), MLVA could represent a useful screening method to
660 alleviate PFGE workload. Within the context of an outbreak, MLVA could
661 advantageously fill the gap between the throughput needed to characterize high
662 number of isolates in a short period of time, and the high discrimination level needed
663 for informed epidemiological decisions. MLVA may also represent a suitable first line
664 assay in listeriosis surveillance, with PFGE efforts being focused on common MLVA
665 genotypes. A two-step MLVA-PFGE strategy could save significant workload and
666 would position MLVA as an important new tool in listeriosis surveillance.
667

668 **Acknowledgements**

669 We thank M. Wiedmann for providing strains of the ILSI collection and Marie Ragon
670 and Alban Le Monnier for contributing to the initiation of this work. We acknowledge
671 Nathalie Tessaud-Rita and Arnaud Bertel for technical help.

672

673 **Funding**

674 This work was supported financially by Institut Pasteur and Institut de Veille Sanitaire
675 (Saint Maurice, France).

676

677 **Figure legends**

678

679 **Figure 1**. Approximate location of VNTR loci along the chromosome of strain EGDe
680 (accession number, NC_003210). For loci identified and analyzed by multiple
681 authors, alternative names are indicated in boxed areas. Underlined loci were
682 identified in this study. The 11 VNTR loci selected for use in the present study are
683 indicated in bold type. Open circles locate loci that were not retained in this study.
684 Circles with checkerboard patterns were included in multiplex PCR group M1. Circles
685 with grey shade were included in multiplex PCR group M2.

686

687 **Figure 2**. MLST-based minimum spanning tree of the 728 *L. monocytogenes* isolates
688 of lineages 1 and 2 characterized by MLST either in previous work (1, 8, 39) or in this
689 study. For each lineage separately, each color represents isolates with an identical
690 MLVA type (not taking locus Lm11 into account). Note that as panels A and B were
691 colored independently, identical colors in panel A and in panel B represent distinct
692 MLVA types. There was no single MLVA type shared among isolates of both
693 lineages. Each circle represents a sequence type (ST). The diameter of circles is
694 related to the number of isolates with this ST. Colored sectors indicate the proportion
695 of isolates with a given MLVA type; white correspond to isolates that were not
696 included in the present study. Bold lines between circles correspond to links with a
697 single allelic mismatch; plain lines to those with two allelic mismatches. Grey zones
698 around circles delineate clonal complexes. Links corresponding to more than two
699 allelic mismatches were not represented, as several equally likely alternative links
700 exist; therefore, the relative position of clonal complexes or single STs should not be
701 taken as evidence of phylogenetic proximity. Numbers inside or just above circles

indicate the ST number of the central ST of numerically important clonal complexes. Left panel, lineage 1; right panel, lineage 2. All data are publicly available from http://www.pasteur.fr/mlst.

Figure 3. UPGMA dendrogram of the 66 MLVA patterns identified in this study. The dendrogram was based on the 11 markers, whereas the MLVA type was defined based on 10 markers, after excluding Lm11. Absence of this locus is denoted with allele code '99' in most strains of clones CC7 and CC8. The stars in the MLVA type column indicate the patterns that are discriminated solely by Lm11. Lineages 1 and 2 are highlighted by dark and light grey zones in the dendrogram, respectively.

