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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

Viviane Chenal-Francisque, Laure Diancourt, Thomas Cantinelli, Virginie Passet, Coralie Tran-Hykes, H el ene Bracq-Dieye, Alexandre Leclercq, Christine Pourcel, Marc Lecuit, Sylvain Brisse

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

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5 Viviane Chenal-Francisque^{1,2,3}, Laure Diancourt⁴, Thomas Cantinelli^{1,2,3}, Virginie
6 Passet^{4,5}, Coralie Tran-Hykes⁴, H el ene Bracq-Dieye^{1,2,3}, Alexandre Leclercq^{1,2,3},
7 Christine Pourcel^{5,6}, Marc Lecuit^{1,2,3,7,8*} and Sylvain Brisse^{4,9*}

8
9 ¹ Institut Pasteur, National Reference Centre and World Health Organisation
10 Collaborating Centre for *Listeria*

11 ² Institut Pasteur, Biology of Infection Unit, Paris, France

12 ³ Inserm U1117, Paris, France

13 ⁴ Institut Pasteur, Genotyping of Pathogens and Public Health, Paris, France

14 ⁵ Univ Paris-Sud, Institut de G en etique et Microbiologie, UMR 8621, Orsay, France

15 ⁶ CNRS, Orsay, France

16 ⁷ Paris Descartes University, Sorbonne Paris Cit e, Institut Imagine, Paris, France

17 ⁸ Necker-Enfants Malades University Hospital, APHP, Division of Infectious Diseases
18 and Tropical Medicine, Paris, France

19 ⁹ Institut Pasteur, Microbial Evolutionary Genomics, Paris, France

20 * Corresponding authors

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

23 M. Lecuit. Biology of Infection Unit, Institut Pasteur, 25 rue du Dr Roux, F-75724
24 Paris, France. E-mail: marc.lecuit@pasteur.fr; Phone +33 1 40 61 30 29

25

26 **Abstract**

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

43

44

Introduction

45

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

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

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

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

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

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

117 (iii) to evaluate and compare to PFGE, the added value of MLVA in terms of strain
118 discrimination within clonal groups of particular interest.
119

120

Materials and Methods

121

122 **Bacterial isolates.** A total of 255 isolates were included in the study (**Table S1**
123 **strains**). First, 217 *L. monocytogenes* isolates were selected from the National
124 Reference Centre for Listeria (NRC-L) and from the World Health Organization
125 Collaborative Centre for Listeria (WHO-CC-L) collections. This included 155 isolates
126 previously characterized by us: 58 isolates from the study of Ragon et al. (39) and 97
127 isolates from the study of Chenal-Francisque et al. (8). We also included 38 isolates
128 selected to represent additional epidemiologically unrelated isolates of clones CC3,
129 CC4, CC5 and CC8, which were represented only by few isolates in our previous
130 studies. To assess the epidemiological concordance of MLVA, we also included 24
131 strains collected during the period of a large outbreak that occurred in 1992 in France
132 linked a ready-to-eat meat product, pork tongue in jelly (20). The 217 above isolates
133 were epidemiologically unrelated, except for some of the 24 human or food isolates
134 corresponding to the period of the 1992 French outbreak (see results). Overall, the
135 217 isolates were recovered from human infection cases (n=130), food (n=47),
136 animal (n=18), the environment (n=9), vegetation (n=1), and 12 were of
137 undocumented origin. They were collected from 31 countries across 6 continents and
138 were isolated between 1933 and 2010.

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

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

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

150

151 **DNA preparation and PCR amplification.** Total genomic DNA was extracted using
152 the Promega Wizard Genomic DNA purification kit (Promega, Madison, WI, USA)
153 according to the manufacturer's instructions. DNA samples diluted in water at
154 50 ng/μl were used as template for PCR amplifications.

155

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

158

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

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

171

172 **MLVA assay development.** A preliminary screening was performed on a genetically
173 diversified subset of isolates of major clonal groups of phylogenetic lineages 1 and 2.
174 Each VNTR region was separately amplified by PCR using Isis DNA polymerase (MP
175 Biomedicals, Santa Ana, Ca.). PCR run conditions included a denaturation step at
176 94°C for 4 min, followed by 35 cycles including denaturation at 94°C for 30 s,
177 elongation at a temperature depending on the primer pair (**Table 1**) for 30 s, and an
178 extension step of 72°C for 1 min. A final extension of 72°C for 10 min was performed.
179 The finalized protocol consisted of two multiplexed PCR mixes called M1 (containing
180 six primer sets) and M2 (five primer sets). The Qiagen Multiplex PCR kit (Valencia,
181 CA, USA) was used. The concentrations of the primers were adjusted to obtain even
182 intensities for all fragments, with the forward primers being fluorescently labelled at
183 their 5' end (**Table 2**). The same PCR cycle was used for M1 and M2 multiplex
184 PCRs: 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 56°C for 90 s and
185 72°C for 90 s, with a final extension at 72°C for 10 min.

