Original Article

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

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ARTICLE INFO

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 (Multiple-Locus Variable-number tandem repeats Analysis – High-Resolution Melting) 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 Variable Numbers of Tandem Repeat (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.

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Introduction

Listeria monocytogenes is a Gram-positive, motile, non-spore forming, and food-borne pathogeindustry, an 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 concentrations.[3] *L. monocytogenes* is an environmental organism that typically infects food in the 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 became 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 Multiple-Locus Variable-number tandem repeats Analysis (MLVA) method requires electrophoresis of PCR products 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 48h. 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).

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

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

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 30s, 54°C for 22s, and 72°C for 40s for VNTR locus 1 (Lm_10), 95°C
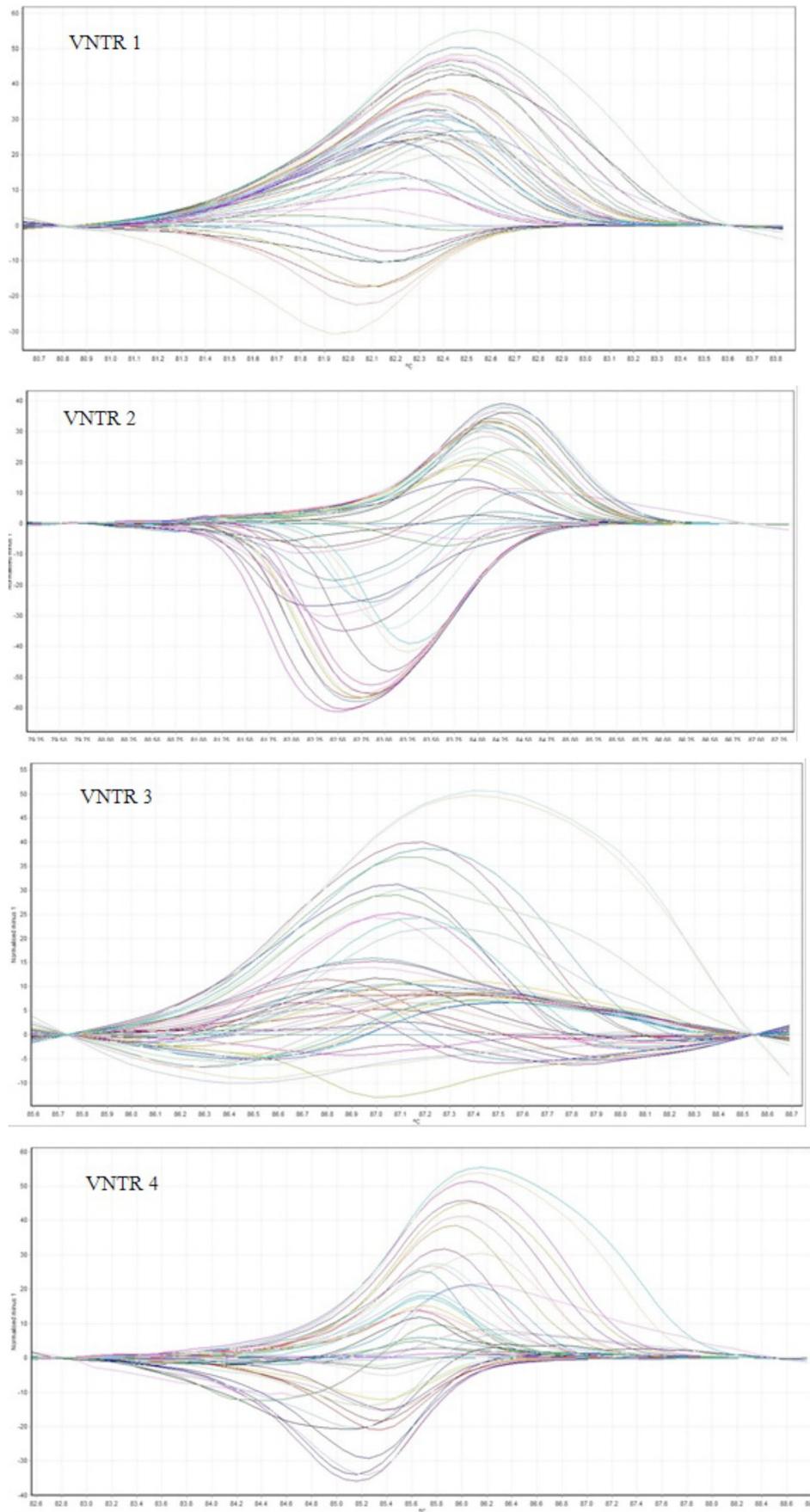
Fig. 1 – Different plots of 48 *Listeria monocytogenes* strains for 5 VNTR regions.

