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The Use of Pulsed Field Gel Electrophoresis in *Listeria monocytogenes* Sub-Typing – Comparison with MLVA Method Coupled with Gel Electrophoresis

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1. Introduction

Out of the several molecular methods currently available, pulsed field gel electrophoresis (PFGE) is one of the most discriminatory and reproducible methods for the sub-typing of *Listeria monocytogenes (L. monocytogenes*) (Kerouanton *et al*., 1998; Brosch *et al*., 1996). The combination of restriction endonucleases *Asc*I and *Apa*I has shown excellent discrimination for *L. monocytogenes* (Brosch et al., 1996). Thus, the PFGE method, using these two enzymes, is considered to be the international standard for sub-typing (Graves and Swamminathan, 2001). However, although the protocol has been shortened to 30 hours from the time a pure culture of the bacteria has been obtained (Graves and Swamminathan, 2001), PFGE remains a manual, time-consuming and labor intensive subtyping method. It also requires highly skilled operators and does not offer standardized reagents.

ANSES Maisons-Alfort Laboratory for Food Safety has been the European Union Reference Laboratory (EURL) for *L. monocytogenes* in the food chain since 2006. One of the main EURL activities is to develop relevant subtyping methods that are faster than the reference subtyping method, PFGE and that can be easily be implemented in the National Reference Laboratories (NRLs) of European countries.

Multiple-locus variable-number tandem-repeat analysis (MLVA) is a rapid subtyping method based on (PCR) amplification and size analysis of regions of DNA containing variable numbers of tandem repeats (VNTRs). MLVA has been successfully developed for subtyping various bacterial genera. The amplification products are measured using either a capillary electrophoresis system (CE) or a simple agarose gel electrophoresis system. However, with the latter, it is necessary to select VNTR loci with repeat sizes large enough

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(greater than or equal to 9 bp) that the difference between two alleles is clearly visible on the gel (Vergnaud and Pourcel (2006)).

For *L. monocytogenes*, a standardized PulseNet USA MLVA protocol has recently been developed based on the detection of nine VNTR loci, with a VNTR size between 6 and 15 bp. The panel of strains was composed of 250 epidemiologically unrelated strains and most of the tested isolates were of human origin. The clusters obtained correlate with isolate serotypes (Hyytia-Trees, 2010; Sperry et al., 2008). At the Serum Statens Institute (SSI) in Copenhagen, Larsson et al. (2010) developed another scheme using 10 VNTR loci based on the analysis of 20 genome sequences. Seven loci were common to the PulseNet protocol. For five of these loci, degenerate primers were designed to match genome diversity. The results demonstrated better discriminatory power for MLVA compared to combined *Apa*I/*Asc*I PFGE. This scheme has been successfully used for the surveillance of *L. monocytogenes* in Denmark (Larsson et al., 2010). Of the nine loci used by PulseNet, four have been previously described: Lindstedt et al. (2008) developed an MLVA scheme based on the use of five VNTR loci to characterize 140 isolates, mainly from human and environmental sources and belonging to various serotypes. The discriminatory power of MLVA is similar to that of *Asc*I-PFGE. Another MLVA scheme using only three described VNTR loci has been developed to type 60 serotype 4b isolates from various sources. Simpson's diversity index has been shown to be higher than that of *Apa*I-PFGE, MLST, and *Eco*RI ribotyping (Miya et al., 2008).

In the MLVA schemes developed so far, fragment detection is performed by CE. Nevertheless, Murphy et al (2007) demonstrated that it is possible to detect VNTR loci on agarose gels. However, most of the 45 isolates tested in this study had the same origin (food origin) and the same serotype (1/2a). Moreover, out of the six VNTR loci described by Murphy et al. (2007), four were excluded from the PulseNet USA MLVA protocol because two loci have low diversity and two others display sequence variability in flanking regions. The purpose of the present study was to evaluate the feasibility of a MLVA protocol coupled with conventional gel electrophoresis. The results were compared with those obtained by PFGE.

2. Materials and methods

2.1 Strain panel

This study was conducted on 72 strains (Figure 1): 45 isolated from clinical samples and 18 isolated from different food products. This panel included also nine reference strains with six CLIP strains (CLIP 74903 (1/2b), CLIP 74904 (1/2c) , CLIP 74905 (3a), CLIP 74906 (3b), CLIP 74907 (3c), CLIP 74912 (4d)) and three fully sequenced strains: EGDe (1/2a), F2365 (4b) and CLIP 80459 (4b). Twenty human strains came from SSI. Twelve non-human field isolates came from the EURL and were collected from French food analysis laboratories, as part of their monitoring, surveillance sampling activities or research projects. Thirty-three strains had previously been used in the WHO international multicenter *L. monocytogenes* subtyping study (Bille & Rocourt, 1996). These strains were labeled TS ("Test study") (Schönberg et al. (1996). Twenty of the 72 strains were related to nine different epidemiological groups (02, 03, 05, 11, 15, 16, 19, 21, 22) (Bille & Rocourt, 1996). Four strains

were represented by two duplicates each (TS32,TS72;TS56,TS77;TS35,TS75;TS63,TS73). A panel of 40 strains (20 SSI human strains, 8 ANSES food strains and 12 TS strains) was typed both at ANSES and SSI.

