International Journal of Food Microbiology 310 (2019) 108289

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Characterization of *Listeria monocytogenes* serovar 1/2a, 1/2b, 1/2c and 4b by high resolution melting analysis for epidemiological investigations

Manuela Tamburro, Michela Lucia Sammarco, Incoronata Fanelli, Giancarlo Ripabelli*

Department of Medicine and Health Sciences "Vincenzo Tiberio", University of Molise, Italy

ARTICLE INFO

Keywords:
Molecular epidemiology
Lineages
Listeria pathogenicity island-1
Internalins A and B
Surveillance

ABSTRACT

This study aimed to characterize serovar 1/2a, 1/2b, 1/2c and 4b of *Listeria monocytogenes* cultures based on High Resolution Melting (HRM) profiles, targeting 53 fragments in the region comprising *prs*, Listeria Pathogenicity Island-1 (LIPI-1) and *ldh* loci, and 28 fragments of *inl*AB operon. Fifty *L. monocytogenes* cultures (28 of lineage I, 22 of lineage II) were tested. Real time PCR and EvaGreen-based HRM assays were performed, and the HRM profile for each amplicon was compared to that of *L. monocytogenes* EGD-e strain. Considering all fragments tested, 45 HRM profiles were identified (Diversity Index = 99.35). Similarity analysis identified two main clusters: the first consisted of 25 cultures, including all 1/2a and 1/2c strains, except for three isolates from food of serovar 4b; the second group only included 1/2b and 4b isolates. Eighteen out of targeted amplicons showed constant HRM characteristics irrespective of the serovar/lineage, and *hly*A displayed the most stable melting behavior. Conversely, thirteen amplicons were specific for 1/2b and 4b isolates, showing major variations within *act*A, followed by *prs*, *prf*A, *mpl* and *plc*B genes. A fragment targeting an intragenic region (part of *plc*A and part of an unknown gene) had a melting profile allowing differentiation between 4b and 1/2b isolates showing different Tm variants. Amplicons of *inl*A and *inl*B exhibited the largest intra-strain melting differences, and one *inl*B fragment could allow discriminating between 4b and 1/2b cultures, as well as between lineages. A greater level of genetic diversity amongst 1/2a cultures compared to 1/2c, 1/2b and 4b isolates was observed, with variations identified in LIPI-1, as well as within *inl*A and *inl*B genes. Sequencing analysis of amplicons with differential HRM profile from EGD-e confirmed point mutations. The study findings underlines that HRM-based approach may be useful for bacterial subtyping for epidemiological purposes when sequencing-based methods are not available.

1. Introduction

Listeriosis is a severe foodborne infection with a high fatality rate, particularly amongst susceptible population groups, including the elderly, unborn and newly delivered infants, pregnant women and the immunocompromised people (Charlier et al., 2017; Comandatore et al., 2017).

From a meta-analysis, the worldwide burden of listeriosis for 2010 was estimated as 23,150 cases, 5,463 deaths and 172,823 disability-adjusted life-years (de Noordhout et al., 2014). The annual incidence of listeriosis in Italy is lower (0.19–0.27 cases/100,000 inhabitants per year) than that estimated in other areas of Europe (0.34–0.52/100,000 inhabitants per year), but the observed rate may be biased by under-diagnosis or under-reporting (Zolin et al., 2017).

The implementation of strategies for effectively tracking foodborne pathogens is essential for prevention and control of infectious diseases (Sammarco et al., 2014), including listeriosis. Bioinformatics tools are nowadays available to facilitate the comparison of *L. monocytogenes* isolates, revealing differences at the genomic, transcriptomic, and pathogenic levels (Bécavin et al., 2014; Tamburro et al., 2015a). However, these tools, including Whole Genome Sequencing (WGS), are not yet universally available, and expensive to implement.

Identification of *L. monocytogenes* serovar, lineage and clonal groups represent the first approach for understanding intra-species genetic differences, particularly related to the pathogenic potential (Doijad et al., 2015; Tamburro et al., 2015b). Amongst the 13 well-recognized serovars, 4b, 1/2a, 1/2b and 1/2c are associated with the majority of human cases (den Bakker et al., 2010; Yin et al., 2015). Furthermore, four evolutionary serovar-related lineages (I-IV) have been described (Orsi et al., 2008; Rawool et al., 2016; Ward et al., 2008). Lineage I includes serovar 4b, 1/2b and 3b, and the major epidemic clones associated with large outbreaks, as well as with sporadic human cases; lineage II includes serovar 1/2a, 1/2c, 3a and 3c, and groups isolates

* Corresponding author at: Department of Medicine and Health Sciences "Vincenzo Tiberio", University of Molise, Via De Sanctis, 86100 Campobasso, Italy.
E-mail addresses: manuela.tamburro@unimol.it (M. Tamburro), sammarco@unimol.it (M.L. Sammarco), inc.fanelli@unimol.it (I. Fanelli), ripab@unimol.it (G. Ripabelli).

https://doi.org/10.1016/j.ijfoodmicro.2019.108289
Received 30 October 2018; Received in revised form 24 May 2019; Accepted 4 August 2019
Available online 06 August 2019
0168-1605/

from cases of human listeriosis (particularly serovar 1/2a) and the majority of isolates from food and environment; lineage III comprises 4a and 4c strains isolated from animal hosts, and lineage IV is represented by a divergent 4a subgroup (Lomonaco et al., 2015; Orsi et al., 2011; Ward et al., 2008).

Molecular methods have progressively improved the discrimination between strains according to variation in virulence-associated determinants (Camargo et al., 2016; Takahashi et al., 2017; Tamburro et al., 2010, 2015b).

High resolution melting (HRM) analysis can rapidly discriminate DNA sequence variants using real time PCR amplification and melting temperature (Tm) or curve analysis, without sequencing or hybridization procedures (Tamburro and Ripabelli, 2017; Tamburro et al., 2018; Tong and Giffard, 2012). Therefore, HRM analysis can improve *L. monocytogenes* molecular characterization by exploiting sequence differences within PCR fragments through direct melting and high-density data acquisition (Tamburro and Ripabelli, 2017; Tong and Giffard, 2012).

HRM assays have been previously applied to *Listeria* species identification (Forghani et al., 2016; Jin et al., 2012; Ohshima et al., 2014; Wang et al., 2010), and *L. monocytogenes* genotyping (Ohshima et al., 2017; Pietzka et al., 2011; Sakaridis et al., 2014). We recently reported the development and evaluation of a novel HRM approach for typing *L. monocytogenes* cultures of serovar 1/2a and 1/2c (lineage II) (Tamburro et al., 2018), targeting the region comprised between the *prs* and *ldh* housekeeping genes, including Listeria Pathogenicity Island-1 (LIPI-1) genes, and the *inl*AB operon.

In the present study, we describe the evaluation of that assay to map sequence variations within *L. monocytogenes* cultures of serovar 1/2b and 4b (lineage I), and to assess its usefulness for comparisons and discrimination with the 1/2a and 1/2c (lineage II) isolates. The application of the HRM approach here described is proposed as an alternative tool for the molecular characterization of *L. monocytogenes* applicable to discriminate between isolates of serovars 1/2a, 1/2b, 1/2c and 4b belonging to the two main lineages associated with human listeriosis, and may be a useful tool for the epidemiological investigations.

2. Materials and methods

2.1. L. monocytogenes cultures, DNA extraction and PCR-based serotyping classification

Fifty *L. monocytogenes* cultures were analyzed (Table 1): 27 isolates were provided from Gastrointestinal Bacteria Reference Unit, Food Safety Microbiology Laboratory (GBRU, Public Health England, UK), and the remaining from Laboratory of Hygiene culture collection (University of Molise, Campobasso, Italy). Thirty-eight of the *L. monocytogenes* were isolated from human listeriosis cases and 12 were isolated from food.

Pure cultures of *L. monocytogenes* were subcultured on Trypticase Soy agar (Oxoid, Basingstoke, Hampshire, UK), and incubated at 37 °C for 24 h. DNA was extracted by Maxwell® 16 Cell DNA Purification kit (Promega, Enterprise Rd, Southampton, UK) using automated Maxwell® 16 Instrument (Promega), according to manufacturers' instructions. Serogroup was identified by Multiplex-PCR assay (Doumith et al., 2004; Tamburro et al., 2010), while PCR-based lineage classification was carried out in other studies (unpublished data), as previously described (Ward et al., 2004).

2.2. Real time PCR and HRM assays

For this study, 81 primer pairs, previously designed (Tamburro et al., 2018) for the analysis of 1/2a and 1/2c cultures by using the published genome sequence of *L. monocytogenes* EGD-e strain (serovar 1/2a, lineage II, NCBI Reference Sequence: NC_003210.1), were utilized. Oligonucleotides amplified fragments within the *prs* and *ldh* loci, including LIPI-1 genes (markers from LPAI-1 to LPAI-53), and the *inl*AB operon (markers from LINT-1 to LINT-28). Both real time PCR and HRM were previously developed and standardized (Tamburro et al., 2018). Briefly, PCRs were performed in 20 µl, with 2 µl of 1:10 diluted genomic DNA, 1× PCR Gold Buffer (Applied Biosystems, Thermo Fisher Scientific, UK), 0.25 mM dNTP mix (Invitrogen, Thermo Fisher Scientific), 2 mM $MgCl_2$ (Applied Biosystems), 0.5× EvaGreen™ (Biotium, Hayward, CA), 1 U/µl AmpliTaq® Gold DNA polymerase (Applied Biosystems), and 0.5 µM of forward and reverse primers (Eurogentec Ltd., Hampshire, UK).