for 30s, 52°C for 22s, and 72°C for 40s for VNTR locus 2 (Lm_11), 95°C for 30s, 55.5°C for 30s, and 72°C for 30s for VNTR locus 3 (Lm_23), 95°C for 30s, 54°C for 30s, 72°C for 45s for VNTR locus 4 (Lm_32) and 95°C for 30s, 57°C for 22s, and 72°C for 40s 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 were 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 30s, annealing at 52°C for 22s, and extension at 72°C for 40s, 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
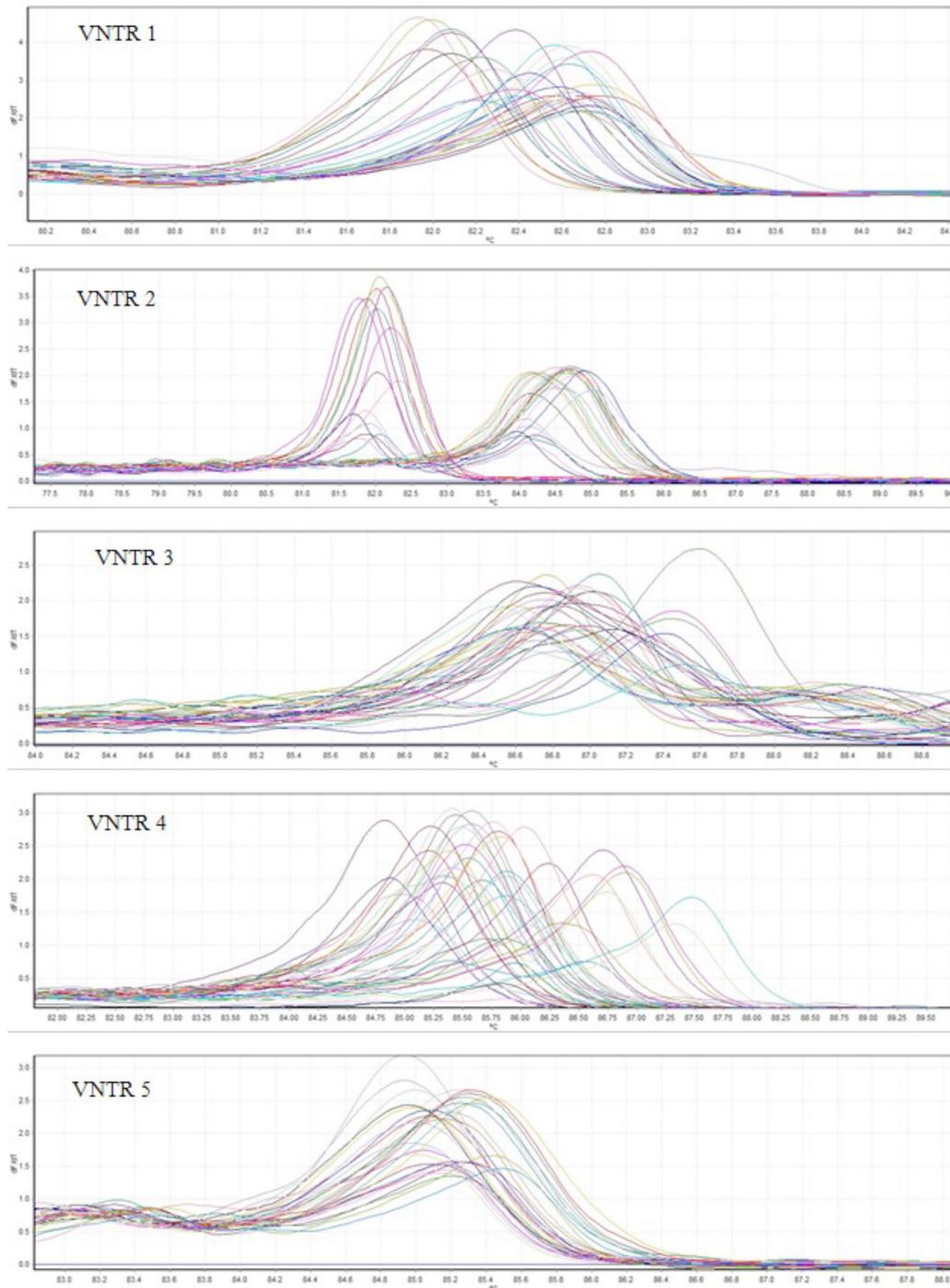