186

187 **PCR amplicon sequence verification.** The purified PCR products of single PCR
188 reactions performed with unlabeled primers were sequenced to control the molecular
189 origin of size variation. The DNA sequence for every distinct allele of all VNTR loci
190 was determined. Nucleotide sequence data were edited and compared using
191 BioNumerics version 6.6 (Applied Maths, Sint-Martens Latem, Belgium) and Multalin
192 (<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 10th 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 *Ascl* and *Apal* restriction
201 enzymes (16). Data analysis was performed using BioNumerics version 6.5. *Apal*
202 and *Ascl* 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

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

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

228

229

Results

230

231 **MLST analysis of the 255 study isolates.** To determine their diversity and whether
232 they belong to major MLST clones, the 255 isolates were analyzed by MLST. Data
233 for 155 isolates were derived from our previous studies (8, 39), and we performed
234 MLST analysis of 100 additional isolates for the purposes of the present study. These
235 100 isolates included 38 strains from the reference ILSI collection, 24 isolates
236 collected during the large 1992 outbreak in France (20), and 38 isolates representing
237 clones CC3, CC4, CC5 and CC8 (see Materials and Methods). The 7 MLST genes
238 could be amplified for the 100 isolates and their ST was determined. The 20 STs that
239 were not previously described (ST326 or above in **Table S1**) were incorporated into
240 the Institut Pasteur MLST database at www.pasteur.fr/mlst.

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

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

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

255 MLST analysis of the ILSI reference strains representing past outbreaks
256 revealed that all outbreaks except three were caused by strains that belong to major
257 clones as defined by MLST (**Figure S1**). The three exceptions were the 2000 Queso
258 fresco North Carolina outbreak, caused by a strain that belong to ST558, which
259 differs by three MLST genes from ST2, and the 1988 turkey franks case and the
260 2000 sliced turkey deli meat multistate outbreak, caused by a strain that belongs to
261 ST11. The two latter events were traced to the same food processing facility and
262 corresponding strains were shown to be highly similar, which led to the definition of
263 epidemic clone (EC) III (24). ST1, the central genotype of MLST clone CC1, included
264 strains of the 1986 Los Angeles Jalisco outbreak, the 1981 Nova Scotia coleslaw
265 outbreak and the 1983-1987 Switzerland Vacherin Mont d'Or outbreak. These strains
266 were previously defined as belonging to ECI (24). ST2, the central genotype of clone
267 CC2, comprised the reference strains of the Massachusetts pasteurized milk
268 outbreak and the 1987 UK and Ireland pâté outbreak. These isolates were assigned
269 to EC1a, which was later renamed ECIV (7). ST3, the central genotype of CC3,
270 included the strain from the 1994 Illinois pasteurized milk chocolate outbreak. Finally,
271 ST6, the central ST of clone CC6, included the 1988-1989 multistate hotdog outbreak
272 and the 2002 multistate deli turkey outbreak. Strains involved in these two later
273 outbreaks were assigned to ECII (24). Note that the 2008 Canadian outbreak was
274 assigned to a newly defined ECV, which belongs to MLST clone CC8 (15, 25). The
275 ILSI strains from the diversity set were scattered across the MStree (**Figure S1**),
276 consistent with the fact that they were selected to represent a diversity of ribotypes or
277 PFGE types. However, two of these strains (FSL J2-064 and FSL J1-169) belong to

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

283

284 **PCR amplification assays of 18 VNTR loci on a diverse sample of isolates.**