2.2 Methods

2.2.1 Serotyping

Species identification was performed using agar Listeria according to Ottaviani & Agosti (ALOA) plates (AES, Combourg, France) and the CAMP Test (McKellar 1994). Each strain was serotyped by agglutination using commercially available antisera (Denka, Eurobio, Les Ulis, France), after adapting the manufacturer's instructions and using the procedures outlined by Seeliger & Hohne (1979). Our laboratory has been certified by the French Accreditation Committee (COFRAC) for this serotyping method as an internal method (accreditation no. 1-22465, Section Laboratories, www.cofrac.fr). Determination of the O-antigen was performed from a pure culture [instead of a bacterial suspension]. Determination of the H-antigen was performed using semi-liquid brain heart infusion (BHI) media with 0.5% agar [instead of 0.2%].

2.2.2 Molecular serotyping

Molecular serotyping was performed using the protocol developed by Kerouanton et al. (2010).

2.2.3 PFGE

PFGE was performed using the standard CDC PulseNet protocol (Graves & Swamminathan, 2001) with minor modifications. Each strain was grown overnight on tryptone soya agar with yeast extract (TSAYE) plates instead of BHI. For the DNA digestion step in agarose plugs using *Apa*I and *Asc*I enzymes, 10 units of enzyme were used per plug [instead of 25 units of enzyme per plug for *Asc*I] and 160-200 units of enzyme per plug for *Apa*I in the PulseNet protocol. Plugs were incubated with restriction enzymes for 4 h [instead of 5 h]. Gels were then stained with ethidium bromide and banding patterns were visualized under UV light, using the Gel Doc EQ system and Quantity One software (Bio-Rad). DNA patterns were analyzed with BioNumerics software (ver. 6.5, Applied Maths, Kortrijk, Belgium). The recommendations of Barrett et al. (2006) were followed for gel analysis: gels including partial digestions, or unclear bands were not analyzed. All bands with sizes lower than 33 kb were systematically removed. A similarity value of 97.0% was established as a cut-off to consider two profiles as indistinguishable in UPGMA dendrograms using the Dice coefficient, with a 1% tolerance limit and 1% optimization. If the similarity value was strictly less than 97%, the two profiles were considered as different. The dendrogram settings used were chosen according to PulseNet Europe recommendations (Martin et al., 2006). The similarity value taken as the cut-off was established according to the EURL database settings. Each PFGE profile was arbitrarily assigned a number. Our laboratory has been certified by COFRAC for PFGE analyses (Accreditation no. 1-22465, Section Laboratories, www.cofrac.fr).

2.2.4 MLVA

2.2.4.1. Strain isolation and DNA extraction

Bacterial cultures were revived by plating onto TSAYE plates (Humeau, La Chapelle-sur-Erdre, France). Species confirmation was performed by isolation on ALOA plates (AES, Combourg, France). DNA extraction was performed using the InstaGene kit (Bio-Rad, Marnes-la-Coquette, France) according to the manufacturer's recommendations. Extracts were adjusted to approximately 100 ng/µl using a spectrophotometer (Biophotometer, Eppendorf, ville, France).

2.2.4.2 Locus selection

VNTR loci found in the literature with a repeat size greater than or equal to 9 bp were selected. New VNTR loci were selected from the complete genome of the three reference strains. The genomes of strains EGDe (1/2a), F2365 (4b) and CLIP 80459 (4b) were individually screened using the Tandem Repeat Finder (TRF) program (http://tandem.bu.edu/). The tandem repeat databases http://mlva.u-psud.fr and http://www.hpa-bionum.org.uk/VNTRUK/ were then used to compare the genomes.

2.2.4.3 Primer design

The primer sets were either similar to those described in the literature (Table 2), or designed in regions flanking the VNTR locus, (Table 3), using AlleleID® software (Premier Biosoft International, USA). All the primers were synthesized by Eurogentec (France).

2.2.4.4 Amplification of VNTR loci

The VNTR loci were amplified on DNA from strains EGDe and F2365. The amplification products were electrophoresed on two gels run independently.

For each primer set, the final mix contained 1 U HotStart Taq Polymerase (Roche), 2 or 3 mM $MgCl_2$, 0.2 mM desoxynucleotide triphosphate, 1X PCR buffer, PCR grade water, 0.3 µM each primer, and 1 µl of DNA in a 25 µl reaction mixture. PCR was performed on a thermal cycler (GeneAmp PCR System, 9700, PE, Applied Biosystems). For Lm-8, the parameters used were those described by Sperry et al. (2008): initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 50°C for 20 s, extension at 72°C for 20 s and a final extension at 72°C for 5 min. For LMCEB 02, 06, 12, 14 and Lm-26: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 57°C for 30 s, extension at 72°C for 30 s and a final extension at 72°C for 7 min. For LMCEB 05 the annealing was performed at 54°C. For JLR-4, the parameters used were those described by Larsson et al. (2010). At SSI, amplification for JLR-4 and Lm-8 was performed according to Larson et al. (2010).

