Genotyping of *Listeria monocytogenes* isolates by high-resolution melting curve (HRM) analysis of tandem repeat locus

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Short Report

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Abstract

Listeria monocytogenes is responsible for causing listeriosis, a type of food poisoning with high mortality. This bacterium is mainly transmitted to humans through the consumption of contaminated foods. Detection of *L. monocytogenes* through molecular methods is crucial for food safety and clinical diagnosis. Present techniques are characterized by low discrimination power and high cost, as well as being time-consuming and taking several days to give the final result. In our study, MLVA-HRM was investigated as an alternative method for a fast and precise method for the genotyping of *L. monocytogenes* isolates. Forty-eight isolates of *L. monocytogenes* obtained from the microbial bank of Department of Microbiology, Iran University of Medical Sciences, were typed by MLVA-HRM analysis using five VNTR loci. A total of 43 different types were obtained. This research demonstrated the usefulness of the MLVA-HRMA method and its ability to discriminate *L. monocytogenes* isolates. Since this method is easier and more efficient than existing methods, it can be widely used in food processing plants and diagnostic laboratories as a fast and accurate method.

Highlights

- *Listeria monocytogenes* is responsible for causing listeriosis, a type of food poisoning.
- Present techniques are characterized by low discrimination power and high cost, as well as being time-consuming.
- MLVA-HRM was investigated as an alternative method for a fast and precise method for the genotyping of *monocytogenes* isolates.

Introduction

Listeria monocytogenes is a Gram-positive, motile, non-spore forming, and food-borne pathogen that can cause listeriosis in high-risk individuals, including infants, the elderly, and immunocompromised patients [1, 2]. *L. monocytogenes* is widely distributed in the environment and has the ability to survive and grow in harsh conditions, such as low temperatures and high saline levels [3]. *L. monocytogenes* is an environmental organism that typically infects the food processing industry and it is estimated that 99% of human listeriosis infections are caused by the consumption of contaminated foods [4]. Listeriosis is linked with a high rate of hospitalization (85–90%) and the mortality rate for this disorder is 20-30%, which is higher than infections caused by other food poisoning pathogens [5, 6].

Thus, in recent decades, *L. monocytogenes* has become a major concern in the food industry and public health, and the identification of *L. monocytogenes* species for food safety, epidemiological studies, and clinical diagnosis is very critical [7].

There are several methods for the classification of bacterial isolates, such as multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), and restriction enzyme analysis [8, 9].

Although each of these methods has its advantage in isolating different bacterial species, the typing technique should be simple and easy. PFGE is the current gold standard for typing of *L. monocytogenes* isolates, however, it is time-consuming and hard to standardize, which prevents the exchange of typing results between laboratories [10]. The MLVA method requires capillary electrophoresis and the MLST method requires a large sequence analysis [11, 12]. Therefore, considering the importance of typing food-related bacteria, it is very crucial to use a fast, cheap, and easy method to differentiate the strains [13].

High-resolution melting (HRM) is a quantitative PCR (QPCR)-based method developed to detect changes in nucleic acid sequences. This method monitors changes in DNA sequences according to the changes of melting temperatures of real-time PCR products [14]. Through our research, five variable numbers of tandem repeat (VNTR) loci were selected for the genotyping of *L. monocytogenes* isolates. VNTR is a region in DNA where a short nucleotide sequence called tandem repeats (TRs) with various numbers are located in different strains of bacteria [15, 16]. Today, this difference in the number of VNTR is used as a suitable target for assessing bacterial genotyping [17]. In this study, we used the HRM method, which is a simple and fast method for the analysis of VNTR and genotyping of *L. monocytogenes* strains.

Materials And Methods

Bacterial Strains and Growth Conditions

In this study, *L. monocytogenes* ATCC 19115 and 47 clinical *L. monocytogenes* strains from the Microbial bank of Department of Microbiology affiliated to Iran University of Medical Sciences, Tehran, Iran, were used (Table 1) [18]. Isolates were cultured on Brain Heart Infusion (BHI) agar (Merck, Darmstadt, Germany) at 37°C for 48 h. DNA of the samples was extracted with a DNA Extraction kit (Roche, Germany) according to the manufacturer's protocol. DNA purity and concentration were assessed using a NanoDrop Spectrophotometer (Thermo Scientific, USA).