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

303 each of these loci (**Table 1, Figure 1**): LisTR881 (locus A), Lis-TR1317 (locus B),
304 LMV1 (locus C), LMV6 (locus E), JLR1 (locus F), JLR2 (locus G), Lm11 (locus H),
305 LM-TR4 (locus I) and LMV9 (locus J). For loci L and R, a single primer pair defined
306 previously gave a unique expected band for all screened isolates. In contrast, none
307 of five PCR primer sets defined for locus D were satisfactory, as some did not amplify
308 all the tested isolates or non-specific PCR products were observed. Besides, this
309 locus exhibits size variation due to insertion-deletions in the sequences flanking the
310 tandem repeat region (see below), rendering fragment size variation difficult to
311 interpret in terms of tandem repeat numbers. Similarly, a lack of PCR amplification
312 was observed for some screening isolates with LisTR-495 (locus Q), LM-TR5 (locus
313 N) and LM-TR6 (locus O). The latter is consistent with the initial report of PCR failure
314 for many strains for locus LM-TR6 (35). For locus K, the two PCR primer sets also
315 turned out to be unsatisfactory: TR3 gave non-specific or no amplification depending
316 on the strain, and primer pair Lm8 yielded a unique PCR fragment but this fragment
317 was of the same size for nearly all strains, indicating a lack of discriminatory power,
318 as reported previously (43). Likewise, LM-TR2 (locus M) and Lis-TR357 (locus P)
319 provided low discrimination. Loci D, K, M, N, O, P and Q were thus eliminated from
320 the assay optimization. The 11 loci that were retained (**Figure 1, Tables 2 and 3**)
321 included one novel locus identified herein (Lis-TR1869), one locus (JLR4) uniquely
322 described by Larsson (26) and 9 loci previously identified by various authors. Among
323 the latter, two novel primer pairs were used (Lis-TR881 for locus A and Lis-TR1317
324 for locus B). Notably, the 11 retained loci included 7 out of 8 loci selected for
325 inclusion in the Sperry scheme (43) but only 2 out of 6 loci proposed by Murphy et al.
326 (35) and 1 of the three loci used by Miya et al. (34).
327

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

353 255 isolates, with only three missing data points out of 2550: locus JLR1 for FSL J2-
354 063 (ST16, serotype 1/2a), locus Lis-TR1317 for FSL C1-115 (ST370, serotype 3a),
355 and locus LMV6 for strain LM70290 (ST251, serotype 4b). All amplified PCR
356 products were sized and the corresponding tandem repeat number was determined,
357 indicating nearly complete typeability using these ten loci (**Table 3**). However, we
358 observed no Lm11 PCR product for 38 isolates. Interestingly, these isolates all
359 belonged to MLST clonal complexes CC7 or CC8, with the only exception of ST376
360 (strain FSL J2-066, serotype 1/2a). Locus Lm11 could be amplified and sized for two
361 isolates of CC7 but none of CC8. This result suggests a loss of this locus in isolates
362 of CC8 and in the majority of isolates of CC7. Search for Lm11 locus in genomic
363 sequences confirmed its absence in SLCC5850 (CC7) and 08-5578 and 08-5923
364 (CC8). Regarding strains other than CC7 and CC8, locus Lm11 had allele 5 in most
365 strains of lineage 1 and allele 4 in most lineage 2 strains. However, strains of CC4
366 (lineage I) had Lm11-4, and a subset of CC1 strains had allele Lm11-6, which was
367 specific for these strains. Likewise, only strains of ST11 (ECIII) had allele Lm11-1.
368 This locus is therefore very useful for characterization of strains from these specific
369 groups.

370

371 **Identification of major MLST clones by MLVA.** MLVA analysis using the two
372 combined multiplexed PCR assays M1 (excluding Lm11) and M2 resolved the 255
373 isolates into 66 different MLVA types. In contrast, the M1 multiplexed PCR generated
374 44 MLVA M1 types, whereas M2 multiplexed PCR distinguished 35 MLVA M2 types.
375 **Figure 2** illustrates the distribution of the distinct MLVA types onto the MLST
376 diversity (note that the same color in the lineage 1 and 2 panels represent distinct
377 MLVA types). It was remarkable that all major clones were characterized by specific

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

382 Although less informative than when used together, the two multiplexed PCRs
383 M1 and M2 also provided useful information when considered independently. Using
384 M2 PCR, most major clones showed clone-specific patterns that could prove useful
385 for their identification. For example, CC1 isolates were characterized by M2 profile 8-
386 2-21-2-11, within only four exceptions that had profile 8-2-21-2-12 due to variation at
387 locus LMV9 (**Figure 3, Table S1**). Likewise, CC2 isolates had profile 7-1-21-3-12,
388 with a single exception (7-1-21-3-11). In particular, whereas most clones showed
389 several patterns, clones CC3 and CC7 showed only one M2 type that was specific of
390 either CC. Cluster analysis of M2 patterns recovered all major clones as single
391 branches, except CC9 (data not shown). In contrast, due to higher variability of M1
392 multiplex PCR markers, cluster analysis of M1 data did not recover the MLST clones
393 in general.