Fig. 2 – Melting curves of 48 *Listeria monocytogenes* strains for 5 VNTR region.

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 different plot and melting curves of 48 *L. monocytogenes* strains for 5 VNTR regions were drawn (Figs. 1 and 2). HRM was performed in three replications, which gave very similar melting curves, thus confirming the reproducibility of the data. 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 genotypes. Of these, 38 were single genotypes and each of the 33, 35, 36, 39 and 4 genotypes were determined in 2 isolates. While the genotype 33 in strain originated from vaginal swab and neonatal blood, genotype 4 in strain originated from cheese and neonatal blood, genotype 36 in strain
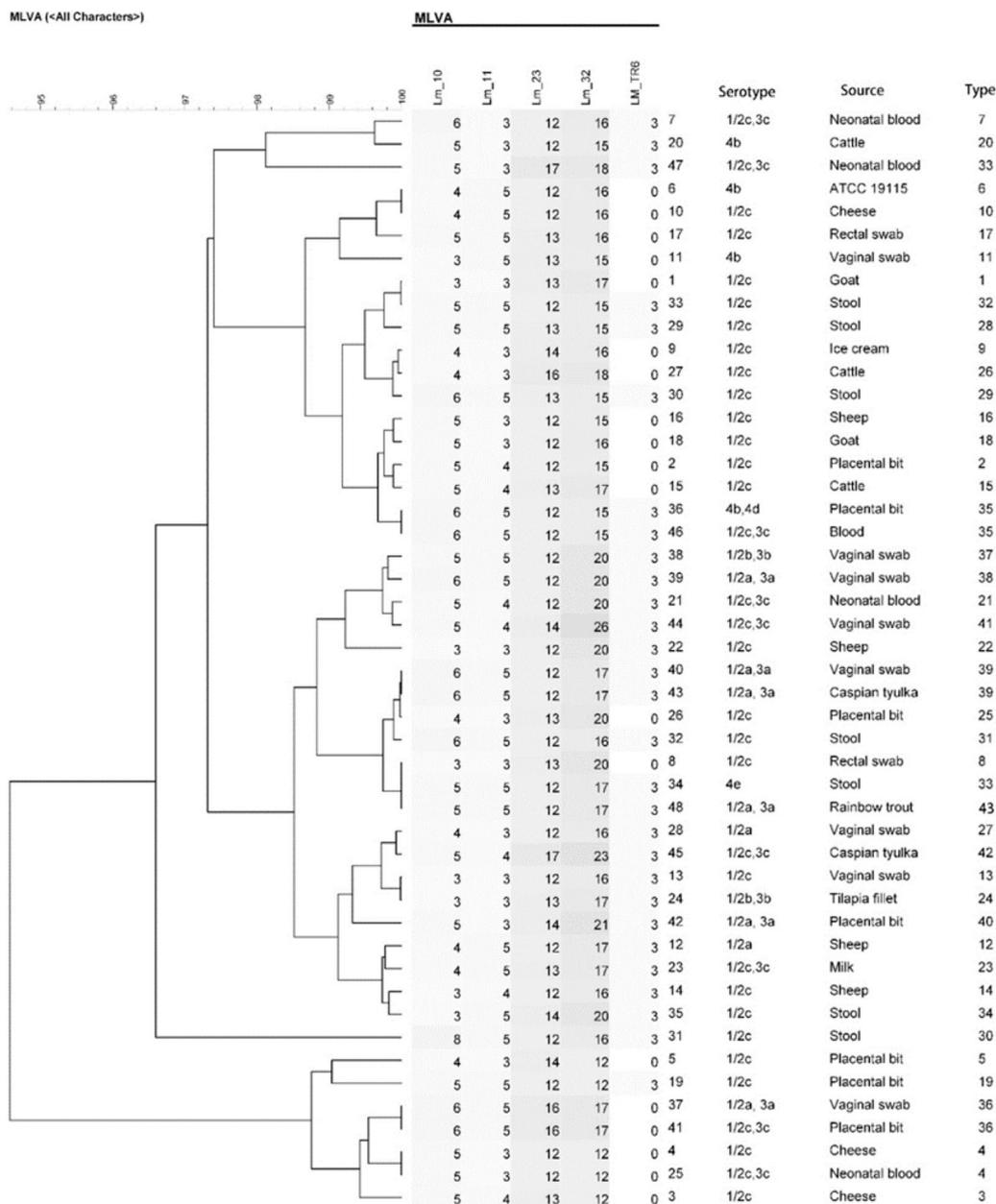