MLVA-HRM

In this study, we used 5 VNTR loci for MLVA-HRMA based on the previously designed primers [19]. The PCR amplification and HRMA were performed using a Rotor-Gene thermal cycler (Corbett Life Sciences, Sydney, Australia). The PCR reaction solution was performed with a total of 20 µL, containing 1 µL of each forward and reverse primers, 1 µL of template DNA (0.5 µg), 4 µl of 5× Hot Firepol Eva Green HRM Mix (Solis BioDyne), and 13 µL of sterile distilled water.

The thermal program was performed by an initial denaturation at 95 °C for 5 min followed by 40 cycles of 95 °C for 30 s, 54 °C for 22 s, and 72 °C for 40 s for VNTR locus 1 (Lm_10), 95 °C for 30 s, 52 °C for 22 s, and 72 °C for 40 s for VNTR locus 2 (Lm_11), 95 °C for 30 s, 55.5 °C for 30 s, and 72 °C for 30 s for VNTR locus 3 (Lm_23), 95 °C for 30 s, 54 °C for 30 s, 72 °C for 45 s for VNTR locus 4 (Lm_32) and 95 °C for 30 s, 57 °C for 22 s, and 72 °C for 40 s for VNTR locus 5 (LM_TR6).

After PCR amplification, the HRM step was performed. For HRM step the fluorescence was measured by increasing the temperature from 75° to 95°C with a rate of 0.1 °C/s. In case of nucleotide changes in different strains, various curves are produced.

The melting curve and different plots of strain 6 were used as the baseline control. Each strain with equal waveform was grouped. Then, two strains were randomly selected from each group and the PCR reaction was performed for the studied genes using a DNA thermal cycler (PeqLab, Germany) with the following profile: initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 22 s, and extension at 72°C for 40 s, with a final extension step at 72°C for 5 min. The PCR product was sent for sequencing to Takapozist Company, Iran (on behalf of Bioneer Company, Korea). The allele number was determined and imputed for each locus into BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium) to draw the UPGMA dendrogram and the minimum spanning tree for all the studied *L. monocytogenes.*

Results

MLVA-HRM

For the 48 *L. monocytogenes* strains, we performed HRM on 5 VNTR loci to determine the STs. The difference plot and melting curves of 48 *L. monocytogenes* strains for 5 VNTR regions were drawn (Figure 1, Figure 2). According to similar peak waveforms for 48 *L. monocytogenes* strains, the Lm_10, Lm_11, Lm_23, Lm_32, and LM_TR6 loci were divided into 6, 3, 5, 7, and 2 types, respectively. After sequencing and calculating the number of alleles and imputing each locus into BioNumerics and combining the allele numbers of the 5 loci, UPGMA dendrogram and the minimum spanning tree for 48 strains of L. monocytogenes were drawn and 48 strains of L. monocytogenes were finally divided into 43 HRM groups (Figure 3, Figure 4).

Discussion

Molecular typing of *L. monocytogenes* isolates plays an important role in identifying the source of infection and preventing the spread of this pathogen [20]. Currently, various methods are used for molecular typing of *L. monocytogenes* from various sources, including PFGE, MLST, and more recently WGS, which requires a lot of work and cost [21, 22]. MLVA (multilocus variable-number tandem-repeat analysis) is another molecular typing method that comes with practical benefits such as speed and ease of use. This method uses capillary electrophoresis for diagnosis [23]. VNTR loci in the bacterial genome often mutate, leading to changes in the number of tandem repeats and successive changes in nucleic acid. Changes in the number of VNTR repeats are used by the MLVA method for the molecular typing of different isolates [13, 24]. To evaluate the appropriateness and the suitability of the MLVA method for routine monitoring and subtyping of *L. monocytogenes* isolates from meat products, Belén Martín and colleagues collected 113 isolates of *L. monocytogenes* from meat [25]. Their study showed that MLVA is a reliable method for *L. monocytogenes* typing with a higher discriminating power than MLST, especially

for serotype 1/2c isolates [25]. Lindstad et al. compared MLVA with the PFGE method to evaluate the resolution power of the MLVA method for molecular typing of *L. monocytogenes* isolates and their results showed that the MLVA method was slightly more discriminatory for Norwegian isolates (28 MLVA profiles and 24 PFGE profiles) [26].