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Figure 1

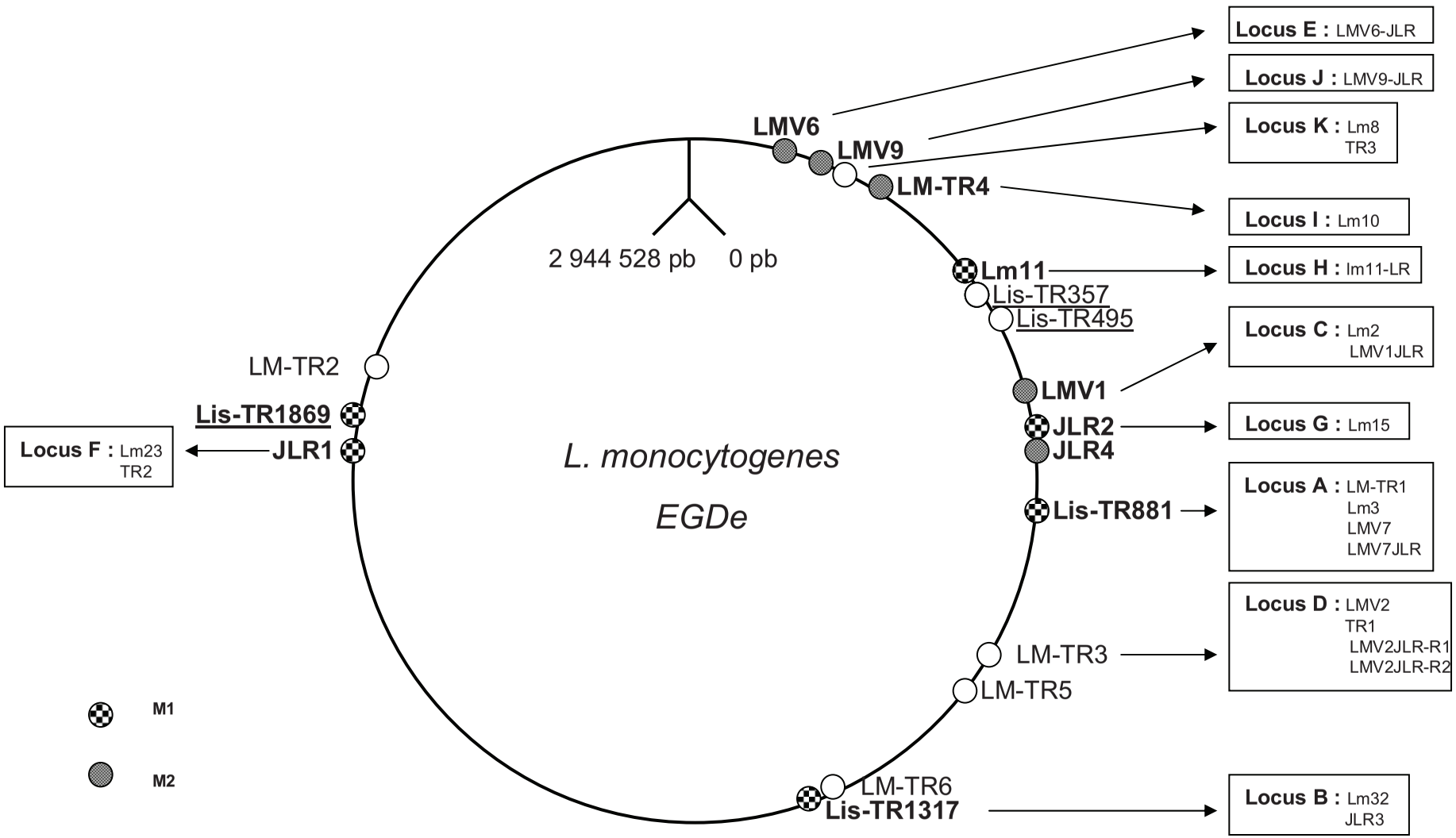
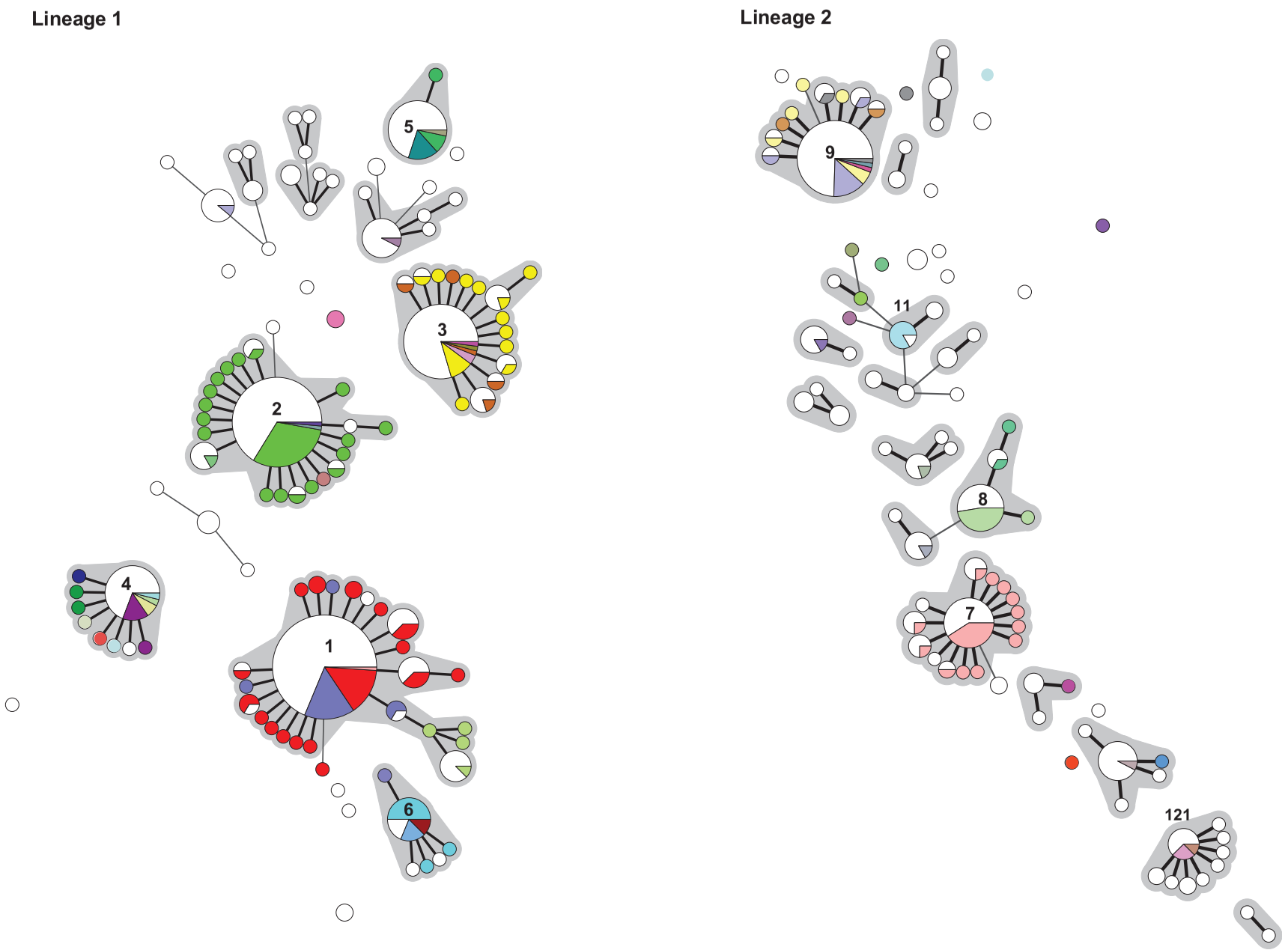