2.2.4.5 Detection of VNTR loci

Aliquots (5 µl) of amplified products were electrophoresed on 2% agarose gels (Resophor, Eurobio, France) in 1X TBE buffer (0.45 mM Tris-HCl, 0.45 mM boric acid, 1 mM EDTA, pH 8). Electrophoresis was performed in 12 cm long gels and run at 80 V for 30 min followed by 90 V for 4 h. In each run, the 20 bp DNA Ladder (Bio-Rad, France) and the PCR products from the two strains EGDe and F2365 were systematically included at least twice to facilitate the sizing of amplified DNA fragments. Each run included a negative/water control to ensure the absence of contamination.

The gels were stained in 2 µg/ml ethidium bromide for 90 min and photographed under UV illumination (Gel Doc EQ^R Bio-Rad, France). The length of each amplified VNTR locus was measured using Quantity One software (Bio-Rad, France). An allele number string based on the estimated number of tandem repeats at each locus was assigned to the amplified DNA fragments from each isolate. Detection of PCR products by capillary electrophoresis was performed according to Larsson et al. (2010).

2.2.4.6 Data analysis

The allele strings were imported into BioNumerics software. Dendrograms were constructed using a categorical coefficient and UPGMA clustering. Allele nomenclature was that recommended by PulseNet USA. No amplification was coded as negative (–1). Efficient amplification with no VNTR detected was coded as "zero" (0). Partial repeats were rounded down to the closest whole number.

2.2.4.7 Sequence verification

The loci and flanking regions were amplified in both directions with high-fidelity HotStart Taq Polymerase (Roche). Amplification products were sequenced by Eurofins (MWG Operon, France). The sequence analysis was performed with the CodonCode Aligner software (CodonCode Corporation, USA).

2.2.4.8 Stability determination

The stability test was performed according to Sperry et al. (2008): the strains EGDe, F2365 and CLIP 80459 were tested 45 times. All DNA were tested for MLVA.

2.2.4.9 Reproducibility

The reproducibility of the MLVA method was determined from the results obtained from the two reference strains included in each run, and with the four TS strains represented in duplicate and the epidemiologically related strains included in this study. Moreover, amplification products were systematically run on two independent gels. At least two independent PCRs were performed from a given DNA extract from the reference strains.

3. Results

3.1 Serotyping data

The agglutination serotyping distribution was as follows: 27 serotype 1/2a strains, 10 serotype 1/2b strains, 5 serotype 1/2c strains, 25 serotype 4b strains, 1 strain of each serotype 3a, 3b, 3c, 4d and 1 autoagglutinable strain.

3.2 Subtyping data

3.2.1 Development of an MLVA assay

3.2.1.1 Selection of VNTR loci from the literature

A total of 16 VNTRs have been described in the literature (Table 1). Although some VNTRs are common to different MLVA schemes, their nomenclature is different. Moreover, the primer pairs used for the amplification of a given locus can differ among studies.

The number in brackets indicates the size of the tandem repeat motif in the VNTR locus; [1] Excluded due to low diversity (Hyytia-Trees, 2010); [2] Excluded due to sequence variability in flanking region (Hyytia-Trees, 2010); [3] Excluded due to short repeat unit length (3 bp) and low diversity (Hyytia-Trees, 2010); [4] Degenerate primers.

PulseNet USA (Hyttia-Trees, 2010)	Sperry et al. (2008)	Larsson et al. (2010)	Lindstedt et al. (2008)	Murphy et al. (2007)	Miya et al. (2008)
LM-2 (6 bp)	Lm-2	LMV1-JLR[4]	LMV1		
LM-3 (9 bp)	Lm-3	LMV7-JLR[4]	LMV7	LMTR-1	
LM-8 (15 bp)	Lm-8				
LM-10 (12 bp)	Lm-10			LM-TR-4	
LM-11 (12 bp)	Lm-11	LM11-LR			
LM-15 (12 bp)	Lm-15	JLR2[4]			
LM-23 (6 bp)	Lm-23	JLR1			TR2
LM-32 (6 bp)	Lm-32	JLR3[4]			
LMV09 (9 bp)		LMV9-JLR[4]	LMV9		
				LM-TR-2[1] (18 bp)	
				LM-TR-3[2] (9 bp)	TR1[2]
		LMV2-JLR (9 bp)	LMV2	LM-TR-5[2]	
				LM-TR-6[1] (12 bp)	
					TR3[3] (3 bp)
		LMV6-JLR (15 bp)	LMV6		
		JLR4 (9 bp)			

Table 1. Comparison of 16 MLVA VNTR loci described in the literature and used for subtyping *L. monocytogenes*.