394

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

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

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

409

410 **MLVA analysis of representative isolates of 'epidemic clones'**. The 38 isolates of
411 the ILSI collection, including the 23 isolates representing outbreaks, were divided into
412 22 MLVA types. The clone CC1 reference strains representing the 1981 Nova Scotia
413 coleslaw outbreak and the 1983-1987 Vacherin Mont d'Or Switzerland outbreak
414 shared the same MLVA type, which differed by a single marker (Lm11) from the
415 1986-1987 California Jalisco soft cheese outbreak (**Figure 3, Table S1**). Clone CC2
416 strains from the 1983 Massachusetts milk outbreak and the 1987-1989 UK and
417 Ireland outbreak shared the same pattern except for variation at locus Lis-TR881
418 (**Figure 3, Table S1**). Clone CC6 isolates from the two represented outbreaks could
419 be distinguished by two loci, Lis-TR1869 and LMV6. Finally, the 4 isolates of CC11
420 (ECIII) from 1988/1989 and 2000 were all identical by MLVA. The CC1, CC2, CC6
421 and CC11 strains previously assigned to different epidemic clones (ECI to ECIV)
422 were clearly distinguished by MLVA, as were the isolates of the ILSI diversity set
423 (**Figure 3, Table S1**).

424

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

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

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

444

445 **Comparison of MLVA with PFGE.** PFGE analysis was performed with *Apal* and
446 *Ascl* enzymes for all isolates. The 255 isolates displayed 123 combined *Ascl*-*Apal*
447 PFGE types (**Table S1**). To estimate the respective discriminatory power of MLVA
448 and PFGE methods using only unrelated isolates (45), we excluded 9 of the 10
449 isolates shown to be identical by MLVA and PFGE and that were associated to the
450 1992 French outbreak (see below). Based on the 246 remaining isolates, Simpson's
451 index of discrimination was 98.5% for *Apal*+*Ascl* PFGE (95% confidence interval,

452 0.980-0.990). In contrast, Simpson's index was only 93.6% (CI, 0.921-0.951) for
453 MLVA based on the 10 loci (excluding Lm11). When taking into account locus Lm11,
454 69 distinct MLVA profiles were observed and Simpson's index increased only to
455 94.5% (0.932-0.959), still well below PFGE discriminatory power (**Table S1**). As
456 expected, several PFGE profiles were observed for isolates that shared the same
457 MLVA type. However, in some cases, the reverse was observed: several MLVA
458 types were found for isolates of the same PFGE profile. Interestingly, the relative
459 discrimination of MLVA and PFGE appeared to depend on the MLST clone (**Table 4**).
460 The most notable difference between MLVA and PFGE was found for CC7 isolates,
461 with Simpson' indexes of 0.10 and 0.90, respectively. Isolates of this clone had only
462 two distinct MLVA types, whereas they corresponded to 13 distinct PFGE types.
463 Similarly, CC1 isolates showed only 4 MLVA types, in contrast to 28 PFGE profiles,
464 whereas CC2 was subtyped into 5 MLVA and 19 PFGE patterns, and CC6 showed 6
465 MLVA and 11 PFGE patterns (**Table 4**). CC1, CC2 and CC6 include strains included
466 in epidemic clones I, IV and II, respectively. Therefore, they correspond to clonal
467 groups that have caused multiple outbreaks. These results show that MLVA has
468 limited discriminatory power in epidemiologically important clones. However,
469 interestingly, MLVA was more discriminatory than PFGE among isolates of CC4 (11
470 types versus 7; Simpson 0.91 versus 0.75) and CC9 (8 types versus 5; Simpson 0.78
471 versus 0.32). Therefore, MLVA may be useful for subtyping isolates that belong to
472 these particular clones.

473

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

477 isolates that had been collected during the period of the 1992 French outbreak of
478 listeriosis. These isolates included 13 human clinical isolates that spanned the July-
479 October 1992 period, as well as 11 food isolates collected during epidemiological
480 investigations of this outbreak. The 24 isolates displayed 13 distinct combined
481 *Apal/Ascl* PFGE types and only three distinct MLVA patterns. The most frequent
482 PFGE pattern (*Apal/Ascl*-38/41) was represented by 10 isolates, among which the
483 LM25703 strain, which was isolated from the incriminated source, pork tongue in
484 jelly. These 10 isolates displayed MLVA type 0025, showing that MLVA did not
485 exclude any isolate that was associated to the outbreak PFGE pattern. However, out
486 of the 14 isolates with other PFGE types, 9 also had MLVA pattern 0025, whereas 4
487 had MLVA pattern 0022, differing by a single repeat unit at both JLR1 and Lis-
488 TR1869), and one had MLVA pattern 0031, differing from MLVA pattern 0025 at 6
489 out of 11 loci (**Figure 3**). The 9 isolates with MLVA pattern 0025 but with a PFGE
490 type that was distinct from *Apal/Ascl*-38/41, differed from the latter pattern by up to 8
491 bands with *Ascl* and up to 5 bands with *Apal*. These results showed that MLVA did
492 not distinguish from the source isolate, several isolates that were clearly
493 distinguished from the source isolate based on PFGE.
494