Figure 2



Multiplex M1						Multiplex M2								
JLR1	JLR2	Lis-TR1317	Lis-TR1869	Lis-TR881	Lm11 *	JLR4	LM-TR4	LMV1	LMV6	LMV9	MLVA type	M1 type	M2 type	Clonal complex	Sequence type
16	7	4	8	7	5	8	2	25	3	11	0020	0021	0003	CC3	3
16	7	4	8	7	5	8	2	22	3	11	0017	0021	0001	CC3	3
16	7	4	8	6	5	8	2	22	3	11	0016	0020	0001	CC3	3
16	7	7	8	7	5	8	2	22	3	11	0021	0022	0001	CC3	3
14	7	4	7	6	5	8	2	22	3	11	0019	0015	0001	CC3	3
18	6	3	9	5	5	8	1	22	2	11	0029	0029	0006	CC5	5
18	6	3	9	5	5	8	1	22	3	11	0028	0029	0002	CC5	409
16	6	3	8	5	5	8	1	22	3	11	0018	0031	0002	CC5	5
17	5	4	8	4	5	8	2	21	2	11	0025	0003	0008	CC1	63
15	5	4	7	4	5	8	2	21	2	11	0031	0011	0008	CC1	1
19	5	4	9	4	5	8	2	21	2	11	0023	0007	0008	CC1	422
18	5	4	9	4	5	8	2	21	2	11	0022*	0006	0008	CC1	1
18	5	4	9	4	6	8	2	21	2	11	0022*	0006	0008	CC1	1
18	6	4	9	4	5	8	2	22	2	11	0024	0005	0005	CC558	558
18	6	4	9	4	5	8	2	21	2	12	0026	0005	0007	CC1	104
14	5	4	7	4	5	8	2	18	7	11	0034	0012	0009	CC6	81
11	5	4	5	4	5	8	2	18	7	11	0033	0004	0009	CC6	6
23	5	4	11	4	5	8	2	18	5	11	0064	0001	0010	CC6	6
22	5	4	11	4	5	8	2	18	5	11	0065	0002	0010	CC6	443
18	5	4	9	4	5	8	2	18	5	11	0066	0006	0010	CC6	254
11	5	4	5	4	5	8	2	23	3	14	0015	0004	0004	CC288	288
13	8	4	6	4	5	7	1	21	3	12	0014	0017	0033	CC2	2
13	6	4	6	4	5	7	1	21	3	12	0012	0016	0033	CC2	145
13	7	4	6	4	5	7	1	21	3	11	0013	0018	0034	CC2	246
13	7	4	6	4	5	7	1	21	3	12	0010	0018	0033	CC2	2
13	7	4	6	3	5	7	1	21	3	12	0011	0019	0033	CC2	2
15	7	4	7	4	4	8	4	21	4	12	0001	0013	0012	CC4	4
15	7	4	7	4	4	8	4	21	4	15	0008	0013	0011	CC4	125
15	7	3	7	4	4	8	4	21	4	13	0004	0014	0014	CC4	408
15	7	4	7	4	4	8	4	21	4	13	0003	0013	0014	CC4	4
15	7	4	7	4	4	8	4	15	4	13	0032	0013	0016	CC4	4
15	7	4	7	4	4	8	3	21	4	13	0005	0013	0013	CC4	4
15	7	4	7	4	4	8	3	21	4	12	0002	0013	0019	CC4	4
15	7	4	7	4	4	8	2	21	4	13	0006	0013	0015	CC4	4
13	7	4	6	4	4	8	4	21	4	13	0009	0018	0014	CC4	146
15	6	2	7	4	4	7	4	21	99	12	0068	0010	0035	CC4	251
18	6	3	9	5	4	8	2	21	3	13	0030	0029	0017	CC59	59
15	6	4	7	5	4	8	4	21	3	13	0007	0009	0018	CC4	242
18	4	3	9	5	99	4	2	22	3	8	0060	0027	0032	CC376	376
19	4	3	9	5	1	4	2	22	2	8	0058	0026	0025	CC11	11
16	4	3	8	5	4	4	2	22	1	8	0036	0030	0023	CC155	155
22	4	3	10	8	4	4	2	22	4	8	0057	0033	0021	CC18	18
22	4	3	11	7	99	4	2	23	2	8	0055	0038	0026	CC8	8
22	4	3	11	8	99	4	2	23	2	8	0054	0037	0026	CC8	232
99	4	3	4	7	99	4	2	23	2	8	0067	0039	0026	CC16	16
21	4	3	10	8	99	4	2	23	2	8	0056	0034	0026	CC8	8
21	4	3	10	11	99	4	2	22	2	8	0063	0035	0025	CC412	412
10	4	4	5	5	99	4	2	23	2	8	0047	0025	0026	CC19	19
16	4	3	8	5	4	4	2	23	2	8	0035	0030	0026	CC556	556
22	4	3	10	5	4	4	2	23	2	8	0061	0032	0026	CC415	394
11	8	3	5	5	4	4	2	23	2	8	0052	0023	0026	CC101	101
11	10	3	5	5	4	4	2	23	2	8	0053	0024	0026	CC101	101
21	6	3	10	5	4	4	2	21	2	8	0062	0036	0024	CC121	108
12	4	3	6	8	4	4	2	25	3	8	0041	0042	0031	CC9	9
12	4	3	6	8	4	4	2	25	2	8	0043	0042	0027	CC9	9
12	4	3	6	8	4	4	2	25	4	8	0046	0042	0022	CC9	9
12	4	3	6	7	4	4	2	25	2	8	0040	0040	0027	CC9	9
12	4	3	6	7	4	4	2	25	3	8	0039	0040	0031	CC9	9
12	4	3	6	7	4	4	2	24	3	8	0038	0040	0030	CC9	34
12	4	3	6	8	4	4	2	24	3	8	0042	0042	0030	CC9	9
12	4	3	6	7	4	4	2	24	4	8	0045	0040	0020	CC9	35
12	4	3	6	5	99	4	2	21	3	8	0048*	0041	0029	CC7	7
12	4	3	6	5	4	4	2	21	3	8	0048*	0041	0029	CC7	7
12	4	3	6	5	5	4	2	21	3	8	0048*	0041	0029	CC7	7
12	5	3	6	5	99	4	2	21	3	8	0049	0043	0029	CC7	7
13	5	3	6	6	4	4	2	24	4	8	0051	0046	0020	CC26	93
13	5	3	6	6	4	4	2	24	3	8	0050	0046	0030	CC26	26
13	5	3	10	5	4	4	2	24	2	8	0037	0047	0028	CC121	121
18	5	99	9	6	99	4	2	21	2	8	0069	0008	0024	CC370	370

Table 1. VNTR loci assayed during the initial screening. VNTRs in bold type were selected for the multiplex MLVA assay.