Fig. 3 – UPGMA dendrogram of MLVA-HRM results for the 48 *L. monocytogenes* isolates. The columns represent the serotype, source, and bacterial type.

from vaginal swab and placental bit, genotype 35 in strain from placental bit and blood and genotype 39 in strain originated from Caspian tyulka and vaginal swab were observed (Figs. 3 and 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 Whole Genome Sequencing (WGS), which requires a lot of work and cost.[21,22] MLVA is another molecular typing method that comes with practical benefits such as speed and ease of use. This method uses 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 et al. 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 discriminant 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 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 Lüdeke 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 study, MLVA-HRM method with five VNTR loci used as a simple and fast typing method was able to differentiate *L. monocytogenes* strains, which we believe can be a valuable alternative to costly and time-consuming techniques. Also, due to the characteristics of this method, it can be used in routine bacterial diagnoses.
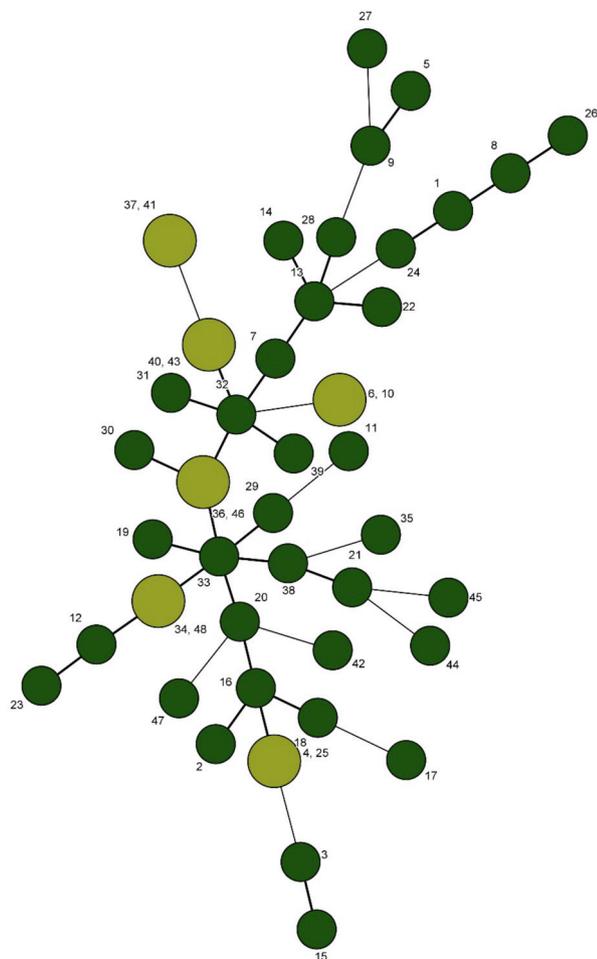
Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