Amplifications were carried out in a single run in ten replicate experiments on EGD-e using Fast Real-time 7500 PCR instrument (ABI FAST 7500 SDS, Applied Biosystems) version 2.0.1, at the following conditions: 95 °C for 10 min; 30 cycles at 95 °C for 10 s, 58 °C for 15 s, and 72 °C for 40 s. Products were heated at 95 °C for 5 s, annealed at 50 °C for 30 s, and HRM was performed from 60 °C to 95 °C (Tamburro et al., 2018), rising at 1 °C/s with 25 acquisitions per 1 °C step (i.e. when the temperature at which such product melts is determined, the melting interval should be reduced to a maximum of 25 °C ensuring that the melting programs start/end at least 10 °C before/5 °C after the

Table 1
L. monocytogenes cultures isolated from human cases and food analyzed in this study.

Strain	Country	Source	Lineage	Serovar
LM1	UK	Food	I	4b
LM2	UK	Food	I	4b
LM4	UK	Food	I	4b
LM6	UK	Human	I	4b
LM8	UK	Food	I	4b
LM9	UK	Human	I	4b
LM11	UK	Human	I	4b
LM12	UK	Food	I	4b
LM13	UK	Food	I	4b
LM15	UK	Food	I	1/2b
LM18	UK	Human	I	1/2b
LM21	UK	Human	I	4b
LM29	UK	Human	I	1/2b
LM32	UK	Food	I	4b
LM34	UK	Human	I	1/2b
LMO01	Italy	Human	I	1/2b
LMO02	Italy	Human	I	1/2b
LMO04	Italy	Human	I	4b
LMO05	Italy	Human	I	4b
LMO07	Italy	Human	I	1/2b
LMO08	Italy	Human	I	4b
LMO09	Italy	Human	I	4b
LMO10	Italy	Human	I	1/2b
LMO11	Italy	Human	I	1/2b
LMO13	Italy	Human	I	4b
LMO14	Italy	Human	I	4b
LMO20	Italy	Human	I	4b
LMO28	Italy	Human	I	4b
LM3	UK	Human	II	1/2a
LM5	UK	Human	II	1/2a
LM7	UK	Human	II	1/2c
LM14	UK	Human	II	1/2a
LM16	UK	Food	II	1/2a
LM17	UK	Food	II	1/2a
LM19	UK	Human	II	1/2a
LM20	UK	Food	II	1/2a
LM22	UK	Food	II	1/2a
LM23	UK	Human	II	1/2a
LM24	UK	Human	II	1/2a
LM35	UK	Human	II	1/2c
LMO06	Italy	Human	II	1/2a
LMO12	Italy	Human	II	1/2a
LMO15	Italy	Human	II	1/2a
LMO16	Italy	Human	II	1/2a
LMO17	Italy	Human	II	1/2a
LMO18	Italy	Human	II	1/2a
LMO19	Italy	Human	II	1/2a
LMO21	Italy	Human	II	1/2a
LMO23	Italy	Human	II	1/2a
LMO26	Italy	Human	II	1/2a

expected Tm value; twenty-five acquisitions/°C are considered sufficient to result in a resolution appropriate for HRM analysis; https://lifescience.roche.com/documents/High-Resolution-Melting-Optimization-Strategies.pdf). Each *L. monocytogenes* culture was tested at least in duplicate for all markers, and a negative control (water without DNA) was included in all batches of reactions. The EGDE-e strain was used as the internal positive control, tested 10 times to evaluate the assay reproducibility and repeatability. At least two previously amplified markers each for *prs*-LIPI-1-*ldh* region and *inl*AB operon were included in all reactions. *L. innocua* NCTC 11288 provided by GBRU (Public Health England, UK) was also analyzed to verify the absence of LIPI-1 targets.

Tm intervals using these primers for the 81 markers on the EGD-e strain were calculated as previously reported (Tamburro et al., 2018).

2.3. Data analysis

From ten replicate experiments on EGD-e, Tm mean, standard deviations (SD) and small-number standard deviations (z = SD multiplied by 1.05, which corresponds to the square root of number of assays on EGD-e/ number of assays on EGD-e minus 1), were calculated. The highest z value amongst all the fragments was multiplied by 3 (obtaining Q), and this result was applied to Tm mean of all amplicons (± Q), as previously reported (Tamburro et al., 2018). This approach was used for the estimation of Tm interval comprised between a lower (Tm mean minus Q) and an upper (Tm mean plus Q) limit for each fragment. The z value was multiplied by 3 according to the normal (Gauss) distribution of the quantitative variables, where the area comprised between ± 1, 2, and 3 standard deviations from the mean is 68%, 95%, and 99.7%, respectively. In particular, 99.7% of the area of any normal distribution is within three standard deviations of the mean (there is 0.3% chance that the assay would give a Tm outside the range, with the assumption that DNA sequence is different).

The HRM analysis on 50 *L. monocytogenes* cultures was performed in comparison to the results on EGD-e, and a sequence divergence (variant) was estimated when Tm was outside these limits. A HRM profile was assigned to each isolate considering the Tm values and their variants amongst the tested markers.

A Simpson's Index of Diversity based on the targeted fragments, including the region encompassing *prs*/LIPI-1/*ldh*, and the *inl*AB operon, was determined to assess the discriminatory power of the method (Hunter and Gaston, 1988).

Furthermore, using Tm values and related variants for each amplicon, a tree of relatedness (dendrogram) for a character-based clustering on the 50 *L. monocytogenes* cultures was generated through BioNumerics software (Applied Maths, Belgium) v6.1, with the assumptions that data were categorical and independent. Hence, the Unweighted Pair Group Method with Arithmatic Mean UPGMA algorithm was used for cluster analysis.

A subset of PCR fragments showing Tm variants were sequenced to confirm point mutations. Sanger sequencing was carried out using the forward and reverse primers of specific targets amplification. FASTA sequences provided by Eurofins Genomics (Ebersberg, Germany) were evaluated using BioNumerics software (Applied Maths) and compared against EGD-e strain by clustalW multiple alignment.

2.4. Key resources table

Resource	Source	Identifier
Chemical		
Gold		
MgCl2		
Organism		
EDGE	N/A	N/A

3. Results

3.1. PCR-serogrouping classification

Nineteen (38.0%) and 9 (18.0%) strains were of serovar 4b (serogroup IV) and 1/2b (serogroup III) respectively, while 20 (40.0%) and 2 (4.0%) belonged to serovar 1/2a (serogroup I) and 1/2c (serogroup II). Hence, twenty-eight (56.0%) cultures were classified into lineage I and 22 into lineage II (Table 1).

3.2. HRM profiling of L. monocytogenes cultures

The *L. innocua* strain NCTC 11288 tested for the HRM assay did not show any amplification for all LIPI-1 fragments, while were detected the *prs* (LPAI-1, 2, and 3) and *ldh* (LPAI-50, 51, 52, and 53) housekeeping genes in Listeria genus, and their melting profiles were comprised within the EGD-e limits.

HRM profiles amongst the 50 *L. monocytogenes* cultures (Fig. 1) were forty-five; a Simpson's Index of Diversity (D) of 99.35 was obtained. The majority of the cultures segregated into two groups, which corresponded to the lineage classification. Considering Tm values for all targets, 25 HRM profiles out of 28 lineage I cultures and 21 profiles out of 22 lineage II cultures were identified; D = 0.998 for lineage I and D = 0.996 for lineage II. Cultures of serovar 1/2a had a greater level of genetic diversity than all other serovars. Amongst the targeted genes, the *hly*A fragments had the most stable Tms, irrespectively of the lineage and serovar compared to the other fragments showing typical Tms within lineage or serovar. In particular, the main differences between lineage I and II were observed for the fragments targeting the *prs*, *prf*A, *plc*A, *mpl*, *act*A and *plc*B genes.

HRM results identified eighteen (LPAI-1, 3, 4, 6, 8, 9, 13–16, 21, 24, 27, 40, 46, 48, and INT-25, 26) out of the 81 targets, which generated stable Tm characteristics irrespectively of the lineage.

Generally, targets generated a single different Tm from the EGD-e strain, although 17 (LPAI-12, 19, 20, 31, 32, 35, 41, and 51; LINT-2, 3, 4, 6, 13, 14, 16, 20, and 24) of them provided two variants, and one (LINT-28 targeting *inl*B) produced three variants (Fig. 1).

The similarity analysis for the 81 markers identified two main clusters (Fig. 2). Cluster A consisted of 25 cultures (from LM24 to LM20), all classified within lineage II (serovar 1/2a and 1/2c), except for LM2, LM4, and LM32, which were isolated from food (Fig. 2), and were classified as serovar 4b by both conventional serotyping and PCR-based serogrouping.

In contrast, cluster B included only cultures within lineage I (from LM15 to LMO14). Identical HRM profiles were generated by LM2 and LM4 cultures from food (serovar 4b), and LM7 and LM35 from human cases (serovar 1/2c). These four isolates had identical HRM patterns for all markers except for LPAI-51 as compared to EGD-e. Amongst the tested cultures of lineage II, 1/2a isolates showed a greater degree of variation than all other serovars, although isolates LM3, LM5, LM23 and LMO21 showed the highest level of similarity (Fig. 2). In comparison with EGD-e, the main differences in melting profiles were observed for markers within the *act*A, *ldh*, and both *inl*A and *inl*B genes (Fig. 1).