In this study, VNTR loci were selected according to the following criteria: (1) a repeat size greater than or equal to 9 bp (2) diversity and (3) no sequence variability in flanking regions. For this reason, four loci used by Murphy et al. (2007), LM-TR-2, LM-TR6, LM-TR-3 and LM-

TR-5, were excluded from this study because Hyytia-Trees (2010) demonstrated low diversity in LM-TR-2 and LM-TR6 and sequence variability in flanking regions in LM-TR-3 and LM-TR-5.

Eight loci, Lm-3 (=LmTR-1 for Murphy et al. (2007)), Lm-8, Lm-10 (=LmTR-4 for Murphy et al. (2007)), Lm-11, Lm-15, JLR4, LMV6-JLR and LMV9-JLR (Table 1) were thus selected. The primers used in the present are shown in Table 2.

Locus name	Primer names	References
Lm-10	Lm-10F-Lm-10R LM-TR-4F-LM-TR-4R	Sperry et al. (2008) Murphy et al. (2007)
Lm-11	Lm-11F-Lm-11R LM11-LR F-LM11-LR-R	Sperry et al. (2008) Larsson et al. (2010)
Lm-3	LMV7-F ; LMV7-R LM-TR-1-F ; LM-TR-1-R Lm-3 F; Lm-3 R LMV7-JLR F ; LMV7-JLR R	Lindstedt et al. (2008) Murphy et al. (2007) Sperry et al. (2008) Larsson et al. (2010)
Lm-8	Lm-8F ; Lm-8R	Sperry et al. (2008)
LMV6-JLR	LMV6-JLR LMV6-F ; LMV6-R	Larsson et al. (2010) Lindstedt et al. (2008)
LMV9-JLR	LMV9-JLR LMV9-F – LMV9-R	Larsson et al. (2010) Lindstedt et al. (2008)
Lm-15	Lm-15F-Lm-15R JLR2 F-JLR2R	Sperry et al. (2008) Larsson et al. (2010)
JLR-4	JLR4 F-JLR4R	Larsson et al. (2010)

Table 2. Primers used for amplification of the eight VNTR loci selected from the literature.

3.2.1.2 Selection of VNTR loci from a bioinformatics-based search

Following a search using TRF in MLVA databases, nine VNTR loci (LMCEB01,02,03,04,05,06,12,14, and Lm-26) were selected (Table 3).

The locus Lm-26 had already been published but has not been used previously due to its low diversity (Sperry et al., 2008). For each of the nine VNTR loci, primers were designed in the regions flanking the locus (Table 4).

3.2.1.3 Amplification and detection of the selected VNTR loci from the two strains EGDe and F2365

For Lm-3, Lm-10, Lm-11, Lm-15, LMV6-JLR and LMV9-JLR, the size of the amplification products obtained with all the primer pairs tested, observed in the same run and in two different runs differed from the true length by up to 18 bp (data not shown). For this reason, other primer pairs were designed and tested. Sizing discrepancies were nevertheless observed (data not shown).

VNTR locus name	Repeat	Repeat motif length (bp)	Identification in EGDe		Identification in F2365		Locus tag and protein description
			Location (nt)	Number of repeats	Location (nt)	Number of repeats	
LMCEB01	TACAGGGTCAACCGGATCAACCGGATT	27	173484-173534	1.8	178831-178897	2.4	lmo0175: peptidoglycan binding protein
LMCEB02	GGAGTTGCTGGATCTGTTGGTGTAGATGGTTCGTCAGGTGTT	42	345133-345213	2.4	358155-358297	3.4	lmo0320: similar to surface protein (peptidoglycan bound, LPXTG motif)
LMCEB03	GATCCAGACCCAGTAAATCCAGATCCAACT	30	589559-589651	2.2	596161-596226	2.2	lmo0551
LMCEB04	ACAGGACTTGATCAAATAGAA	21	1251475-1251565	2.7	1228575-1228632	2.7	lmo1226
LMCEB05	TAAAGTGACTAATACTTGTTCATTT	25	1808069-1808118	2.0	1787712-1787762	2.0	lmo1738: similar to amino acid ABC transporter
LMCEB06	TTCGAATTTCCACCACCACCTACGGATGAAGAGTTAAGACTTGCTTTGCCAGAGACACCAATGCTTCTTGGTTTTAATGCTCCTGCTACATCAGAACCGAGCTCA	105	210255-210498	2.3	215616-215754	1.3	ActA: actin-assembly inducing protein precursor
LMCEB12	CTTCTGGTGTTTCAGGAGTTTCTGGTA	27	695517-695569	2.1	701866-701977	4.2	lmo0652 and lmo0653
LMCEB14	AGAACTTTCAAAATGTACTTTATTTTGATTTAGTTCTTCAATATAAATCTGAGCAAAGCGATGATTTAATCCTTCCC	77	2779641-2779814	2.3	2732284-2732373	1.2	dnaX: highly similar to DNA polymerase III and lmo2705
Lm-26 Sperry et al. (2008)	AATGTATTTTTATTTAAA	18	2169160-2169208	2.7	2157678-2157744	3.7	argG: argininosuccinate synthase

Table 3. Characteristics of the nine VNTR loci selected through a bioinformatics-based search.