In HRM, changes in nucleotide sequences and diversity in the chain length of PCR products are indicated by changes in the melting curve [27]. In a study by Ohshima et al., analysis of MLVA was performed using the HRM method as a simple and rapid method for differentiating *L. monocytogenes* isolates. The study also compared the ability of MLVA-HRMA, MLVA using capillary electrophoresis, and multilocus sequence typing (MLST) to differentiate between strains. This study demonstrated that the MLVA-HRM method was more discriminant than MLST and MLVA using capillary electrophoresis [28]. In a study by Catharina H. et al., for phylogenetic analysis, MLST and MLVA were performed on 58 Vibrio parahaemolyticus isolates, and the results gained by both methods were compared with the PFGE patterns obtained in their previous study. Their results showed that HRM-MLVA was able to separate isolates in the same PFGE cluster with the same ST [29].

In this research, we used the MLVA-HRM method with 5 VNTR loci as a simple and fast typing method that was able to differentiate *L. monocytogenes* strains. The MLVA-HRM method was able to discriminate *L. monocytogenes* strains, which we believe could be a valuable alternative to costly and time-consuming techniques.

Declarations

Acknowledgments:

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Conflict of Interests

The authors declare that they have no competing interests.

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Table 1

Table 1. Serotypes and sources of *Listeria monocytogenes* strains.

strain	serotype	source
1	1/2c	Goat
2	1/2c	Placental bit
3	1/2c	Cheese
4	1/2c	Cheese
5	1/2c	Placental bit
6	4b	ATCC 19115
7	1/2c,3c	Neonatal blood
8	1/2c	Rectal swab
9	1/2c	Ice cream
10	1/2c	Cheese
11	4b	Vaginal swab
12	1/2a	Sheep
13	1/2c	Vaginal swab
14	1/2c	Sheep
15	1/2c	Cattle
16	1/2c	Sheep
17	1/2c	Rectal swab
18	1/2c	Goat
19	1/2c	Placental bit
20	4b	Cattle
21	1/2c,3c	Neonatal blood
22	1/2c	Sheep
23	1/2c,3c	Milk
24	1/2b,3b	Tilapia fillet
25	1/2c,3c	Neonatal blood
26	1/2c	Placental bit
27	1/2c	Cattle
28	1/2a	Vaginal swab
29	1/2c	Stool
30	1/2c	Stool
31	1/2c	Stool
32	1/2c	Stool
33	1/2c	Stool
34	4e	Stool
35	1/2c	Stool
36	4b,4d	Placental bit
37	1/2a, 3a	Vaginal swab
38	1/2b,3b	Vaginal swab
39	1/2a, 3a	Vaginal swab
40	1/2a,3a	Vaginal swab
41	1/2c,3c	Placental bit
42	1/2a, 3a	Placental bit
43	1/2a, 3a	Caspian tyulka
44	1/2c,3c	Vaginal swab
45	1/2c,3c	Caspian tyulka
46	1/2c,3c	Blood
47	1/2c,3c	Neonatal blood
48	1/2a, 3a	Rainbow trout