The HRM method segregated cultures of lineage II (cluster A) from lineage I (cluster B) based on their melting behavior, except for LM2, LM4, and LM32 food isolates. Within lineage I, the inter-strain comparisons revealed that LM12 and LM9, as well as LM11 and LM13 were the only cultures that generated the same Tm profiles for all fragments (Fig. 2), and were all of serovar 4b. Furthermore, cultures of serovar 1/2b were clearly distinguished from 4b.

Compared with EGD-e and the remaining cultures of lineage II, there were consistent differences for 21 markers amongst the 1/2b and 4b isolates, mainly located within region from *prs* to *ldh*, encompassing LIPI-1. Nevertheless, these Tm profiles were conserved within the lineage I, as cultures shared the same Tm irrespectively of the serovar.

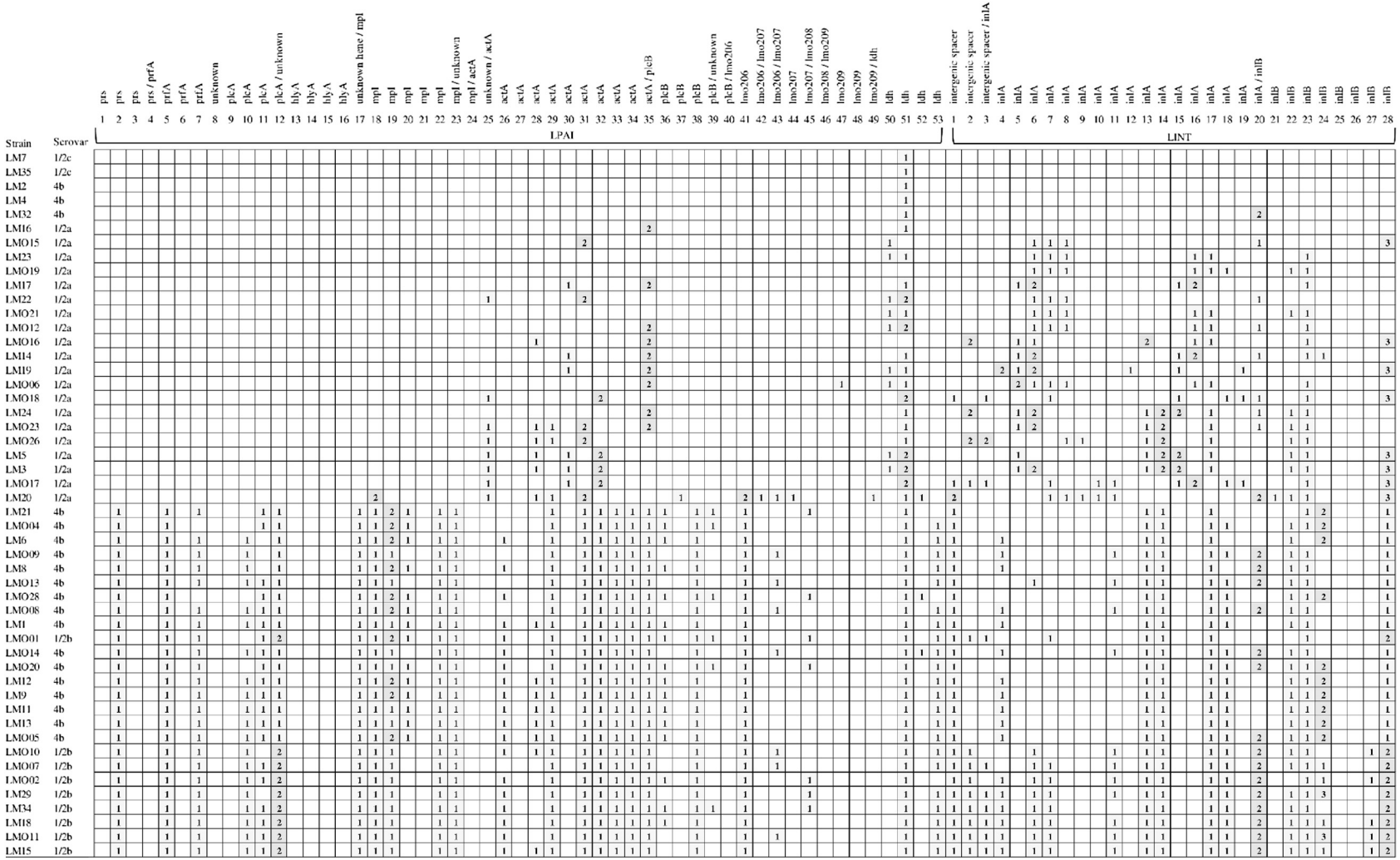

Fig. 1. Synoptic HRM results in terms of Tm variants for the 50 *L. monocytogenes* cultures analyzed respect to EGD-e.
Description: in the figure, the numbers 1, 2 and 3 correspond to Tm variants (value outside from the EGD-e intervals) for a defined amplicon, and in order represent the first, the second and the third most frequent variant observed, respectively.

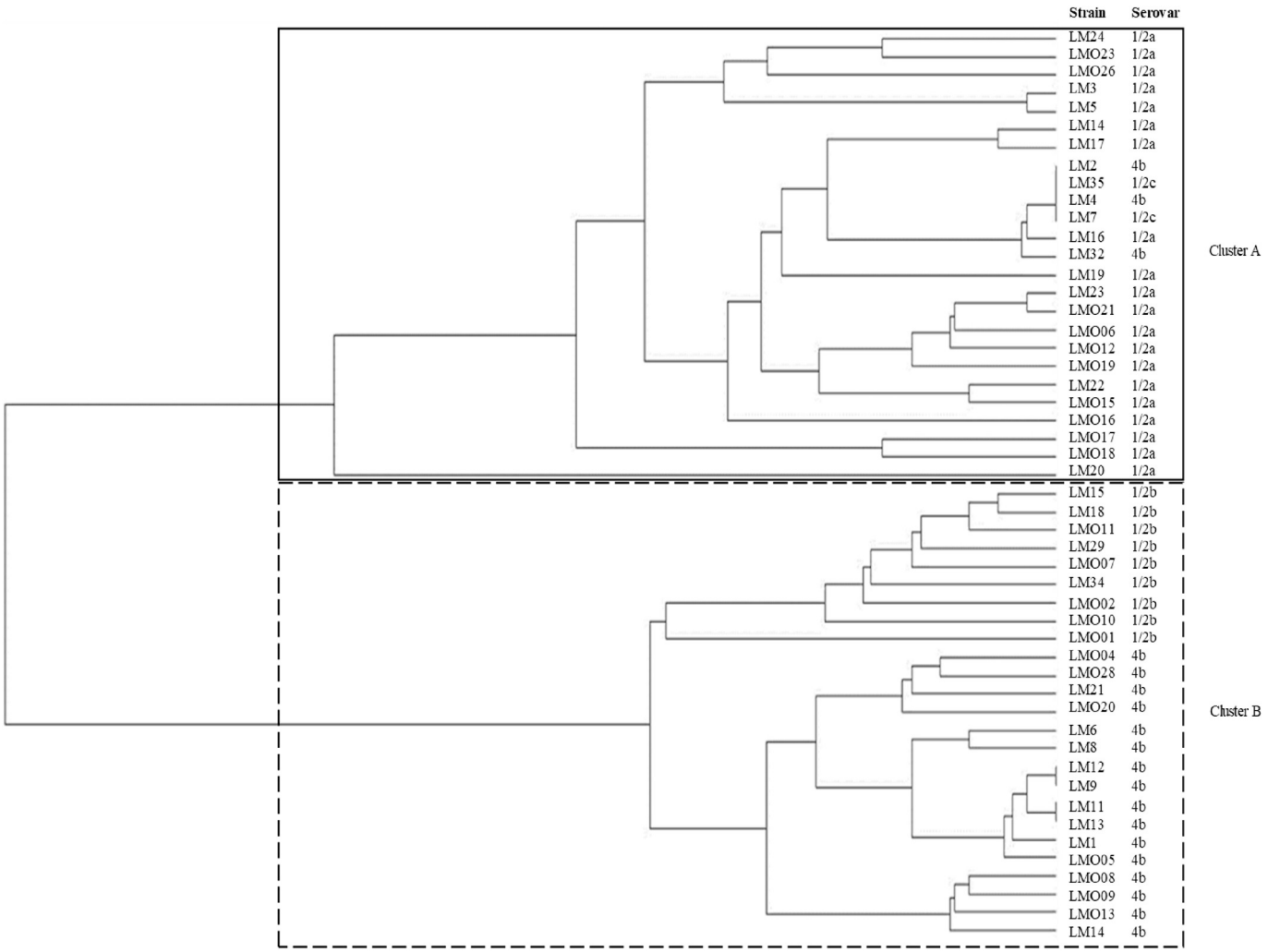

Fig. 2. Dendrogram generated using Tms of 81 gene fragments for each of 50 *L. monocytogenes* cultures.

Specific markers were identified for discriminating isolates within lineage I: LPAI-2 (*prs*), LPAI-5 (*prfA*), LPAI-17 (unknown/*mpl*), LPAI-18 (*mpl*), LPAI-22 (*mpl*), LPAI-23 (*mpl*/unknown), LPAI-31/32/33/34 (*actA*), LPAI-35 (*act*A/*plc*B), LPAI-38 (*plc*B), and LPAI-41 (*lmo206*). Interestingly, the LPAI-12 fragment (targeting part of *plc*A and part of an unknown gene) showed a Tm profile that not only allowed differentiating lineage I from II (Fig. 3), but also discriminated between cultures of serovar 4b and 1/2b, since Tm variants were found to be serovar specific.