Conversely, for Lm-8, Lm-26, JLR4, LMCEB 01,02,03,04,05,06,12,14, the size of the amplification products observed in the same run and in two different runs remained the same. Moreover, the sizes (Table 4) were identical to those predicted by genome sequence analysis. Sequencing of the amplification products demonstrated that the size differences

Primer name	**Locus name**	**Sequence (5'-3')**	**Amplicon location in F2365**		**Amplicon location in EGDe**	
			Position	length (bp)	Position	Length (bp)
LMCEB01F	LMCEB01	ATT AAA AGA AGC AAK GCT CC	178682	297	173343	279
LMCEB01R		AAA YGC AAC TGG TAC TTT CA	178978		173621	
LMCEB02F	LMCEB02	TTG ATT CTG GAT TTT CTG G	358114	193	345088	151
LMCEB02R		CCA CCA AAA AAC GAT CCA GAA	358306		345239	
LMCEB03F	LMCEB03	GTA GAA CAG TAA ARG TAA CA	596015	295	589413	295
LMCEB03R		CCW GAA GAT AAG CTA GAA AC	596290		589707	
LMCEB04F	LMCEB04	AAT CAA GGT ATY CAA CAA CT	1228488	287	1251367	287
LMCEB04R		GTT AAR CCA TCT GTT AAT TG	1228774		1251654	
LNCEB05F	LMCEB05	TAT AAT GTC TGT TAR CAC TT	1787620	210	1807952	210
LNCEB05R		ATT TGG AAT GGW TAT ACT GT	1787829		1808162	
LMCEB06F	LMCEB06	AGA AAA RTG AAG AGG TAA ATG	215594	243	210233	348
LMCEB06R		TAA TAG CAY TTC TCA AAC TA	215836		210581	
LMCEB12F	LMCEB12	RAT TTT ATT TTG GTT CAT TGT	701838	320	695492	308
LMCEB12R		AAG GYA CTT TTA CAG AAG AA	702100		695694	
LMCEB14F	LMCEB14	RTG CGA AGT TTT ATT TTG CA	2732111	316	2779467	393
LMCEB14R		GAT TTT TGR TTT TTG GTG GTG	2732425		2779620	
LMCEB13F	Lm-26	AAT GGA AGT AGA ATR ATC CC	2157557	251	2169040	233
LMCEB13R		TTA TAT TAA CAC YGA TGCT T	2157807		2169273	

Table 4. Primers and characteristics of PCR amplification products in the reference strains for each of the nine VNTR loci selected through a bioinformatics-based search.

observed on the gel of strains EGDe and F2365 were solely related to the differences in repeat number, and not nucleotide variation in the flanking regions. For each locus, the repeat number was very similar to that indicated in the databases.

3.2.1.4 Screening of VNTR loci on the total strain panel

The 11 VNTR loci (Lm-8, Lm-26, JLR4, LMCEB01,02,03,04,05,06,12,14) were tested on the total test strain panel to evaluate the polymorphism of each VNTR locus. The loci LMCEB01, 03 and 04 exhibited no diversity (Table 5) and were therefore removed from the study. The eight remaining VNTR loci displayed between two and six alleles. Locus JLR4 had the highest diversity.

VNTR locus name	No. of alleles	No. of repeats	
		Min	Max
LMCEB01	0	2	2
LMCEB02	3	-1	3
LMCEB03	0	2	2
LMCEB04	0	3	3
LMCEB05	2	-1	2
LMCEB06	2	1	2
LMCEB12	3	1	4
LMCEB14	4	-1	4
Lm-26	3	-1	4
Lm-8	2	3	4
JLR4	6	3	12

Table 5. Numbers of alleles and repeats found at each VNTR locus.

3.2.1.5 Comparison of data obtained with conventional electrophoresis and those obtained with CE

Two loci Lm-8 and JLR-4 were tested at Serun Statens Institute on the common panel of 40 strains using CE. Except for two strains, all showed the same repeat number. For Lm-8, one strain from SSI, 20092474, had a real repeat number of 2.7 in CE and 2.4 in agarose gel electrophoresis. For JLR4, one strain from SSI, 20082357, had a real repeat number of 3 in CE and 3.56 in agarose gel electrophoresis.

3.2.1.6 MLVA stability

The stability of each locus was evaluated to determine the effect of laboratory passage. The copy number was determined to be 100% reproducible (data not shown). Each of the eight loci tested on three reference strains were stable.