Locus	PCR and VNTRs name	Reference	Annealing temperature (°C)	Comment on technical results
A	LM-TR1	Murphy	54	Difficult to calibrate, many alleles < 1
	Lm3	Sperry	50	Satisfactory
	LMV7	Lindstedt	63	Some non-specific PCR fragments
	Lis-TR881	This study	50	Satisfactory; included in M1 multiplex
	LMV7JLR	Larsson	60	Satisfactory
B	Lm32	Sperry	50	Some non-specific PCR fragments
	Lis-TR1317	This study	55	Satisfactory; included in M1 multiplex
	JLR3	Larsson	60	Some negative PCR amplification
C	Lm2	Sperry	50	Satisfactory
	LMV1	Lindstedt	63	Satisfactory; included in M2 multiplex
	LMV1JLR	Larsson	60	Satisfactory
D	LM-TR3	Murphy	54	Some non-specific PCR fragments
	LMV2	Lindstedt	60	Some negative PCR amplification
	LMV2JLR-R1	Larsson	60	Many negative PCR amplification
	LMV2JLR-R2	Larsson	60	Many negative PCR amplification
	TR1	Miya	60	Some non-specific PCR fragments
E	**LMV6**	Lindstedt	60	Satisfactory; included in M2 multiplex
	LMV6-JLR	Larsson	60	Some negative PCR amplification
F	Lm23	Sperry	50	Some non-specific PCR fragments
	JLR1	Larsson	60	Satisfactory; included in M1 multiplex
	TR2	Miya	60	Some negative PCR amplification and non-specific PCR fragments
G	Lm15	Sperry	50	Satisfactory
	JLR2	Larsson	60	Satisfactory; included in M1 multiplex
H	**Lm11**	Sperry	50	Included in M1 multiplex despite some negative PCR amplification (CC7, CC8)
	lm11-LR	Larsson	60	Some negative PCR amplification
I	**LM-TR4**	Murphy	54	Satisfactory; included in M2 multiplex
	Lm10	Sperry	50	Difficult to calibrate
J	**LMV9**	Lindstedt	63	Satisfactory; included in M2 multiplex
	LMV9-JLR	Larsson	60	Some non-specific PCR fragments
K	Lm8	Sperry	50	Only one allele
	TR3	Miya	60	Some negative PCR amplification and non-specific PCR fragments
L	**JLR4**	Larsson	60	Satisfactory; included in M2 multiplex
M	LM-TR2	Murphy	54	Only one allele

N	LM-TR5	Murphy	54	Some non-specific PCR fragments
O	LM-TR6	Murphy	52	Some non-specific PCR fragments
P	Lis-TR357	This study	55	Only 2 alleles
Q	Lis-TR495	This study	60	Some negative PCR amplification
R	**Lis-TR1869**	This study	60	Satisfactory; included in M1 multiplex

Table 2: Information on the 11 VNTR loci selected for the two multiplex M1 and M2 PCR assays.

PCR and VNTR locus name	VNTR location in EGDe	Gene - protein annotation	Primer name	Primer sequence (5'-3')[a]	Final concentration (µM)	Reference
M1						
JLR1	1869662-1870002	lmo1799-peptidoglycan binding protein	JLR1_F JLR1_R	*HEX*-GCGCTATAACCTGAGGAAAGC GTCTTAATCCATGCAGATGGAAC	6	Larsson
JLR2	668711-668944	lmo0627-peptidoglycan bound protein	JLR2_F JLR2_R2	*AT565*-CCTTCCAGAGAAAGACAAAACAG RCTAATCCACCAGCAAATAGC	0.5	Larsson
Lis-TR1317	1317239-1317407	lmo1290-hypothetical protein	LisTR1317_F LisTR1317_R	*AT550*-TGATTTACAAAAAGCTTTGCC ACTTGGCACTTCTGGTTTA	1	This study
Lis-TR1869	1869660-1870086	lmo1789-peptidoglycan bound protein	LisTR1869_F LisTR1869_R	*AT550*-CCGCGCTATAACCTGAGGAAAGC CTGAAATCATTGCAATCAGATGCACC	0.5	This study
Lis-TR881	881441-881713	lmo0842-peptidoglycan binding protein	LisTR881_F LisTR881_R	*FAM*-TGTAAATAAAGCTGGTACGTAC GTATGTTGCTTGTTATCAACTAC	0.5	This study
Lm11	344898-345043	lmo0320-hypothetical protein-cell wall surface anchor family protein	Lm11_F Lm11_R	*HEX*-GAATAAAATGCTAGATGTGG CCGATTCAAAAATAGTAAAC	2	Sperry
M2						
JLR4	695469-695639	lmo0652-hypothetical protein	JLR4_F JLR4_R	*HEX*-AGAAATTCCAGTCCGCCAG GGARCAACAGAAGCTGATCCA	2	Larsson