Based on the *inl*A and *inl*B genes, the 50 *L. monocytogenes* cultures showed the largest Tm differences compared to all markers, with the exception of INT-25 and INT-26 (both targeting *inl*B gene) that always generated a constant Tm behavior.

Considering the *inl*AB operon, the INT-28 fragment represented the most useful marker to discriminate between cultures of lineage I and II, showing typical Tm profiles, which also differentiated serovar 4b from 1/2b isolates.

Although only some fragments with Tm variants were sequenced, the alignments performed in comparison with EGD-e (used as the comparator) showed significant findings in relation to lineage I and II classification, consistent with the dendrogram, drawn on generated Tms. Sequencing results are reported in Table 2, and refer to 11 and 5 fragments targeting *prs*/LIPI-1/*ldh* and *inl*AB, respectively.

Analysis showed that cultures of lineage I with Tm variants compared to EGD-e had sequence differences, with point mutations that in most cases were conserved/constant, and may be considered as stable markers for strains discrimination. Particularly, these variations were found for LPAI-2 (*prs*), LPAI-31, LPAI-32, LPAI-33 (all targeting *act*A), LPAI-35 (*act*A/*plc*B) and LPAI-38 (*plc*B), as well as for LINT-28 (*inl*B). As shown in Table 2, alignments revealed consistent variations within specific fragments valuable for discrimination between lineage I and II, and others suggesting a serovar identification.

4. Discussion

Despite the low incidence, invasive human listeriosis is one of the most serious foodborne diseases under European surveillance causing high hospitalization and mortality (Zolin et al., 2017; European Food Safety Authority and European Centre for Disease Prevention and Control EFSA and ECDC, 2018). Since multistate or multinational outbreaks occur, it is important to generate typing data for characterizing of *L. monocytogenes* from different sources and countries (Allard et al., 2018; Camargo et al., 2016; Lomonaco et al., 2018), integrating conventional with molecular epidemiology (Nyarko and Donnelly, 2015; Sammarco et al., 2014).

A wide range of subtyping methods have been used to study the intra-species genetic diversity, ecological distribution, and differences in virulence potential within *L. monocytogenes* (Poimenidou et al., 2018; Orsi et al., 2011). Pulsed-field gel electrophoresis (PFGE) is considered as the “gold standard” fingerprinting method (Amato et al., 2017) used within PulseNet network established by the Centers for Diseases Control (CDC, Atlanta), which is committed to find and use the best techniques

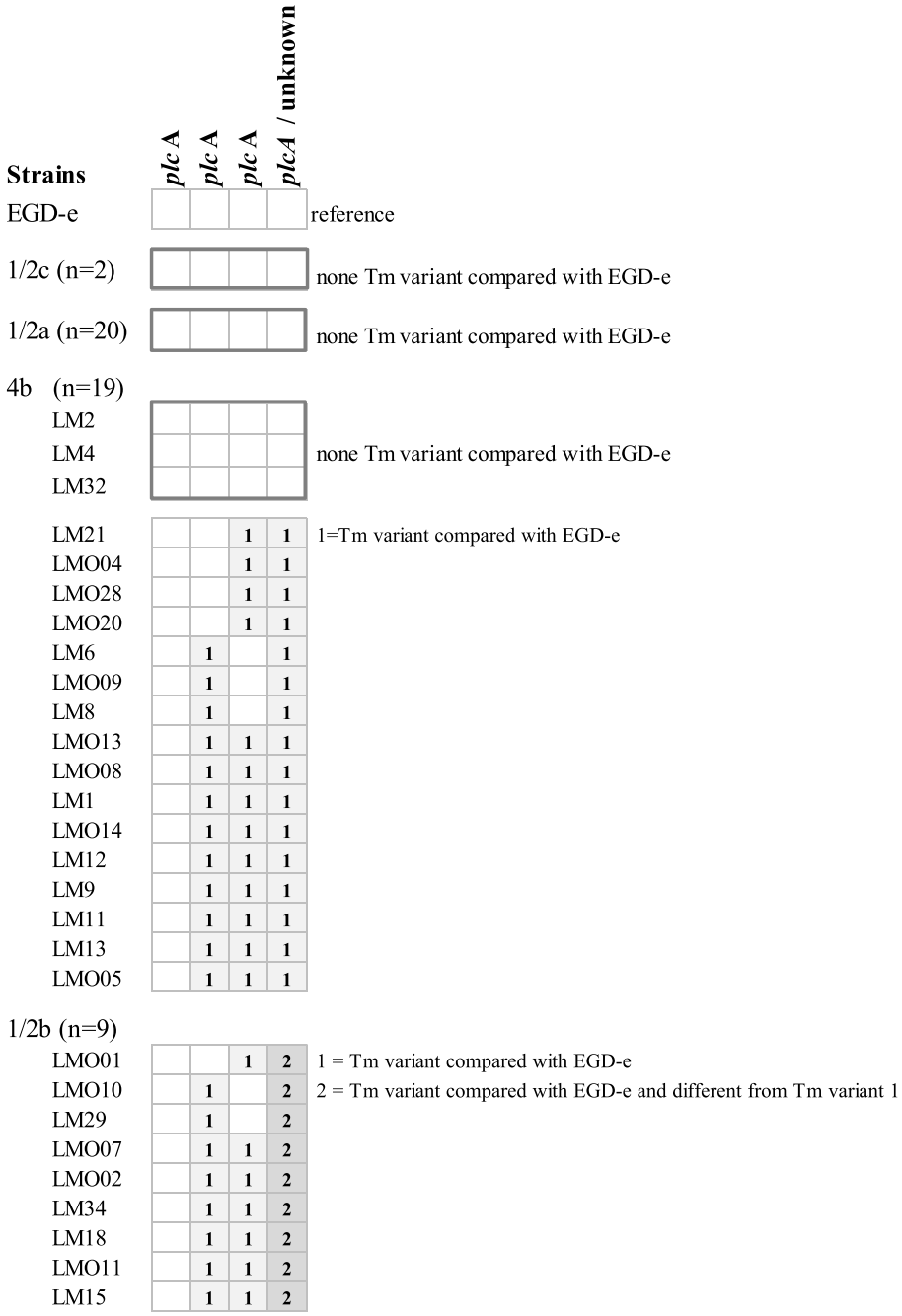

Fig. 3. Representation of Tm variations reflecting sequence divergences within the *plc*A gene in 1/2a, 1/2c, 4b and 1/2b *L. monocytogenes* isolates.

to identify outbreaks. PFGE has several advantages, such as high concordance with epidemiological relatedness; it can be applied as universal generic subtyping method for many different bacteria with the choice of the restriction enzyme and electrophoresis conditions optimized for each species; the DNA-based restriction patterns are stable and reproducible; and it is still more discriminating than multi-locus sequence typing for many bacteria (Sammarco et al., 2014; https://www.cdc.gov/pulsenet/pathogens/pfge.html). However, PFGE does not discriminate between all the unrelated isolates, and shows other limitations, including the change in one restriction site that can result in more than one band change; the relatedness may not represent a true phylogenetic measure, and it does not differentiate isolates to the same degree achieved through WGS. Indeed, at state of the art, PulseNet is transitioning toward the WGS application enhancing the ability to detect and solve outbreaks faster and with more accuracy based on unambiguous, highly detailed data (Amato e et al., 2017; Stasiewicz et al., 2015; https://www.cdc.gov/pulsenet/pathogens/pfge.html).

Although WGS is of a noteworthy power for epidemiological surveillance, infection control and outbreak investigation, some limitations such as investment in next-generation sequencing instrumentation and specialized laboratory personnel, hamper its application in certain instances (Hasnain et al., 2015; Kwong et al., 2016). Indeed, different methods can prove a high level of characterization of the bacterial strains.

In this study, a novel approach based on Tm differences identified by HRM amongst the main virulence genes in *L. monocytogenes* is described to characterize genetic variations without the need of sequencing. This method was previously developed and evaluated for typing 1/2a and 1/2c (lineage II) *L. monocytogenes* cultures (Tamburro et al., 2018), and it was found as reproducible and rapid in identifying

Table 2
Sequencing results amongst *prs*/LIPI-1/*ldh* and *inl*AB fragments showing Tm variants compared to EGD-e and potential markers for lineage classification.

LPAI-2

```
          GCGCTTCCGC AGCAACAATT AATATTGTTA TGCCTTATTA TGGTTATGCC GTCAAGACCG
          TAAAGCAAGA AGTCGTGAAC CAATCACAGC GAAATTAGAG CAAACTTAAT CGAAACTGCT
          GGTGCAACTA GAATGATTAC ACTTGATGCA TGCACCGCAA ATCCAAGGTT TCTTTGATAT
EGD-e     TCCAATTGAT CACTTGAACG CAGTACGCCT TCTAAGTGAC TATTTCAGCG AACGTCATTT
LMO07           C            T       T                          T
LMO08           C            T       T                          T

EGD-e     AGGCGA--GT TTAGTAGTGG TTTCACCTGA CCATGGTGGA GTTACTCGTG CCCGTAAA-T
LMO07        T  TGA  C        A       C                          A   T       A
LMO08        T  TGA  C        A       C                          A   T       A

EGD-e     GGCTGATCGT TTGAAAGCGC CGATTGCTAT TATTGATAAA CGTCGTCCGC GTCCAAACGT
LMO07        G  C                                   G                       A
LMO08        T  C                                   G                       G

EGD-e     GGCTGAAGTA ATGAACATCG TT
LMO07     A
LMO08     A
```

Alignment of LPAI-2 fragments in EGD-e (1/2a used as a comparator), LMO07 (1/2b) and LMO08 (4b) revealed several mutations; of these, 17 were stable nucleotide variants (in bold), useful for lineage discrimination. Tm variants similar to LMO07 and LMO08 were found in all cultures of lineage I, except for LM2, LM4, and LM32 grouped within 1/2c and 1/2a group.