3.2.1.7 MLVA reproducibility

The MLVA types were indistinguishable for the four duplicate TS strains (TS32,TS72; TS56,TS77; TS35,TS75; TS63,TS73). The MLVA types were correlated with the epidemiological groups for the 17 tested TS strains. Two strains (TS 55 and TS 21) of the

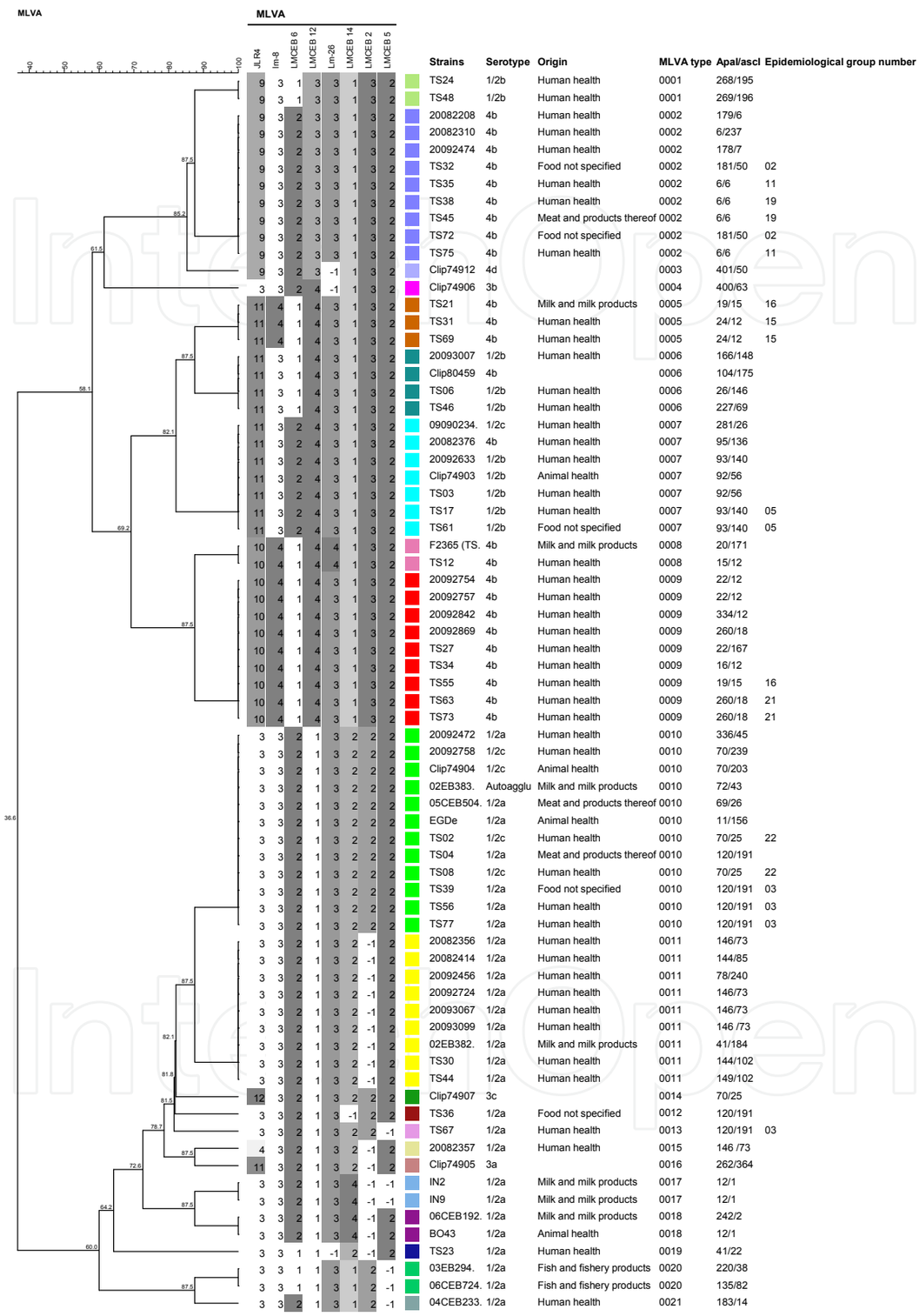

Fig. 1. Cluster analysis of 72 isolates based on MLVA type using the categorical coefficient and UPGMA. Number, origin, serotype, MLVA typing results and combined PFGE results. Each color indicates a distinct MLVA type.