LM-TR4	228768-229245	ftsH-hypothetical protein-ATP-dependent metalloprotease FtsH	LMTR4_F LMTR4_R	*AT565*-TCCGAAAAAGACGAAGAAGTAGCA TGGAACGACGGACGAAATAATAAT	2	Murphy
LMV1	619129-619524	iap-probable endopeptidase p60	LMV1_F LMV1_R	*AT550*-CGTATTGTGCGCCAGAAGTA MAMCAACRCAACAACAAACAG	1	Lindstedt
LMV6	159077-159325	lmo0159-peptidoglycan binding protein	LMV6_F LMV6_R	*FAM*-AAAAGCCCCRATTGGATA CTCGCTGTTTTCTGWTTTCTTAGG	4	Lindstedt
LMV9	161054-161554	lmo0160-peptidoglycan binding protein	LMV9_F LMV9_R	*HEX*-AACGGTKRCKGATTTACTTC CTTGGYGTCGAGGCATTTA	2	Lindstedt

[a] Forward primers were labelled with fluorescent dyes as indicated.

Table 3. Characteristics of the 11 selected VNTR markers.

Name	Repeat sequence	Repeat size (bp)	Multiplex group	Size range (bp)	No. alleles	Allele range (copy no.)	Null alleles (%)	Homoplasy index	Simpson index (%)
JLR1	-CAGCAT-	6	M1	331.8-403.5	14	11-23	0.4	0.41	0.87
JLR2	-CAAAAGATACAC-	12	M1	231.5-301.1	6	4-10	0	0.67	0.72
Lis-TR1317	-AACACCAACACCAGACCCAACACC-	24	M1	148.9-260.1	5	2-7	0.4	0.5	0.47
Lis-TR1869	-TCAGCATCAGCG-	12	M1	403.4-488.3	8	4-11	0	0.59	0.77
Lis-TR881	-AAAACCAATAAAACCATC-	18	M1	189.9-338.7	7	3-11	0	0.60	0.65
Lm11	-TTGCTTGTTTTTG-	12	M1	108.4-173.1	5 (incl. null)	1-6	14.9	0.60	0.59
JLR4	-CTTCTGGAGCTTCTGGTA-	18	M2	170.2-243.1	3	4-8	0	0.50	0.60
LM-TR4	-GAAGAACCAAAA-	12	M2	462.7-498.7	4	1-4	0	0.57	0.42
LMV1	-TTGTAT-	6	M2	348.0-406.8	7	15-25	0	0.57	0.63
LMV6	-AGTACCACCAACACC-	15	M2	212.0-300.9	7	1-7	0.4	0.60	0.63
LMV9	-AGAAAAACC-	9	M2	496.3-550.5	6	8-14	0	0.50	0.67

Table 4. **Ability of three molecular genotyping methods to discriminate isolates within major *L. monocytogenes* clonal complexes (CC) #**

	MLST		MLVA		PFGE	
CC (No. of isolates)	**Number of STs**	**Simpson's index**	**Number of M1+M2 MLVA types (M1 alone, M2 alone)**	**Simpson's index (M1+M2)**	**Number of PFGE profiles**	**Simpson's index**
1 (61)	24	0.76	4 (4,2)	0.54	28	**0.94**
2 (41)	19	0.69	5 (4,2)	0.19	19	0.88
3 (25)	16	**0.85**	5 (4,2)	0.56	11	0.88
4 (17)	8	0.67	11 (5,9)	**0.91**	7	0.75
5 (10)	2	0.20	3 (2,2)	0.64	5	0.76
6 (18)	6	0.49	6 (6,3)	0.68	11	**0.94**
7 (22)	13	0.81	2 (2,1)	0.10	13	0.90
8 (13)	4	0.42	3 (3,1)	0.41	6	0.77
9 (23)	10	0.64	8 (2,5)	0.78	5	0.32

Numbers are given not taking into account locus Lm11, which showed many missing data in clones CC7 and CC8