LPAI-31

```
EGD-e     GAAGAAATTG ATCGCCTAGC TGATTTAAGA GATAGAGGAA CAGGAAAACA CTCAAGAAAT
LMO20     GCGGGTTTTT TACCATTAAA TCCGTTTGCT AGCAGCCCGG TTCCTTCGTT AAGTCCAAAG
LMO08                                   A         C                 G
                                        A         T                 C

EGD-e
LMO20     GTATCGAAAA TAAGCGCACC GGCTCTGATA AGTGACATAA CTAAAAAAAC GCCATTTAAG
LMO08        T              G               C    G             G  A         A
             C              G               C    A             G  A         A

EGD-e
LMO20     AATCCATCAC AGCCATTAAA TGTGTTTAAT AAAAAAACTA CAACGAAAAC AGTGACTAAA
LMO08        GC                         C                        CAC
             GC                         C                        CGC

EGD-e
LMO20     AAACCAACCC CTGTAAAGAC CGCACCAAAG CTAGCAGAAC TTCCTGCCAC AAAACCACAA
LMO08        AT          G  T      G              C        AT    G   G G
             AT          G  T      G              C        AT    G   G G

EGD-e
LMO20     GAAACCGTAC TTAGGGAAAA TAAAACACCC TTTATAGAAA AACAAGCAGA AACAAACAAG
LMO08        T AG    G            G    A                         G         T
             T AG    G            G    A                         G         T

EGD-e
LMO20     CAGTCAATTA ATATGCCGAG CCTACCAGTA ATC
LMO08      G C     G                        A
           G C     G                        A
```

Alignment of LPAI-31 fragments in LMO20 and LMO08 (both 4b) compared with EGD-e revealed 34 stable mutations (in bold) useful for lineage discrimination, since the same Tm variants were also found in the other 1/2b cultures.

(*continued on next page*)

Table 2 (*continued*)

LPAI-32

```
          CCTTCGTTAA GTCCAAAGGT ATCGAAAATA AGCGCACCGG CTCTGATAAG TGACATAACT
          AAAAAAACGC CATTTAAGAA TCCATCACAG CCATTAAATG TGTTTAATAA AAAAACTACA
EGD-e     ACGAAAACAG TGACTAAAAA ACCAACCCCT GTAAAGACCG CACCAAAGCT AGCAGAACTT
LMO08               C C A       AT          G  T      G               C
LMO20               C C A       AT          G  T      G               C

EGD-e     CCTGCCACAA AACCACAAGA AACCGTACTT AGGGAAAATA AAACACCCTT TATAGAAAAA
LMO08        AT   G    G G         T AG    G                  A
LMO20        AT   G    G G         T AG    G                  A

EGD-e     CAAGCAGAAA CAAACAAGCA GTCAATTAAT ATGCCGAGCC TACCAGTAAT CC
LMO08             G         T G  C     G
LMO20             G         T G  C     G
```

Alignment of LPAI-32 fragments in LMO20 and LMO08 (both 4b) compared with EGD-e, revealed 24 stable mutations (in bold) useful for lineage discrimination, since the same Tm variants were found in 1/2b cultures and were different from the 1/2a isolates.

LPAI-33

```
          CGTTAAGTCC AAAGGTATCG AAAATAAGCG CACCGGCTCT GATAAGTGAC ATAACTAAAA
          AAACGCCATT TAAGAATCCA TCACAGCCAT TAAATGTGTT TAATAAAAAA ACTACAACGA
EGD-e     AAACAGTGAC TAAAAAACCA ACCCCTGTAA AGACCGCACC AAAGCTAGCA GAACTTCCTG
LMO08        CGC  A      AT          G                          C       A
LMO20        CAC  A      AT          G                          C       A

EGD-e     CCACAAAACC ACAAGAAACC GTACTTAGGG AAAATAAAAC ACCCTTTATA GAAAAACAAG
LMO08     T   G   G  G        T  AG   G           G     A
LMO20     T   G   G  G        T  AG   G           G     A

EGD-e     CAGAAACAAA CAAGCAGTCA ATTAATATGC CGAGCCTACC AGTAATCCA
LMO08        G         T G C      G
LMO20        G         T G C      G
```

Alignment of LPAI-33 fragments in LMO20 and LMO08 (both 4b) compared with EGD-e revealed 24 stable mutations (in bold) useful for lineage discrimination, since the same Tm variants were found in 1/2b cultures and were different from those of the 1/2a isolates.

LPAI-35

```
          TGCTAAAAGT GCAGAAGACG AAAAAGCGAA GGAAGAACCA GGGAACCATA CGACGTTAAT
EGD-e     TCTTGCAATG TTAGCTATTG GCGTGTTCTC TTTAGGGGCG TTTATCAAAA TTATTCAATT
LMO08                                                G          C
LMO10                                                G          C

EGD-e     AAGAAAAAAT AATTAAAAAC ACAGAACGAA AGAAAAAGTG AGGTGAATGA TATGAAATTC
LMO08              G  G  G   G     T                      G     G    G
LMO10              G  G  G   G     T                      G     G    G

EGD-e     AAAAAGGTGG TTCTAGGTAT GTGCTTGATC GCAAGTGTTC TAGTCTTTC
LMO08        A                        C
LMO10        A                        C
```

Alignment of LPAI-35 fragments in LMO08 (4b) and LMO10 (1/2b) compared with EGD-e revealed 12 stable mutations (in bold) useful for lineage discrimination, since Tm variants were found in 1/2b cultures for this marker and were different from 1/2a cultures.

(*continued on next page*)

Table 2 (*continued*)

LPAI-38

```
          GAGAAGGGAA ATTTGACACA GCGTTTTATA AATTAGGCCT AGCAATCCAT TATTATACGG
EGD-e     ATATTAGTCA ACCTATGCAC GCCAATAATT TTACCGCAAT ATCATACCCT CCAGGCTACC
LMO07                                                A  C  A
LMO20                                                C  T  A

EGD-e     ACTGTGCATA TGAAAATTAC GTAGATACCA TTAAACACAA TTATCAAGCA ACGGAAGACA
LMO07                      T   G     T                           A
LMO20                      C   A     C                           A

EGD-e     TGGTAGCAAA AAGATTTTGC TCAGATGACG TGAAAGACTG GCTCTATGAA AATGCGAAAA
LMO07           TGA               G              A
LMO20           TGC               A              A

EGD-e     GGGCGAAAGC GGACTACCCG AAAATAGTCA ATGCGAAAAC TAAAAAATCA TATTTAGTAG
LMO07                    T                                      C
LMO20                    T                                      C

EGD-e     GAAATTCTGA ATGGAAAAAG GATACAGTGG AACCTACTGG A
LMO07           C
LMO20           C
```

Alignment of LPAI-38 fragments in LMO07 (1/2b) and LMO10 (4b) compared with EGD-e revealed 8 stable mutations (in bold) useful for lineage discrimination, since Tm variants were found in 1/2b cultures and were different from 1/2a isolates.

LPAI-12

```
          ATGACATCGT TTGTGTTTGA GCTAGTGGTT TGGTTAATGT CCATGTTATG TCTCCGTTAT
          AGCTCATCGT ATCATGTGTA CCTGGTATAG AGAGCGCTGC TAGGTTTGTT GTGTCAGGTA
          GAGCGGACAT CCATTGTTTT GTAGTTACAG AGTTCTTTAT TGGCTTATTC CAGTTATTAA
EGD-e     GCGAATATGC TTTTCCGCCT AATGGGAAAG TAAAAAAGTA TAAAATAAAA CAGAGTAATA
LMO08                    A                          C                    C

EGD-e     AAACTAATGT GCGTTGCAAA TAATTCTTAT ACAAAATGGC CCCCTCCTTT GATTAGTATA
LMO08                                                                  A

EGD-e     TTCCTATCTT AAAGTGACTT TTATGTTGAG GCATTAACAT TTGTTAACGA CGATAAAGGG
LMO08                    T           C    T

EGD-e     ACAGC
LMO08
```

Alignment of LPAI-12 fragment in LMO08 (4b) compared with EGD-e revealed 7 mutations that could be useful for serovar discrimination, since Tm variants for this strain and the remaining 4b cultures differed not only from 1/2a and 1/2c but also from 1/2b cultures.