495

Discussion

496

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

512 Previous MLVA schemes have been associated with a number of PCR failures
513 for some VNTR loci, resulting in incomplete characterization of some isolates (28, 30,
514 35, 43) . Missing data lead to lower discrimination and loss of informative characters
515 for phylogenetic placement, and render the interpretation of differences among MLVA
516 profiles problematic. Here, we optimized the selection of VNTR loci and sequence of
517 primers. We thus achieved nearly complete (99.9%) typeability for 10 markers, even
518 when combining these assays into two multiplexed PCR reactions. As previously
519 reported (43), we found null alleles at locus Lm11, but these PCR failures were

520 restricted to isolates of CC7 and CC8, consistent with the absence of the
521 corresponding locus in the genome of representative strains of these two clones.
522 However, we chose to keep this locus in the M1 multiplex assay, because it provides
523 some discrimination within CC1, one of the epidemiologically most important clones
524 of *L. monocytogenes*. Besides, the adjunction of the Lm11 primers in the M1
525 multiplex PCR mix did not affect negatively the assay for the other loci. As our
526 sample covers a large breadth of the clonal diversity of lineages 1 and 2, including all
527 clinically most frequent serotypes and clonal groups, we demonstrate that the MLVA-
528 11 system has broad applicability, an important characteristic of typing systems (45).

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

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

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

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

594 (10, 39). It is also important to use a relatively high number of VNTR markers, as the
595 use of each multiplex individually did not recover phylogenetic placements as
596 accurately (data not shown).

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

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

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

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

639 MLVA has strong potential for inter-laboratory standardization, as this method
640 is highly reproducible and as data scoring into integer numerals provides
641 unambiguous results. This is largely regarded as an advantage over PFGE, which
642 can imply partly subjective decisions during band scoring. Standardization would
643 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 EC1 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

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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