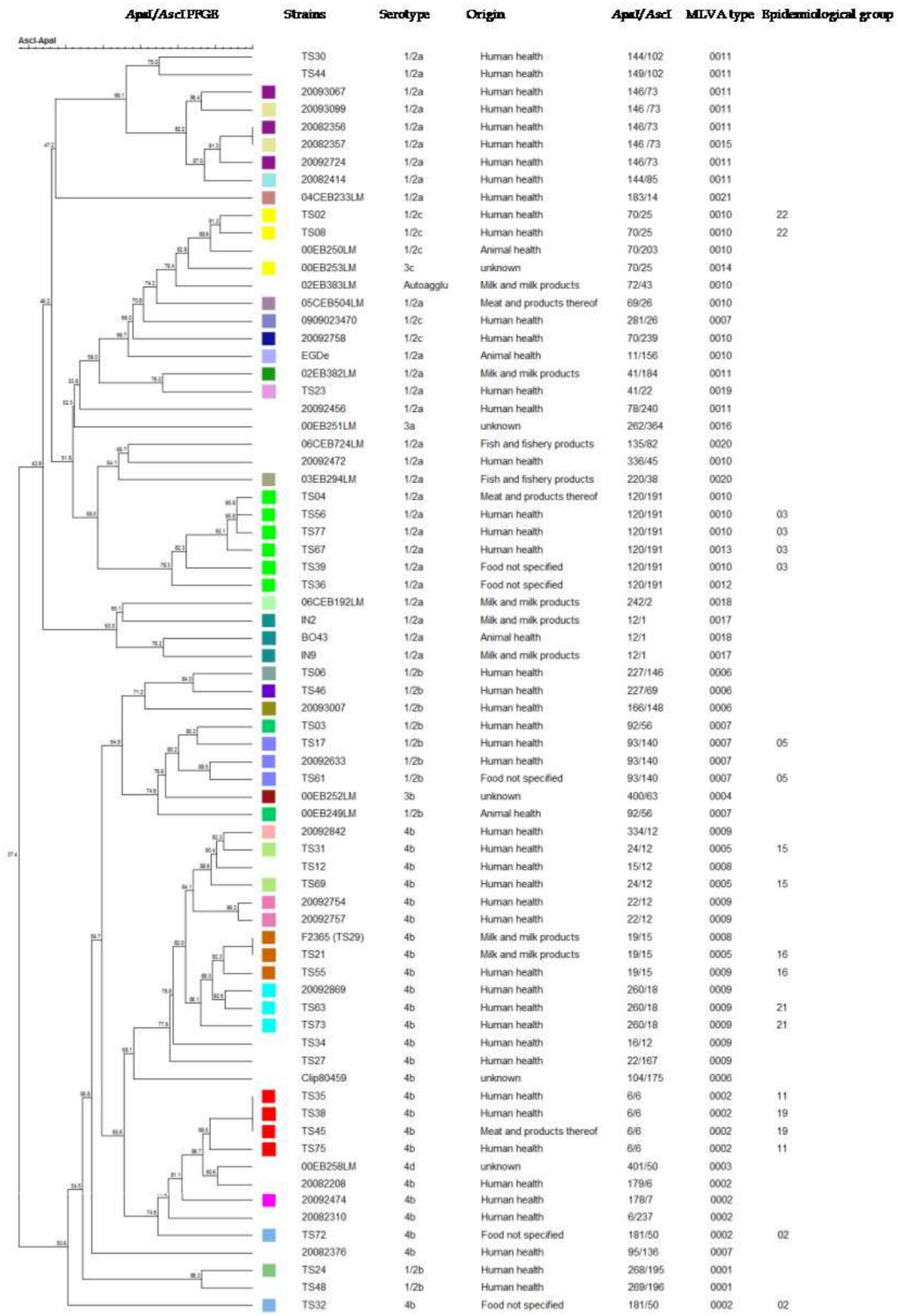

Fig. 2. Cluster analysis of 72 isolates based on combined PFGE using the categorical coefficient and UPGMA. Number, origin, serotype, MLVA typing results and combined PFGE results. The colors (including also white color) indicate distinct combined PFGE types.

same epidemiological group 16 displayed two different MLVA types, 05 and 09. The difference was related to a repeat in the locus JLR-4. Strain TS 67 and three other strains of group 03 displayed two different MLVA types. No amplification was observed for the strain TS 67 at locus LMCEB05.

3.2.2 MLVA assay applied on the test panel of strains

Based on MLVA results, the 72 isolates were divided into 21 types (Figure 1). MLVA types were clustered into two groups. All the isolates of serotypes 1/2a, 3a, 1/2c, 3c were classified in one group, while all the isolates of serotypes 4b, 1/2b, 3b, 4d were in another group (Figure 1). Nineteen of the 21 types contained isolates of the same serotype (Figure 1). Type "10" contained isolates of two serotypes 1/2a and 1/2c and the autoagglutinable strain. Type "7" contained isolates of two serotypes 4b and 1/2b and one isolate of the 1/2c serotype (Figure 1).

3.2.3 PFGE data

For PFGE, the two-enzyme combination divided the isolates into 48 distinct profiles (Figure 2). All the isolates of serotype 1/2a, 3a, 1/2c, 3c were classified in one group, while all the isolates of serotypes 4b, 1/2b, 3b, 4d were in another group. Combined PFGE types contained isolates of the same serotype, except the type "70/25", which contained isolates of serotypes 1/2c and 3c.

3.2.4 MLVA data compared with PFGE data

Six different MLVA types were encountered for nine distinct epidemiological groups. A single *Apa*I/*Asc*I PFGE type was observed for each epidemiological group (Figure 1).

Five MLVA types ("19", "3", "4", "21", "16") contained one unique *Apa*I/*Asc*I PFGE type. The other MLVA types contained at least two different PFGE types. The five *Apa*I/*Asc*I PFGE types "19/15, "120/191", "70/25", "12/1", "146/73" were divided among two MLVA types ("9","5"), three MLVA types ("10","12","13"), two MLVA types ("10","14"), two MLVA types ("17","18") and two MLVA types ("11","15"), respectively (Figure 1).