Fig. 4 – Minimum spanning tree of the 48 *L. monocytogenes* isolates typed by MLVA-HRM.

REFERENCES

1. Rizzi V, Felicio TS, Felix B, et al. The ECDC-EFSA molecular typing database for European Union public health protection. Euroreference. 2017;2:4–12.
2. Kalani BS, Irajian G, Lotfollahi L, et al. Putative type II toxin-antitoxin systems in Listeria monocytogenes isolated from clinical, food, and animal samples in Iran. Microb Pathog. 2018;122:19–24.
3. Narimisa N, Kalani BS, Mohammadzadeh R, et al. Combination of antibiotics – nisin reduces the formation of persister cell in listeria monocytogenes. Microb Drug Resist. 2021;27:137–44.
4. Zhu Q, Gooneratne R, Hussain MA. Listeria monocytogenes in fresh produce: outbreaks, prevalence and contamination levels. Foods. 2017;6:21.

5. Li H, Wang P, Lan R, et al. Risk factors and level of Listeria monocytogenes contamination of raw pork in retail markets in China. Front Microbiol. 2018;9:1090.

6. Kalani BS, Najafi M, Mohammadzadeh R, et al. Targeting Listeria monocytogenes consensus sequence of internalin genes using an antisense molecule. Microb Pathog. 2019;136:103689.

7. Fu J, Li L, Huo D, et al. Meningitic escherichia coli α-hemolysin facilitates blood-brain barrier disruption via targeting Tgfβ1-induced hedgehog signaling. Mol Brain. 2021;14:116.

8. Chen Y, Gonzalez-Escalona N, Hammack TS, et al. Core genome multilocus sequence typing for identification of globally distributed clonal groups and differentiation of outbreak strains of Listeria monocytogenes. Appl Environ Microb. 2016;82:6258–72.

9. Rip D, Gouws PA. PCR-restriction fragment length polymorphism and pulsed-field gel electrophoresis characterization of listeria monocytogenes isolates from ready-to-eat foods, the food processing environment, and clinical samples in South Africa. J Food Prot. 2020;83:518–33.

10. Camargo AC, Woodward JJ, Nero LA. The continuous challenge of characterizing the foodborne pathogen Listeria monocytogenes. Foodborne Pathog Dis. 2016;13:405–16.

11. Lian DS, Zeng HS. Capillary electrophoresis based on nucleic acid detection as used in food analysis. Compr Rev Food Sci Food Saf. 2017;16:1281–95.

12. Yachison CA, Yoshida C, Robertson J, et al. The validation and implications of using whole genome sequencing as a replacement for traditional serotyping for a national Salmonella reference laboratory. Front Microbiol. 2017;8:1044.

13. Martin B, Bover-Cid S, Aymerich T. MLVA subtyping of Listeria monocytogenes isolates from meat products and meat processing plants. Food Res Int. 2018;106:225–32.

14. Tamburro M, Sammarco ML, Fanelli I, Ripabelli G. Characterization of Listeria monocytogenes serovar 1/2a, 1/2b, 1/2c and 4b by high resolution melting analysis for epidemiological investigations. Int J Food Microbiol. 2019;310:108289.