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

705

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

712

713

714 **References**

715

716

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Figure 1

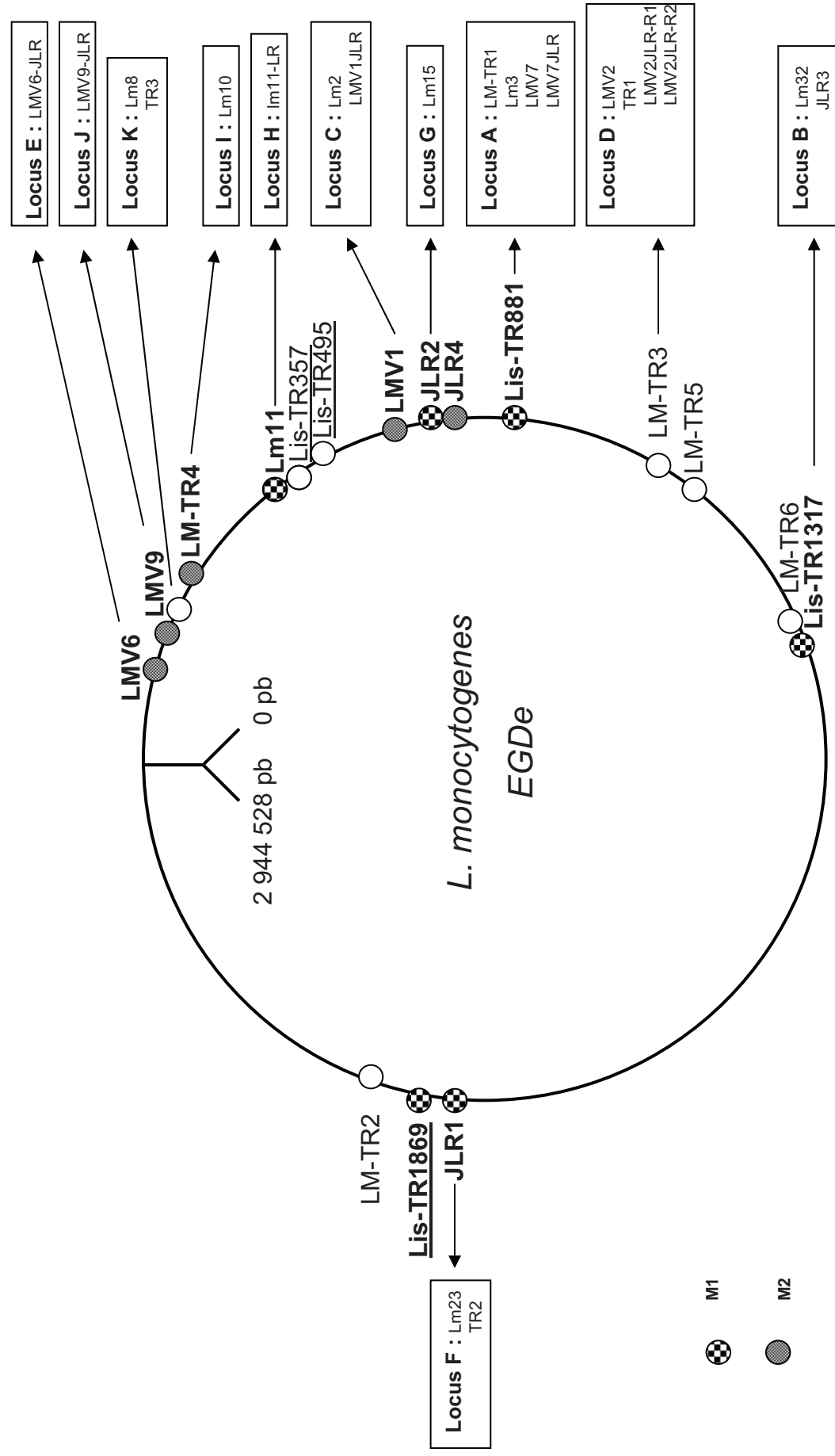
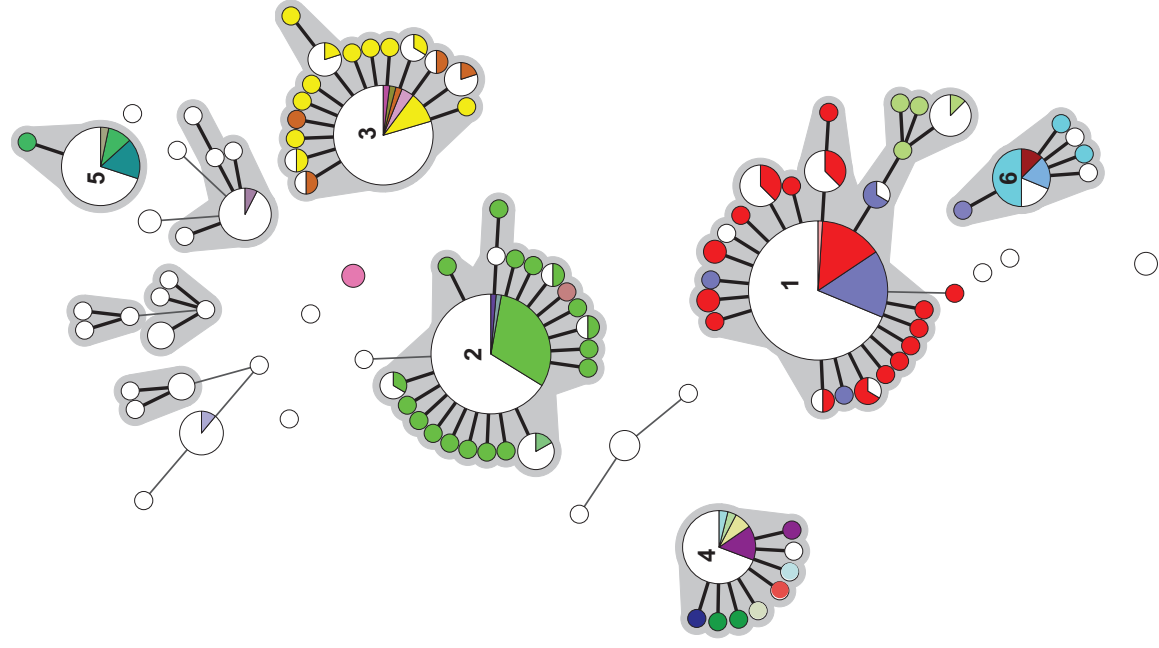
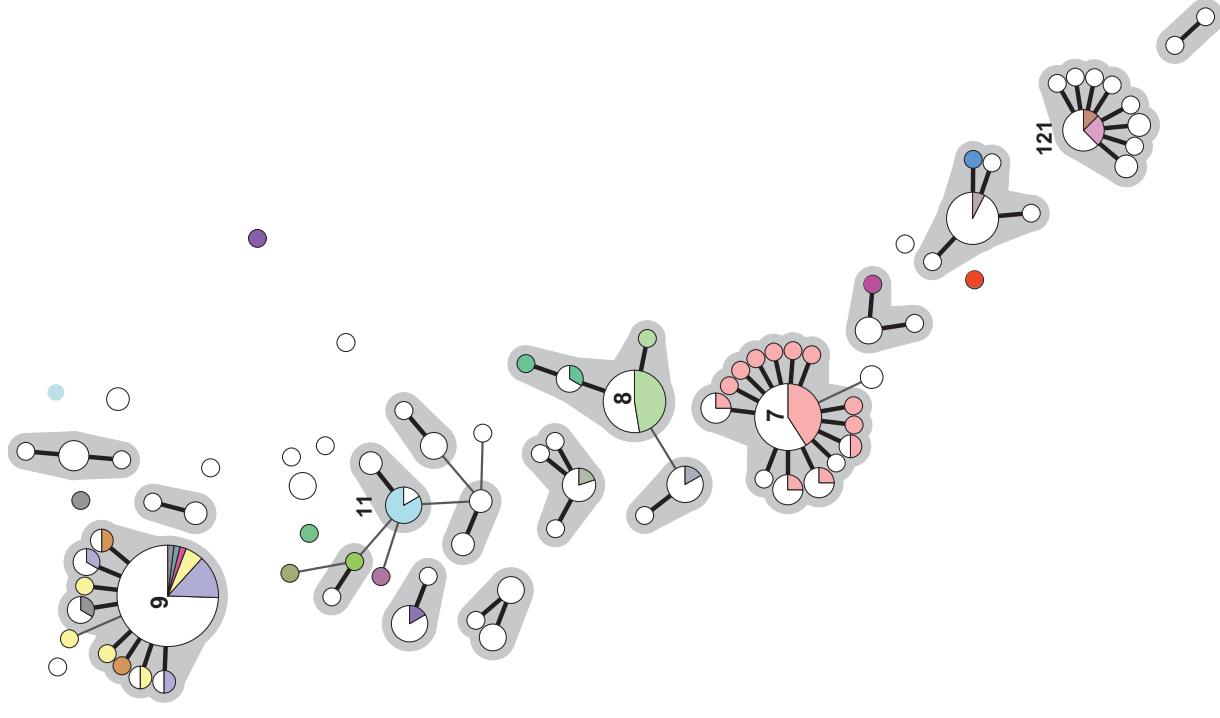