4. Discussion

The objective of this work was to evaluate the feasibility of an MLVA scheme coupled with conventional agarose gel electrophoresis for subtyping *L. monocytogenes*. This type of scheme would be very useful for *L. monocytogenes* surveillance, because it can be implemented by any molecular laboratory and does not require an expensive capillary electrophoresis system.

Out of the 16 VNTRs published, only eight Lm-3, Lm-8, Lm-10, Lm-11, Lm-15, JLR4, LMV6-JLR and LMV9-JLR were selected here because (1) their repeat length was greater than or equal to 9 bp as demonstrated on a large panel of human and food strains (Sperry et al., 2008; Larson et al. 2010; Lindstedt et al., 2008; Murphy et al., 2007). For six out of eight loci (Lm-3, Lm-10, Lm-11, Lm-15, LMV6-JLR and LMV9-JLR), the size of the amplification products observed on the agarose gels differed between the runs. This result was observed

for different primer sets, both previously published and newly designed. This result was surprising, particularly regarding loci Lm-3 and Lm-10, for which Murphy et al. (2007) observed accurate detection on agarose gels. In this study, agarose gel electrophoresis does not appear to be sufficiently accurate for determining repeat number for these six loci. In contrast, agarose gel electrophoresis was suitable for loci Lm-8 and JLR-4. The sizing discrepancies need to be normalized to develop a standardized agarose gel protocol uding all the VNTRs selected here.

For Lm-8, the amplification protocol used here was as similar to that described by Sperry et al. (2008). The repeat number obtained here for the 34 "TS" strains on agarose gel was exactly the same as that obtained on the same panel on a CE Beckman Coulter CEQ 8000 genetic analyzer (Sperry et al., 2008). For Lm-8 and JLR-4, of 39 strains from a panel of 40, the repeat number on agarose gels was exactly the same as that obtained on the ABI 3130 genetic analyzer (Applied Biosystems) at SSI (Larsson et al., 2010). For only one strain, a low difference (maximum 0.56) was observed in the number of base pairs. We demonstrated here that the change in equipment used for the detection of JLR4 and Lm-8 did not affect the determination of repeat number. These data confirm the reliability of these two loci.

However, locus Lm-8 revealed low levels of diversity (2 alleles) on the tested panel of human and food strains. This result corroborates those obtained by Sperry et al. (2008) who report only two alleles from a panel of 193 isolates. Locus JLR-4 showed the highest number of alleles. Locus Lm-26 also showed low diversity (3 alleles), as previously demonstrated by Sperry et al. (2008). This locus overlaps with locus LM-TR2, included in the scheme of Murphy et al. (2007). It had the lowest diversity index in comparison to the five other VNTR loci.

The five VNTR loci found here, LM 02, 05, 06, 12, 14, were identified from the sequenced genomes of three reference strains. They have never been described before. Our results demonstrate that these loci show reliable amplification.

With 71 of 72 strains, our MLVA scheme of eight loci (Lm 02, 05, 06, 12, 14, Lm-8, Lm-26 and JLR-4) confirmed the division of *L. monocytogenes* strains into two distinct genetic lineages. One strain of the 1/2c serotype showed an MLVA type common to strains of serotype 1/2b and 4b. This strain belonged to molecular serogroup IIc and has a combined PFGE profile specific to 1/2c and IIc strains. Other molecular methods are needed to further investigate the genetic profile of this strain.

Five VNTR loci, LM 02, 05, 06, 12, 14, exhibited low diversity on the total test strain panel. These data indicate that the MLVA scheme developed here was less discriminating than *Apa*I/*Asc*I PFGE. However, the eight VNTR loci selected in this study have proved useful and can be included in a larger MLVA scheme coupled with CE, including VNTR loci with shorter repeat motifs and with higher polymorphism. The more polymorphic loci were excluded from this study, either because they are too short to be visible on agarose gels or because sizing discrepancies were observed on agarose gels. It is absolutely necessary to normalize these sizing discrepancies for accurate and standardized detection on agarose gels. Moreover, in the future, it is necessary to compare all the data obtained in different laboratories and to harmonize VNTR loci and allele naming for a standardized *L. monocytogenes* MLVA scheme.

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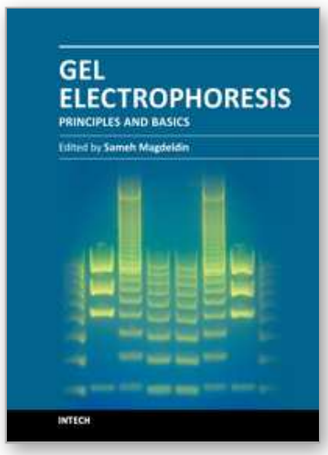

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Most will agree that gel electrophoresis is one of the basic pillars of molecular biology. This coined terminology covers a myriad of gel-based separation approaches that rely mainly on fractionating biomolecules under electrophoretic current based mainly on the molecular weight. In this book, the authors try to present simplified fundamentals of gel-based separation together with exemplarily applications of this versatile technique. We try to keep the contents of the book crisp and comprehensive, and hope that it will receive overwhelming interest and deliver benefits and valuable information to the readers.

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