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

Negar Narimisa (n.narimisa72@gmail.com)
Iran University of Medical Sciences

Fatemeh Amraei
Iran University of Medical Sciences

Mohammad Sholeh
Iran University of Medical Sciences

Shiva Mirkalantari
Iran University of Medical Sciences

Shabnam Razavi
Iran University of Medical Sciences

Lida Lotfollahi
Urmia University of Medical Sciences: Urmia University of Medical Sciences

Behrooz Sadeghi Kalani
Ilam University of Medical Sciences

Faramarz Masjedian Jazi
Iran University of Medical Sciences

Short Report

Keywords: Listeria monocytogenes, MLVA-HRMA, VNTR locus, Molecular typing

DOI: https://doi.org/10.21203/rs.3.rs-183849/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
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 \textit{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 \textit{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 \textit{L. monocytogenes} strains.

**Materials And Methods**

**Bacterial Strains and Growth Conditions**

In this study, \textit{L. monocytogenes} ATCC 19115 and 47 clinical \textit{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:

This study was financially supported by Iran University of Medical Sciences (Tehran, Iran), for which we
are very grateful.

Conflict of Interests

The authors declare that they have no competing interests.

References

1. Rizzi, V., et al., The ECDC-EFSA molecular typing database for European Union public health
protection. Euroreference, 2017. 2: p. 4-12.

2. Kalani, B.S., et al., Putative type II toxin-antitoxin systems in Listeria monocytogenes isolated from
clinical, food, and animal samples in Iran. Microbial pathogenesis, 2018. 122: p. 19-24.

3. Narimisa, N., et al., Combination of Antibiotics—Nisin Reduces the Formation of Persister Cell in
Listeria monocytogenes. Microbial Drug Resistance, 2020.

4. Zhu, Q., R. Gooneratne, and M.A. Hussain, Listeria monocytogenes in fresh produce: outbreaks,
prevalence and contamination levels. Foods, 2017. 6(3): p. 21.
5. Li, H., et al., *Risk factors and level of Listeria monocytogenes contamination of raw pork in retail markets in China.* Frontiers in microbiology, 2018. **9**: p. 1090.

6. Kalani, B.S., et al., *Targeting Listeria monocytogenes consensus sequence of internalin genes using an antisense molecule.* Microbial pathogenesis, 2019. **136**: p. 103689.

7. Fu, J., et al., *Meningitic Escherichia Coli α-Hemolysin Facilitates Blood-Brain Barrier Disruption Via Targeting Tgfβ1-Induced Hedgehog Signaling.* CELL-REPORTS.

8. Chen, Y., et al., *Core genome multilocus sequence typing for identification of globally distributed clonal groups and differentiation of outbreak strains of Listeria monocytogenes.* Applied environmental microbiology, 2016. **82**(20): p. 6258-6272.

9. RIP, D. and P.A. GOUWS, *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.* Journal of Food Protection, 2020. **83**(3): p. 518-533.

10. Camargo, A.C., J.J. Woodward, and L.A. Nero, *The continuous challenge of characterizing the foodborne pathogen Listeria monocytogenes.* Foodborne pathogens disease, 2016. **13**(8): p. 405-416.

11. Lian, D.S. and H.S. Zeng, *Capillary electrophoresis based on nucleic acid detection as used in food analysis.* Comprehensive Reviews in Food ScienceFood Safety, 2017. **16**(6): p. 1281-1295.

12. Yachison, C.A., et al., *The validation and implications of using whole genome sequencing as a replacement for traditional serotyping for a national Salmonella reference laboratory.* Frontiers in Microbiology, 2017. **8**: p. 1044.

13. Martin, B., S. Bover-Cid, and T. Aymerich, *MLVA subtyping of Listeria monocytogenes isolates from meat products and meat processing plants.* Food Research International, 2018. **106**: p. 225-232.

14. Tamburro, M., et al., *Characterization of Listeria monocytogenes serovar 1/2a, 1/2b, 1/2c and 4b by high resolution melting analysis for epidemiological investigations.* International Journal of Food Microbiology, 2019. **310**: p. 108289.

15. Bakhtiari, M., et al., *Targeted genotyping of variable number tandem repeats with adVNTR.* Genome research, 2018. **28**(11): p. 1709-1719.

16. Khodaei, N., et al., *Evaluation of the genetic relatedness of Bacteroides fragilis isolates by TRs analysis.* Iranian Journal of Basic Medical Sciences, 2020. **23**(10): p. 1323-1327.

17. Vergnaud, G., 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.* Frontiers in microbiology, 2018. **9**: p. 1545.

18. Zamani, M., et al., *Corrigendum to á Prevalence of Premature Stop Codons (PMSCs) in Listeria monocytogenes isolated from clinical and food samples in Iraná Gene Rep. volume 17 (2019) 100451 (Gene Reports (2019) 17,(S2452014419300937),(10.1016/j. genrep. 2019.100451)).* Gene Reports, 2020. **19**.
19. Kalani, B.S., et al., *Genotypic characterization, invasion index and antimicrobial resistance pattern in Listeria monocytogenes strains isolated from clinical samples.* Journal of Acute Disease, 2015. 4(2): p. 141-146.

20. Radoshevich, L. and P. Cossart, *Listeria monocytogenes: towards a complete picture of its physiology and pathogenesis.* Nature Reviews Microbiology, 2018. 16(1): p. 32-46.

21. Londero, A., et al., *Comparison of three molecular subtyping techniques for Listeria monocytogenes.* Revista Argentina de Microbiología, 2019. 51(4): p. 359-362.

22. Stessl, B., M. Wagner, and W. Ruppitsch, *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., 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(20): p. 6472-80.

24. Lu, C.-Y., et al., *Multiple-locus variable-number tandem-repeat analysis (MLVA) of macrolide-susceptible and-resistant Mycoplasma pneumoniae in children in Taiwan.* Journal of the Formosan Medical Association, 2020.

25. Martin, B., S. Bover-Cid, and T.J.F.R.I. Aymerich, *MLVA subtyping of Listeria monocytogenes isolates from meat products and meat processing plants.* Food Research International, 2018. 106: p. 225-232.

26. Lindstedt, B.A., et al., *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(2): p. 141-8.

27. Ashrafi, R., et al., *Application of high resolution melting assay (HRM) to study temperature-dependent intraspecific competition in a pathogenic bacterium.* Scientific reports, 2017. 7(1): p. 1-8.

28. Ohshima, C., et al., *A novel typing method for Listeria monocytogenes using high-resolution melting analysis (HRMA) of tandem repeat regions.* International journal of food microbiology, 2017. 253: p. 36-42.

29. Lüdeke, C.H., et al., *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: p. 564

**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|
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.