Figures

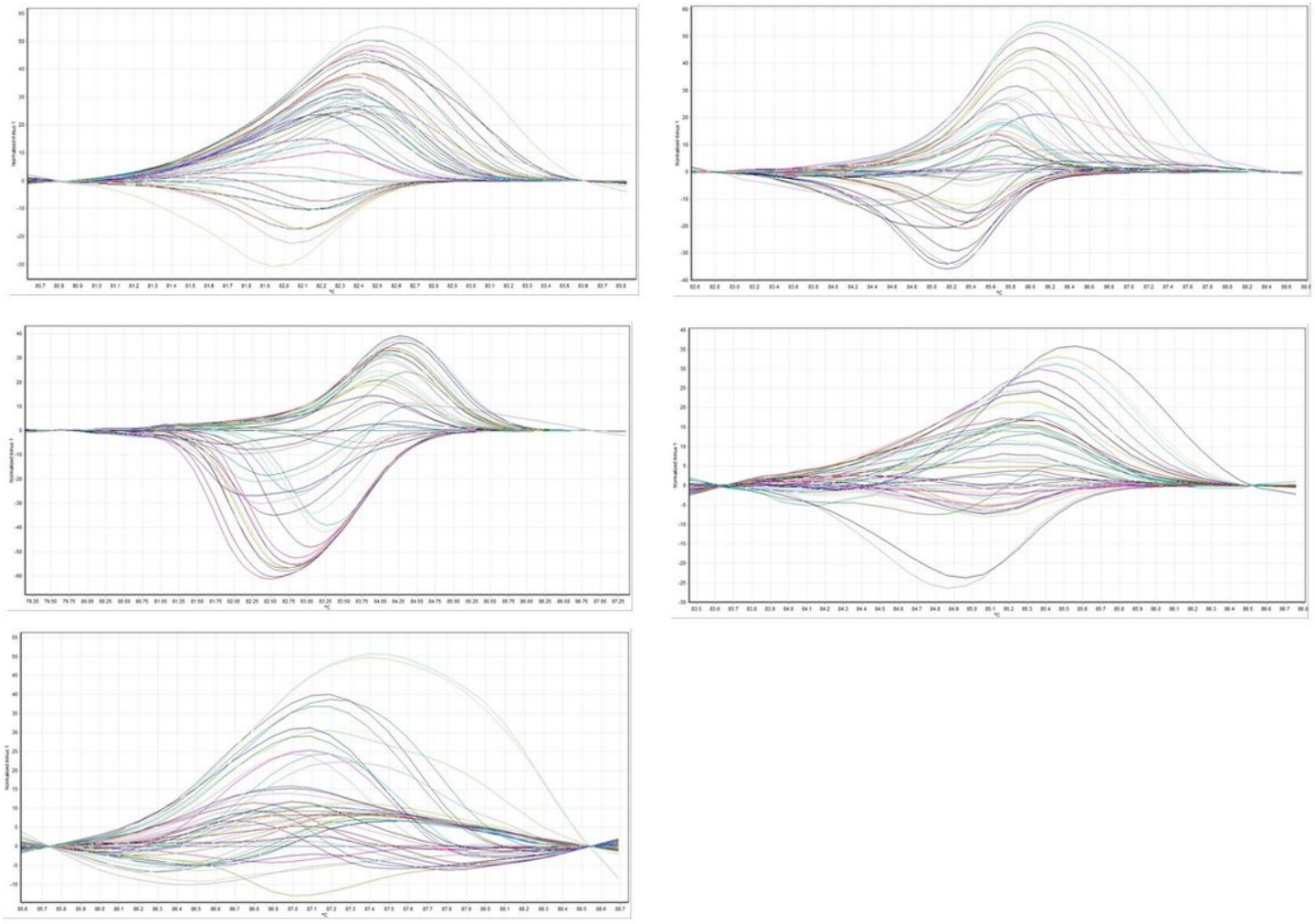

Figure 1

Different plots of 48 Listeria monocytogenes strains for 5 VNTR regions.