Figure 2

Lineage 1



Lineage 2



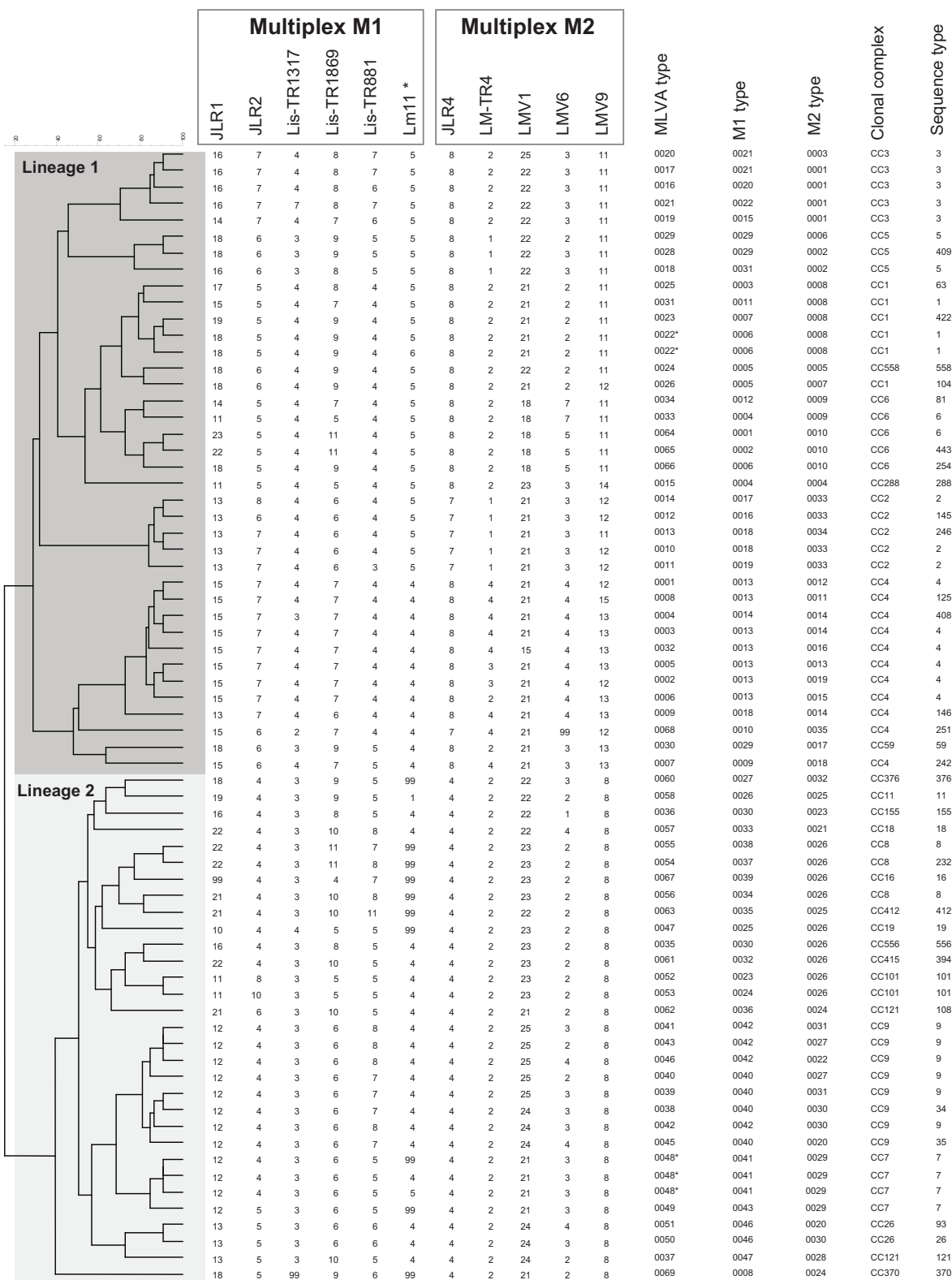


Table 1. VNTR loci assayed during the initial screening. VNTRs in bold type were selected for the multiplex MLVA assay.

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	AT5565-CCTTCCAGAGAAAGACAAAACAG RCTAATCCACCAGCAAATAGC	0.5	Larsson
Lis-TR1317	1317239-1317407	lmo1290-hypothetical protein	LisTR1317_F LisTR1317_R	AT5550-TGATTTACAAAAAGCTTTGCC ACTTGGCACTTCTGGTTTA	1	This study
Lis-TR1869	1869660-1870086	lmo1789-peptidoglycan bound protein	LisTR1869_F LisTR1869_R	AT5550-CCGCGCTATAACCTGAGGAAAGC CTGAAATCATTGCAATCAGATGCACC	0.5	This study
Lis-TR881	881441-881713	lmo0842-peptidoglycan binding protein	LisTR881_F LisTR881_R	FAM-TGTAAATAAAGCTGGTACGTAC GTATGTTGCTTGTATCAACTAC	0.5	This study
Lm11	344898-345043	lmo0320-hypothetical protein-cell wall surface anchor family protein	Lm11_F Lm11_R	HEX-GAATAAAAATGCTAGATGTGG CCGATTCAAAAATAGTAAAC	2	Sperry
M2						
JLR4	695469-695639	lmo0652-hypothetical protein	JLR4_F JLR4_R	HEX-AGAAATTCAGTCCGCCAG GGARCAACAGAAGCTGATCCA	2	Larsson

LM-TR4	228768-229245	ftsH-hypothetical protein-ATP-dependent metalloprotease FtsH	LMTR4_F LMTR4_R	<i>AT565</i> -TCCGAAAAAGACGAAGAAGTAGCA TGGAACGACGGACGAAATAATAAT	2	Murphy
LMV1	619129-619524	iap-probable endopeptidase p60	LMV1_F LMV1_R	<i>AT550</i> -CGTATTGTGCGCCAGAAGTA MAMCAACRCAACAACAAACAG	1	Lindstedt
LMV6	159077-159325	lmo0159-peptidoglycan binding protein	LMV6_F LMV6_R	<i>FAM</i> -AAAAGCCCCRATTGGATA CTCGCTGTTTTCTGWTTTCTTAGG	4	Lindstedt
LMV9	161054-161554	lmo0160-peptidoglycan binding protein	LMV9_F LMV9_R	<i>HEX</i> -AACGGTKRCKGATTACTTC 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	- AACACCAACACCA GACCCAACACC-	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	- AAAACCAATAAAA CCATC-	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	- CTTCTGGAGCTTCT GGTA-	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	- AGTACCACCAACA CC-	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) #

CC (No. of isolates)	MLST		MLVA		PFGE	
	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