LPAI-18

```
          CTTGATATGA CAAAAGATAA GCTAGGCGTT ACGCATTATA CGCTCGCGCT AAGTTCTGGT
          GGTTACTTGA CCGATAATGA TGAAATCAAG GTCCACGTCA CACCGGATAA TAAAATCACT
EGD-e     TTCATAAATG GAGATTTGCA GCAAGGACAG CTTAGGATTA CTAATCAAAT AAAAATTACA
LMO10                              G                                   C

EGD-e     GAAAAAAATG CTATCGAAAA AGCATTTGAA GCCATCGGTC AGAGTGAAGC TCATGTGAAA
LMO10                   T                        T

EGD-e     AGTTATGTCG GAAACCCAGT GAAAGAAAAA GAAATCATCC TCAATTCCAG AACAAAACGC
LMO10           A T       T                          A                G

EGD-e     TTAGTTTATA ATATAAAATT GATTTTTGCT GAGCCGGAAG TTGCGAGTTG G
LMO10
```

Alignment of LPAI-18 fragment in LMO10 (1/2b) compared with EGD-e

(*continued on next page*)

Table 2 (*continued*)

	revealed 9 mutations that could suggest lineage discrimination, since Tm variants for this strain and all 1/2b and 4b cultures differed from EGD-e, as well as from and 1/2a and 1/2c isolates.
LPAI-22	
EGD-e	GCGGTTATCC AGTATTCGGC GGGATTGGAA TACGAAGGGC AATCAGGTGC GCTAAACGAG
LMO10	A
EGD-e	TCGTTCGCCG ATGTTTTTGG TTATTTTATT GCGCCAAATC ATTGGTTGAT TGGTGAGGAT
LMO10	C
EGD-e	GTCTGTGTGC GTGGGTCGCG AGATGGGCGA ATAAGAAGCA TTAAAGATCC TGACAAATAT
LMO10	TA C
EGD-e	AATCAAGCGG CTCATATGAA AGATTACGAA TCGCTTCCAC TCACAGAGGA AGGCGACTGG
LMO10	G T A
	Alignment of LPAI-22 fragment in LMO10 (1/2b) compared with EGD-e revealed 8 mutations suggesting lineage discrimination, since the Tm variants for this strain and all 1/2b and 4b cultures differed from those in 172a and 1/2c isolates.
LPAI-34	
	ATATGCCGAG CCTACCAGTA ATCCAAAAAG AAGCTACAGA GAGCGATAAA GAGGAAATGA
EGD-e	AACCACAAAC CGAGGAAAAA ATGGTAGAGG AAAGCGAATC AGCTAATAAC GCAAACGGAA
LMO20	T
EGD-e	AAAATCGTTC TGCTGGCATT GAAGAAGGAA AACTAATTGC TAAAAGTGCA GAAGACGAAA
LMO20	T
EGD-e	AAGCGAAGGA AGAACCAGGG
LMO20	A T
	Alignment of LPAI-34 fragment in LMO20 (4b) compared with EGD-e revealed 4 mutations suggesting lineage discrimination, since the Tm variants for this strain and all 1/2b and 4b differed from those in 1/2a and 1/2c isolates.
LPAI-36	
	GTGGTTCTAG GTATGTGCTT GATCGCAAGT GTTCTAGTCT TTCCGGTAAC GATAAAAGCA
EGD-e	AATGCCTGTT GTGATGAATA CTTACAAACA CCCGCAGCTC CGCATGATAT TGACAGCAAA
LMO20	A
EGD-e	TTACCACATA AACTTAGTTG GTCCGCGGAT AACCCGACAA ATACTGACGT AAATACGCAC
LMO20	G T
EGD-e	TATTGGCT
LMO20	
	Alignment of LPAI-36 fragment in LMO20 (4b) compared with EGD-e revealed 3 mutations suggesting lineage discrimination, since the Tm variants for this strain and all 1/2b and 4b differed from those in 1/2a and 1/2c cultures.
LINT-14	GGAACGACAA CATTTAGTGG AACCGTGACG CAGCCACTTA AGGCAATTTT TAATGTTAAG
	TTTCATGTGG ACGGCAAAGA AACAACCAAA GAAGTGGAAG CTGGGAATTT ATTGACTGAA
EGD-e	CCAGCTAAGC CCGTAAAAGA AGGTCACACA TTTGTTGGTT GGTTTGATGC CCAAACAGGC
LMO08	T A T T T G C
EGD-e	GGAACTAAAT GGAATTTCAG TA
LMO08	

(*continued on next page*)

Table 2 (*continued*)

Alignment of LINT-14 fragment in LMO08 (4b) compared with EGD-e revealed 7 mutations suggesting lineage discrimination, since the Tm variants for this strain and all 1/2b and 4b cultures differed from those found in 1/2a and 1/2c isolates.

LINT-28

```
EGD-e  CGAAGCCAAA ACACCAATTA CTACATGGTA TCAATTTAGC ATTGGTGGTA AAGTAATTGG
LMO08                     G                     A
LMO07                     T                     G

EGD-e  TTGGGTCGAT ACCCGAGCAC TTAACACATT CTACAAACAA AGCATGGAAA AGCCAACCCG
LMO08             G  T                T  T          C         TA    T  A
LMO07             G  T                C  C          T         AA    C  G

EGD-e  TTTAACTCGT TATGTCAGCG CCAATAAAGC TGGCGAATCG TACTATAAAG TCCCGGTAGC
LMO08  A        A   C         A A     G AAAT   G A               T       T
LMO07  T        T   T         A G     G AAAT   G A               C       C

EGD-e  AGATAATCCA GTCAAAAGGG GTACTTTAG
LMO08       GC    A     T     A
LMO07       AT    G     A     T
```

Alignment of lint-28 fragments in LMO08 (4b) and LMO07 (1/2b) compared with EGD-e revealed several mutations; of these, 11 were commonly found in LMO08 and LMO07 (in bold) that could be useful for lineage discrimination, since Tm variants were different from 1/2a isolates. The other mutations may be useful for discriminating between serovar 1/2b and 4b, because Tm variants in these serovars were always different each other and from 1/2a and 1/2c isolates.

variations in key virulence (comprised in the LIPI-1 and *inl*AB) genes through the analysis of melting profiles of PCR products.

Here, the HRM analysis was further extended to *L. monocytogenes* cultures of serovar 1/2b and 4b (lineage I). HRM identified specific gene fragments for discrimination between cultures of lineage I and II, which may show divergence in terms of virulence potential and origin (Buchanan et al., 2017; Poimenidou et al., 2018). The method further demonstrated the genetic diversity between isolates of the four serovars tested, particularly amongst 1/2a cultures, according to other studies (den Bakker et al., 2008; Camargo et al., 2016; Hadjilouka et al., 2018; Poimenidou et al., 2018; Orsi et al., 2008), as well as with available phylogenetic data, which demonstrated that isolates within lineage II, including serovar 1/2a, can have higher recombination rates than lineage I, possibly facilitating adaptation to different environments (Orsi et al., 2008, 2011). Amongst the four serovars investigated here, the HRM approach revealed that 4b strains are more genetically homogeneous compared to the other serovars, particularly with respect to 1/2a, as revealed with other techniques (den Bakker et al., 2008; Bania et al., 2009; Poimenidou et al., 2018). Comparisons between cultures of different serovars also underlined consistent Tm values from fragments of *hly*A and certain *prf*A and *plc*Bfragments, while the most frequent variations were found in *plc*A, *mpl*, and *act*A, suggesting a different selective pressure acting on these genes within each serovar (Orsi et al., 2008; Poimenidou et al., 2018).

By considering all targeted genes, HRM analysis revealed that the *hly*A and *act*A genes were the most stable and most diverse in terms of Tms respectively, as previously reported (Poimenidou et al., 2018). The variations observed within LIPI-1 were also confirmed in other studies (Zhang et al., 2003), in particular for the *act*A gene, reporting that its polymorphisms can be useful for subtyping and specific amongst lineage (Bania et al., 2009). In this study, non-hemolytic *L. monocytogenes* to evaluate stop codon or mutations in the *hly*A gene were not included. However, the occurrence of these strains is rarely described, and the phylogenetic diversity of isolates with an altered hemolysis phenotype is unclear, as well as their genetic and microbiological characteristics. In a recent study (Maury et al., 2017), the hemolytic activity of listeriolysin O (LLO), encoded by *hly*A, was used as a phenotypic marker to screen for spontaneous virulence-attenuating mutations. The results revealed that amongst 57,820 *L. monocytogenes* isolated from a variety of sources, only sixty (0.1%) non-hemolytic isolates were identified, whose phenotype resulted by *hly*A mutations leading to a single amino acid substitution or premature stop codon causing a decreased virulence (Maury et al., 2017). The HRM approach for *inl*AB was also valuable for typing isolates, according with a previous study, reporting that the internalin sequences cluster into the evolutionary lineages I and II (Tsai et al., 2006). In fact, the *inl*A and *inl*B genes, in addition to *act*A, showed the greatest degree of diversity based on HRM profiles, supporting that these should be considered as hyper-variable possibly due to different host interactions (Cabanes et al., 2002). Furthermore, some strains tend to lose the functionality of the InlA, protein with a crucial role in human infections, leading to a reduced virulence (Ragon et al., 2008) due to the expression of truncated forms, frequently found in *L. monocytogenes* isolated from food products and the environment, especially those of serovar 1/2a and 1/2c (Jacquet et al., 2004). In this study, the *inl*A fragments were not tested in order to identify potential premature stop codons. Anyway, to evaluate whether the obtained HRM profiles for *inl*A can be useful for this purpose, results were compared to those obtained when restriction fragment length polymorphisms (RFLP) of *inl*A amplified products (Tamburro et al., 2010; Rousseaux et al., 2004) has been previously carried for the Italian strains included in the present study. Indeed, by considering RLFP results, these Italian strains had a profile referred to a complete/full-length internalin A, except for LMO17, LMO18, LMO23 and LMO26 for which a profile could not have been determined with this method, and LMO07 with a typical truncated profile that according to the RFLP method indicated a premature stop codon. By analyzing HRM results on

*inl*A, appreciable differences for LMO07 strain were not found. Hence, the evaluation of the HRM method as a tool for identifying truncated internalins could be performed in additional studies by analyzing more isolates with a well-definied truncated profile. Interestingly, the proposed method also showed a good cost-effectiveness, by costing approximately 12.00€ per sample, excluding expenditures for plates and bacteria cultivation, and real time PCR 96-well plates, as well as those for the required instrumentation.