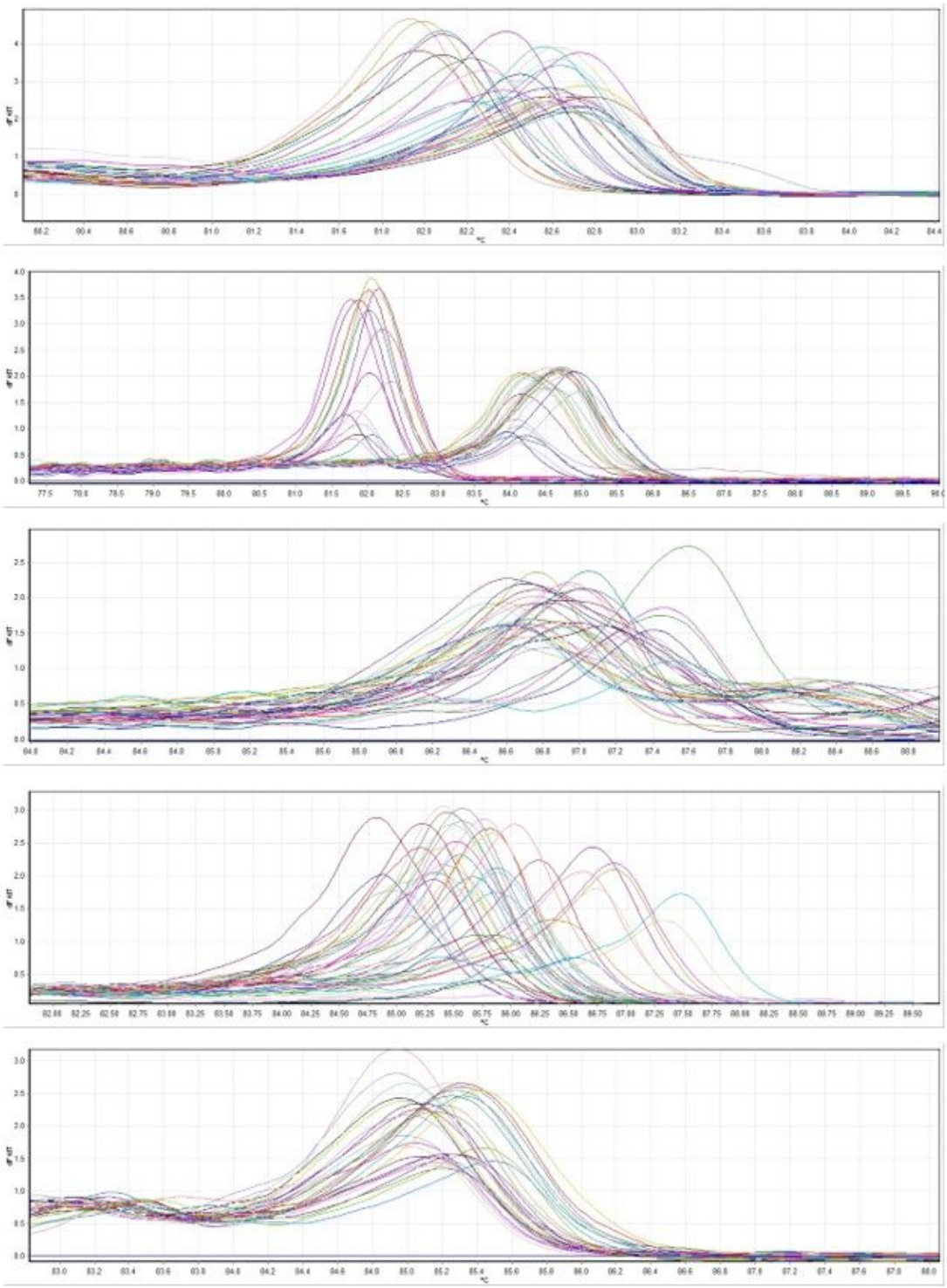

Figure 2

Melting curves of 48 Listeria monocytogenes strains for 5 VNTR regions.