15. Bakhtiari M, Shleizer-Burko S, Gymrek M, Bansal V, Bafna V. Targeted genotyping of variable number tandem repeats with adVNTR. Genome Res. 2018;28:1709–19.

16. Khodaei N, Kalani BS, Zamani M, et al. Evaluation of the genetic relatedness of Bacteroides fragilis isolates by TRs analysis. Iran J Basic Med Sci. 2020;23:1323–7.

17. Vergnaud G, Hauck Y, Christiany D, et al. Genotypic expansion within the population structure of classical Brucella species revealed by MLVA16 typing of 1404 Brucella isolates from different animal and geographic origins, 1974–2006. Front Microbiol. 2018;9:1545.

18. Zamani M, Kalani BS, Khodaei N, Lotfollahi L, Jazi FM. Corrigendum to â Prevalence of Premature Stop Codons (PMSCs) in Listeria monocytogenes isolated from clinical and food samples in Iranâ. Gene Rep. 2019;19:100451.

19. Kalani BS, Pournajaf A, Sedighi M, Bahador A, Irajian G, Valian F. Genotypic characterization, invasion index and antimicrobial resistance pattern in Listeria monocytogenes strains isolated from clinical samples. J Acute Dis. 2015;4:141–6.

20. Radoshevich L, Cossart P. Listeria monocytogenes: towards a complete picture of its physiology and pathogenesis. Nat Rev Microbiol. 2018;16:32–46.

21. Londero A, Costa M, Sucari A, Leotta G. Comparison of three molecular subtyping techniques for Listeria monocytogenes. Rev Argent Microbiol. 2019;51:359–62.

22. Stessl B., Wagner M., Ruppitsch W. Multilocus Sequence Typing (MLST) and Whole Genome Sequencing (WGS) of Listeria monocytogenes and Listeria innocua, in Listeria Monocytogenes. Springer. p. 89-103.

23. Saleh-Lakha S, Allen VG, Li J, et al. Subtyping of a large collection of historical Listeria monocytogenes strains from Ontario, Canada, by an improved multilocus variable-number tandem-repeat analysis (MLVA). Appl Environ Microbiol. 2013;79:6472–80.

24. Lu CY, Yen TY, Chang LY, et al. Multiple-locus variable-number tandem-repeat analysis (MLVA) of macrolide-susceptible and -resistant Mycoplasma pneumoniae in children in Taiwan. J Formos Med Assoc. 2020;119:1539–45.

25. Martin B, Bover-Cid S, Aymerich T. MLVA subtyping of Listeria monocytogenes isolates from meat products and meat processing plants. Food Res Int. 2018;106:225–32.

26. Lindstedt BA, Tham W, Danielsson-Tham ML, Vardund T, Helmersson S, Kapperud G. Multiple-locus variable-number tandem-repeats analysis of Listeria monocytogenes using multicolour capillary electrophoresis and comparison with pulsed-field gel electrophoresis typing. J Microbiol Methods. 2008;72:141–8.

27. Ashrafi R, Bruneaux M, Sundberg LR, Pulkkinen K, Ketola T. Application of high resolution melting assay (HRM) to study temperature-dependent intraspecific competition in a pathogenic bacterium. Sci Rep. 2017;7:1–8.

28. Ohshima C, Takahashi H, Iwakawa A, et al. A novel typing method for Listeria monocytogenes using high-resolution melting analysis (HRMA) of tandem repeat regions. Int J Food Microbiol. 2017;253:36–42.

29. Lüdeke CH, Gonzalez-Escalona N, Fischer M, Jones JL. Examination of clinical and environmental Vibrio parahaemolyticus isolates by multi-locus sequence typing (MLST) and multiple-locus variable-number tandem-repeat analysis (MLVA). Front Microbiol. 2015;6:564.