In conclusion, the proposed HRM approach provides a rapid, cost-effective, accurate and reproducible method for *L. monocytogenes* subtyping, allowing the simultaneous detection and analysis of differences within DNA sequences based on the melting measurements of the virulence genes within LIPI-1, and *inl*A and *inl*B genes. The study findings further underline the usefulness and convenience of certain selected fragments for lineage I and II differentiation, suggesting the application of the method in epidemiological investigations, due to insights on clonal relationships for inter-strain comparisons. Additional research is warranted to evaluate the usefulness and convenience of this method for discrimination of *L. monocytogenes* cultures of lineages III and IV.

Declaration of competing interest

The authors declared do not have any conflict of interest.

Acknowledgements

The Authors acknowledge Dr. Jim McLauchlin, Public Health England, London, UK, for the helpful comments and the support to the study design.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

Allard, M.W., Bell, R., Ferreira, C.M., Gonzalez-Escalona, N., Hoffmann, M., Muruvanda, T., Ottesen, A., Ramachandran, P., Reed, E., Sharma, S., Stevens, E., Timme, R., Zheng, J., Brown, E.W., 2018. Genomics of foodborne pathogens for microbial food safety. Curr. Opin. Biotechnol. 49, 224–229. https://doi.org/10.1016/j.copbio.2017.11.002.

Amato, E., Filipello, V., Gori, M., Lomonaco, S., Losio, M.N., Parisi, A., Huedo, P., Knabel, S.J., Pontello, M., 2017. Identification of a major *Listeria monocytogenes* outbreak clone linked to soft cheese in Northern Italy - 2009-2011. BMC Infect. Dis. 17, 342. https://doi.org/10.1186/s12879-017-2441-6.

Bania, J., Zarczyńska, A., Molenda, J., Dabrowska, A., Kosek-Paszkowska, K., Wieckowska-Szakiel, M., Rózalska, B., 2009. Subtyping of *Listeria monocytogenes* isolates by *act*A gene sequencing, PCR-fingerprinting, and cell-invasion assay. Folia Microbiol. (Praha) 54, 17–24. https://doi.org/10.1007/s12223-009-0003-z.

Bécavin, C., Bouchier, C., Lechat, P., Archambaud, C., Creno, S., Gouin, E., Wu, Z., Kühbacher, A., Brisse, S., Pucciarelli, M.G., García-del Portillo, F., Hain, T., Portnoy, D.A., Chakraborty, T., Lecuit, M., Pizarro-Cerdá, J., Moszer, I., Bierne, H., Cossart, P., 2014. Comparison of widely used *Listeria monocytogenes* strains EGD, 10403S, and EGD-e highlights genomic variations underlying differences in pathogenicity. MBio 5https://doi.org/10.1128/mBio.00969-14. (e00969–14).

Buchanan, R.L., Gorris, L.G.M., Hayman, M.M., Jackson, T.C., Whiting, R.C., 2017. A review of *Listeria monocytogenes*: an update on outbreaks, virulence, dose-response, ecology, and risk assessments. Food Control 75, 1–13. https://doi.org/10.1016/j.foodcont.2016.12.016.

Cabanes, D., Dehoux, P., Dussurget, O., Frangeul, L., Cossart, P., 2002. Surface proteins and the pathogenic potential of *Listeria monocytogenes*. Trends Microbiol. 10, 238–245.

Camargo, A.C., Woodward, J.J., Nero, L.A., 2016. The continuous challenge of characterizing the foodborne pathogen *Listeria monocytogenes*. Foodborne Pathog. Dis. 13, 405–416. https://doi.org/10.1089/fpd.2015.2115.

Charlier, C., Perrodeau, É., Leclercq, A., Cazenave, B., Pilmis, B., Henry, B., Lopes, A., Maury, M.M., Moura, A., Goffinet, F., Dieye, H.B., Thouvenot, P., Ungeheuer, M.N., Tourdjman, M., Goulet, V., de Valk, H., Lortholary, O., Ravaud, P., Lecuit, M., MONALISA study group, 2017. Clinical features and prognostic factors of listeriosis: the MONALISA national prospective cohort study. Lancet Infect. Dis. 17, 510–519. https://doi.org/10.1016/S1473-3099(16)30521-7.

Comandatore, F., Corbella, M., Andreoli, G., Scaltriti, E., Aguzzi, M., Gaiarsa, S., Mariani, B., Morganti, M., Bandi, C., Fabbi, M., Marone, P., Pongolini, S., Sassera, D., 2017. Genomic characterization helps dissecting an outbreak of listeriosis in Northern Italy. PLoS Curr 9. https://doi.org/10.1371/currents.outbreaks.633fd8994e9f06f31b3494567c7e504c.

de Noordhout, C.M., Devleesschauwer, B., Angulo, F.J., Verbeke, G., Haagsma, J., Kirk, M., Havelaar, A., Speybroeck, N., 2014. The global burden of listeriosis: a systematic review and meta-analysis. Lancet Infect. Dis. 14, 1073–1082. https://doi.org/10.1016/S1473-3099(14)70870-9.

den Bakker, H.C., Didelot, X., Fortes, E.D., Nightingale, K.K., Wiedmann, M., 2008. Lineage specific recombination rates and microevolution in *Listeria monocytogenes*. BMC Evol. Biol. 8, 277. https://doi.org/10.1186/1471-2148-8-277.

den Bakker, H.C., Bundrant, B.N., Fortes, E.D., Orsi, R.H., Wiedmann, M., 2010. A population genetics-based and phylogenetic approach to understanding the evolution of virulence in the genus *Listeria*. Appl. Environ. Microbiol. 76, 6085–6100. https://doi.org/10.1128/AEM.00447-10.

Doijad, S., Weigel, M., Barbuddhe, S., Blom, J., Goesmann, A., Hain, T., Chakraborty, T., 2015. Phylogenomic grouping of *Listeria monocytogenes*. Can. J. Microbiol. 61, 637–646. https://doi.org/10.1139/cjm-2015-0281.

Doumith, M., Buchrieser, C., Glaser, P., Jacquet, C., Martin, P., 2004. Differentiation of the major *Listeria monocytogenes* serovars by multiplex PCR. J. Clin. Microbiol. 42, 3819–3822.

European Food Safety Authority and European Centre for Disease Prevention and Control (EFSA and ECDC), 2018. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017. EFSA J. 16, 5500. https://doi.org/10.2903/j.efsa.2018.5500.

Forghani, F., Wei, S., Oh, D.H., 2016. A rapid multiplex real-time PCR high-resolution melt curve assay for the simultaneous detection of *Bacillus cereus*, *Listeria monocytogenes*, and *Staphylococcus aureus* in food. J. Food Prot. 79, 810–815. https://doi.org/10.4315/0362-028X.JFP-15-428.

Hadjilouka, A., Paramithiotis, S., Drosinos, E.H., 2018. Genetic analysis of the Listeria pathogenicity island 1 of *Listeria monocytogenes* 1/2a and 4b isolates. Curr. Microbiol. 75, 857–865. https://doi.org/10.1007/s00284-018-1458-4.

Hasnain, S.E., O'Toole, R.F., Grover, S., Ehtesham, N.Z., 2015. Whole genome sequencing: a new paradigm in the surveillance and control of human tuberculosis. Tuberculosis (Edinb.) 95, 91–94. https://doi.org/10.1016/j.tube.2014.12.007.

Hunter, P.R., Gaston, M.A., 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J. Clin. Microbiol. 26, 2465–2466.

Jacquet, C., Doumith, M., Gordon, J.I., Martin, P.M., Cossart, P., Lecuit, M., 2004. A molecular marker for evaluating the pathogenic potential of foodborne *Listeria monocytogenes*. J. Infect. Dis. 189, 2094–2100. https://doi.org/10.1086/420853.

Jin, D., Luo, Y., Zhang, Z., Fang, W., Ye, J., Wu, F., Ding, G., 2012. Rapid molecular identification of *Listeria* species by use of real-time PCR and high-resolution melting analysis. FEMS Microbiol. Lett. 330, 72–80. https://doi.org/10.1111/j.1574-6968.2012.02535.x.

Kwong, J.C., Mercoulia, K., Tomita, T., Easton, M., Li, H.Y., Bulach, D.M., Stinear, T.P., Seemann, T., Howden, B.P., 2016. Prospective whole-genome sequencing enhances national surveillance of *Listeria monocytogenes*. J. Clin. Microbiol. 54, 333–342. https://doi.org/10.1128/JCM.02344-15.

Lomonaco, S., Nucera, D., Filipello, V., 2015. The evolution and epidemiology of *Listeria monocytogenes* in Europe and the United States. Infect. Genet. Evol. 35, 172–183. https://doi.org/10.1016/j.meegid.2015.08.008.

Lomonaco, S., Gallina, S., Filipello, V., Sanchez Leon, M., Kastanis, G.J., Allard, M., Brown, E., Amato, E., Pontello, M., Decastelli, L., 2018. Draft genome sequences of 510 *Listeria monocytogenes* strains from food isolates and human listeriosis cases from Northern Italy. Genome Announc. 6, e01276–e01317. https://doi.org/10.1128/genomeA.01276-17.

Maury, M.M., Chenal-Francisque, V., Bracq-Dieye, H., Han, L., Leclercq, A., Vales, G., Moura, A., Gouin, E., Scortti, M., Disson, O., Vázquez-Boland, J.A., Lecuit, M., 2017. Spontaneous loss of virulence in natural populations of *Listeria monocytogenes*. Infect. Immun. 85https://doi.org/10.1128/IAI.00541-17. pii: e00541-17.