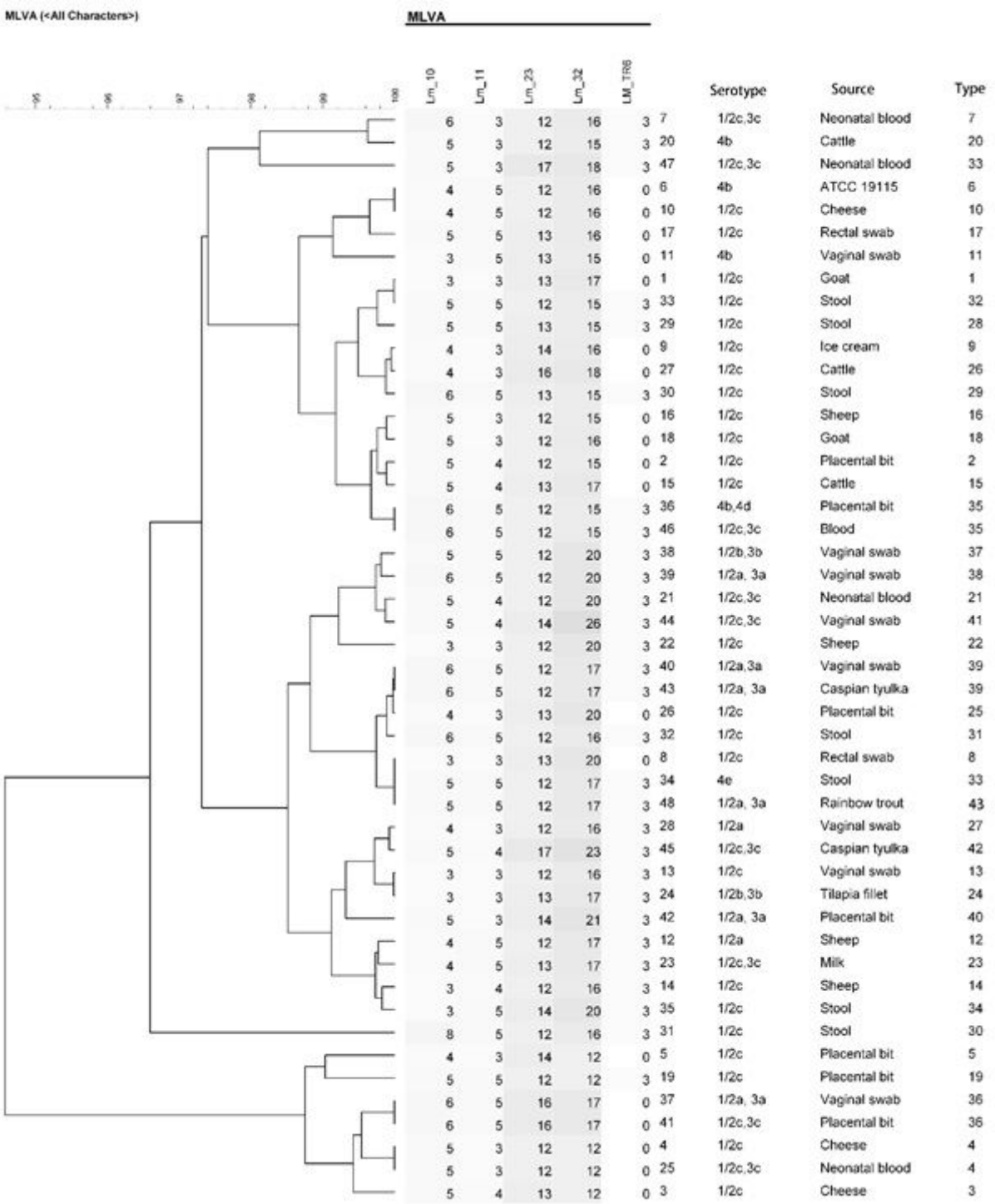

Figure 3

UPGMA dendrogram of MLVA-HRM results for the 48 L. monocytogenes isolates.

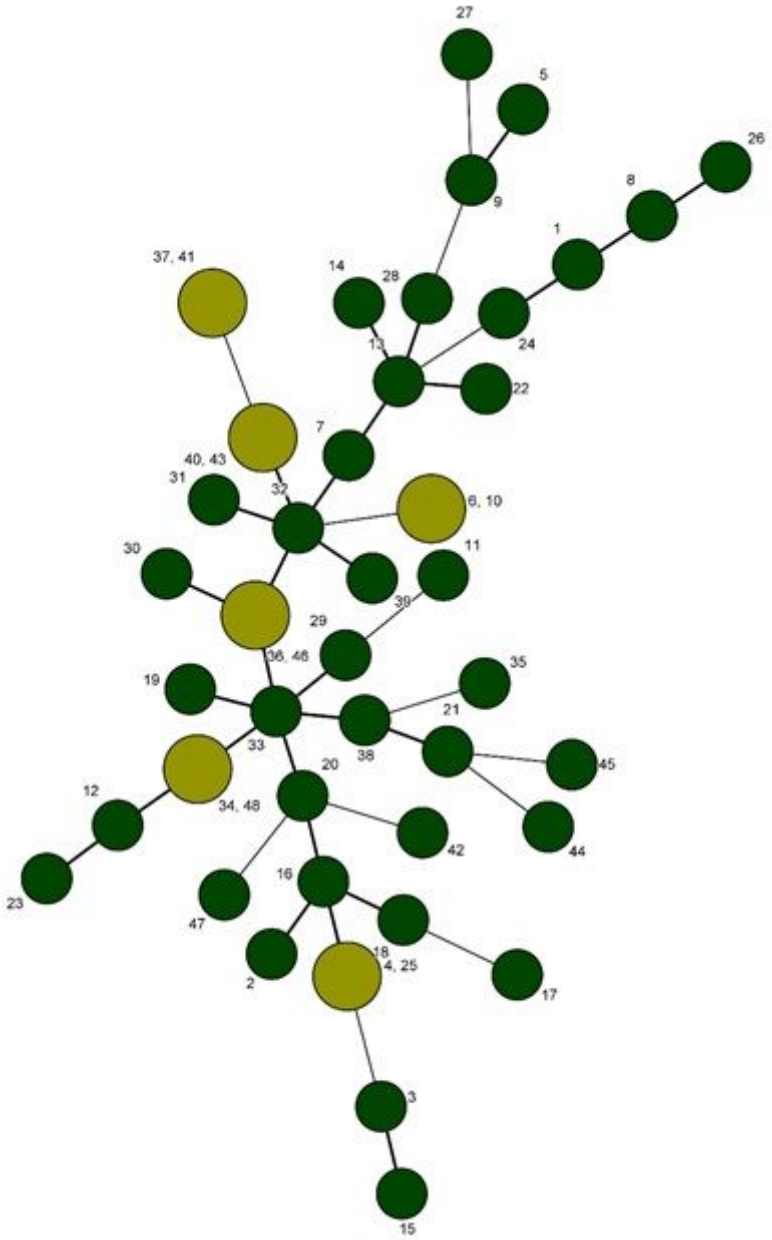

Figure 4

Minimum spanning tree of the 48 L. monocytogenes isolates typed by MLVA-HRM.