Nyarko, E.B., Donnelly, C.W., 2015. *Listeria monocytogenes*: strain heterogeneity, methods, and challenges of subtyping. J. Food Sci. 80, M2868–M2878. https://doi.org/10.1111/1750-3841.13133.

Ohshima, C., Takahashi, H., Phraephaisarn, C., Vesaratchavest, M., Keeratipibul, S., Kuda, T., Kimura, B., 2014. Establishment of a simple and rapid identification method for *Listeria* spp. by using high-resolution melting analysis, and its application in food industry. PLoS One 9, e107342. https://doi.org/10.1371/journal.pone.0099223.

Ohshima, C., Takahashi, H., Iwakawa, A., Kuda, T., Kimura, B., 2017. A novel typing method for *Listeria monocytogenes* using high-resolution melting analysis (HRMA) of tandem repeat regions. Int. J. Food Microbiol. 253, 36–42. https://doi.org/10.1016/j.ijfoodmicro.2017.04.015.

Orsi, R.H., Maron, S.B., Nightingale, K.K., Jerome, M., Tabor, H., Wiedmann, M., 2008. Lineage specific recombination and positive selection in coding and intragenic regions contributed to evolution of the main *Listeria monocytogenes* virulence gene cluster. Infect. Genet. Evol. 8, 566–576. https://doi.org/10.1016/j.meegid.2008.04.006.

Orsi, R.H., den Bakker, H.C., Wiedmann, M., 2011. *Listeria monocytogenes* lineages: genomics, evolution, ecology, and phenotypic characteristics. Int. J. Med. Microbiol. 301, 79–96. https://doi.org/10.1016/j.ijmm.2010.05.002.

Pietzka, A.T., Stöger, A., Huhulescu, S., Allerberger, F., Ruppitsch, W., 2011. Gene scanning of an internalin B gene fragment using high-resolution melting curve analysis as a tool for rapid typing of *Listeria monocytogenes*. J. Mol. Diagn. 13, 57–63. https://doi.org/10.1016/j.jmoldx.2010.11.002.

Poimenidou, S.V., Dalmasso, M., Papadimitriou, K., Fox, E.M., Skandamis, P.N., Jordan, K., 2018. Virulence gene sequencing highlights similarities and differences in

sequences in *Listeria monocytogenes* serotype 1/2a and 4b strains of clinical and food origin from 3 different geographic locations. Front. Microbiol. 9, 1103. https://doi.org/10.3389/fmicb.2018.01103.

Ragon, M., Wirth, T., Hollandt, F., Lavenir, R., Lecuit, M., Le Monnier, A., Brisse, S., 2008. A new perspective on *Listeria monocytogenes* evolution. PLoS Pathog. 4, e1000146. https://doi.org/10.1371/journal.ppat.1000146.

Rawool, D.B., Doijad, S.P., Poharkar, K.V., Negi, M., Kale, S.B., Malik, S.V., Kurkure, N.V., Chakraborty, T., Barbuddhe, S.B., 2016. A multiplex PCR for detection of *Listeria monocytogenes* and its lineages. J. Microbiol. Methods 130, 144–147. https://doi.org/10.1016/j.mimet.2016.09.015.

Rousseaux, S., Olier, M., Lemaître, J.P., Piveteau, P., Guzzo, J., 2004. Use of PCR-restriction fragment length polymorphism of *inl*A for rapid screening of *Listeria monocytogenes* strains deficient in the ability to invade Caco-2 cells. Appl. Environ. Microbiol. 70, 2180–2185.

Sakaridis, I., Ganopoulos, I., Madesis, P., Tsaftaris, A., Argiriou, A., 2014. Genotyping of *Listeria monocytogenes* isolates from poultry carcasses using high resolution melting (HRM) analysis. Biotechnol. Biotechnol. Equip. 28, 107–111.

Sammarco, M.L., Ripabelli, G., Tamburro, M., 2014. Molecular epidemiology of infectious diseases: analytical methods and results interpretation. Ann. Ig. 26, 10–45. https://doi.org/10.7416/ai.2014.1956.

Stasiewicz, M.J., Oliver, H.F., Wiedmann, M., den Bakker, H.C., 2015. Whole-genome sequencing allows for improved identification of persistent *Listeria monocytogenes* in food-associated environments. Appl. Environ. Microbiol. 81, 6024–6037. https://doi.org/10.1128/AEM.01049-15.

Takahashi, H., Iwakawa, A., Ohshima, C., Kyoui, D., Kumano, S., Kuda, T., Kimura, B., 2017. A rapid typing method for *Listeria monocytogenes* based on high-throughput multilocus sequence typing (Hi-MLST). Int. J. Food Microbiol. 243, 84–89. https://doi.org/10.1016/j.ijfoodmicro.2016.12.009.

Tamburro, M., Ripabelli, G., 2017. High resolution melting as a rapid, reliable, accurate and cost-effective emerging tool for genotyping pathogenic bacteria and enhancing molecular epidemiological surveillance: a comprehensive review of the literature. Ann. Ig. 29, 293–316. https://doi.org/10.7416/ai.2017.2153.

Tamburro, M., Ripabelli, G., Fanelli, I., Grasso, G.M., Sammarco, M.L., 2010. Typing of *Listeria monocytogenes* strains isolated in Italy by *inl*A gene characterization and evaluation of a new cost-effective approach to antisera selection for serotyping. J. Appl. Microbiol. 108, 1602–1611. https://doi.org/10.1111/j.1365-2672.2009.04555.x.

Tamburro, M., Ripabelli, G., Vitullo, M., Dallman, T.J., Pontello, M., Amar, C.F.L., Sammarco, M.L., 2015a. Gene expression in *Listeria monocytogenes* exposed to sublethal concentration of benzalkonium chloride. Comp. Immunol. Microbiol. Infect. Dis. 40, 31–39. https://doi.org/10.1016/j.cimid.2015.03.004.

Tamburro, M., Sammarco, M.L., Ammendolia, M.G., Fanelli, I., Minelli, F., Ripabelli, G., 2015b. Evaluation of transcription levels of *inl*A, *inl*B, *hly*, *bsh* and *prf*A genes in *Listeria monocytogenes* strains using quantitative reverse-transcription PCR and ability of invasion into human CaCo-2 cells. FEMS Microbiol. Lett. 362, fnv018. https://doi.org/10.1093/femsle/fnv018.

Tamburro, M., Sammarco, M.L., Ripabelli, G., 2018. High resolution melting analysis for the characterization of lineage II *Listeria monocytogenes* serovars 1/2a and 1/2c based on single nucleotide polymorphisms identification within the *Listeria* Pathogenicity Island-1 and *inl*AB operon: a novel approach for epidemiological surveillance. J. Appl. Microbiol. https://doi.org/10.1111/jam.14100. (Epub ahead of print).

Tong, S.Y., Giffard, P.M., 2012. Microbiological applications of high-resolution melting analysis. J. Clin. Microbiol. 50, 3418–3421. https://doi.org/10.1128/JCM.01709-12.

Tsai, Y.H., Orsi, R.H., Nightingale, K.K., Wiedmann, M., 2006. *Listeria monocytogenes* internalins are highly diverse and evolved by recombination and positive selection. Infect. Genet. Evol. 6, 378–389.

Wang, J., Yamada, S., Ohashi, E., 2010. Rapid identification of *Listeria* species and screening for variants by melting curve and high-resolution melting curve analyses of the intergenic spacer region of the rRNA gene. Can. J. Microbiol. 56, 676–682. https://doi.org/10.1139/w10-054.

Ward, T.J., Gorski, L., Borucki, M.K., Mandrell, R.E., Hutchins, J., Pupedis, K., 2004. Intraspecific phylogeny and lineage group identification based on the prfA virulence gene cluster of *Listeria monocytogenes*. J. Bacteriol. 186, 4994–5002. https://doi.org/10.1128/JB.186.15.4994-5002.2004.

Ward, T.J., Ducey, T.F., Usgaard, T., Dunn, K.A., Bielawski, J.P., 2008. Multilocus genotyping assays for single nucleotide polymorphism-based subtyping of *Listeria monocytogenes* isolates. Appl. Environ. Microbiol. 74, 7629–7642. https://doi.org/10.1128/AEM.01127-08.

Yin, Y., Tan, W., Wang, G., Kong, S., Zhou, X., Zhao, D., Jia, Y., Pan, Z., Jiao, X., 2015. Geographical and longitudinal analysis of *Listeria monocytogenes* genetic diversity reveals its correlation with virulence and unique evolution. Microbiol. Res. 175, 84–92. https://doi.org/10.1016/j.micres.2015.04.002.

Zhang, C., Zhang, M., Ju, J., Nietfeldt, J., Wise, J., Terry, P.M., Olson, M., Kachman, S.D., Wiedmann, M., Samadpour, M., Benson, A.K., 2003. Genome diversification in phylogenetic lineages I and II of *Listeria monocytogenes*: identification of segments unique to lineage II populations. J. Bacteriol. 185, 5573–5584.

Zolin, A., Amato, E., D'Auria, M., Gori, M., Huedo, P., Bossi, A., Pontello, M., 2017. Estimating the real incidence of invasive listeriosis through an integrated surveillance model in use in Lombardy (Italy, 2006–2014). Epidemiol. Infect. 145, 2072–2080. https://doi.org/10.1017/S0950268817000711.