

Occurrence and molecular characteristics of *Listeria monocytogenes* isolated from ready-to-eat meats in Hanoi, Vietnam

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Abstract

Listeria monocytogenes represents one of the most serious threats to food safety. Several studies have shown that Ready-To-Eat (RTE) meats are an important vehicle responsible for listeriosis in human. In Vietnam, little is known about the occurrence and molecular characteristics of *L. monocytogenes* in meat products, which are essential for developing monitoring plans and control measures. In the present study, we investigated the occurrence of *L. monocytogenes* in 258 sausage and sliced meat samples collected during the period of 2013-2015 and determined the genetic diversity of the isolates using multi-locus sequence typing (MLST). Overall, *L. monocytogenes* was present in 19/129 (14.7 %) and 40/129 (31.0 %) sausage and sliced meat samples respectively, with the peak of occurrence being in summer. Furthermore, a minimum spanning tree was constructed based on MLST data of 47 isolates. A total of 15 sequence types were found, with five being novel. Notably, the majority of the isolates (34/47) belonged to the hypervirulent clonal complexes 1, 2, and 3.

Introduction

Listeria monocytogenes is a gram-positive, facultative intracellular bacterium that causes listeriosis (Rogalla and Bomar, 2019). Ubiquitous in nature, *L. monocytogenes* may cross-contaminate food products during handling processes (Di Bonaventura *et al.*, 2008; Tabit, 2018). Unlike other common food pathogens, *L. monocytogenes* can survive during cold storage and grow to significant numbers to cause listeriosis. Once the infection has occurred, especially in immunocompromised individuals, pregnant women or the elderly, *L. monocytogenes* may cause severe and life-threatening complications such as bacteremia, meningitis, seizures, miscarriage or even

death (Schlech, 2000).

Ready-To-Eat (RTE) foods, which are often refrigerated for extended periods and consumed without further treatment, are a relevant route of *L. monocytogenes* infection in human. Sliced meats and RTE sausages were among the most common food vehicles in recorded outbreaks and sporadic cases of food associated listeriosis from 1996 to 2018 (Desai *et al.*, 2019). Recently, a *L. monocytogenes* outbreak in Spain and a multi-country outbreak in Netherlands and Belgium totaling 243 cases, six deaths and seven miscarriages, were linked to RTE meat products (ECDC-EFSA, 2019; WHO, 2019).

In Vietnam, previous studies have evidenced the presence of *L. monocytogenes* in raw and processed meats. Thuc *et al.* (2008) showed that 21.7 % of sausage samples collected during the period of 2006-2007 were contaminated with *L. monocytogenes*; Ngo Chung Thuy *et al.* (2008) revealed that up to 34.3% of the refrigerated minced pork sold in Hanoi were positive for *L. monocytogenes*; Xuan Binh *et al.* (2017) reported a contamination rate of approximately 10% in beef, pork, and poultry samples collected between 2015 and 2016 in Northern Vietnam. Meanwhile, Vietnam has enforced a zero tolerance policy (0/25g) regarding *L. monocytogenes* for RTE meats (Vietnam Ministry of Health's Decision No 46/2007/QD-BYT). However, the occurrence of *L. monocytogenes* in RTE meats in Vietnam has not been studied extensively, and the epidemiological characteristics of the *L. monocytogenes* isolates have not been assessed. Therefore, the aim of this study was to determine the occurrence of *L. monocytogenes* in RTE meat products collected in Hanoi during the period of 2013-2015 and characterize the isolated strains using Multi-locus Sequence Typing (MLST).

Materials and Methods

Sample collection

RTE sausage and sliced meat products were collected at retail stores and supermarkets in Hanoi during the period of 2013-2015, once per season per product. At each sampling, at least ten products per category (sausage and sliced meat) were collected, for a total of 129 sausage and 129 sliced meat samples. Samples were ensured to be less than 30 days old (from manufacture dates). Three packages of the same lot were pooled and minced using an A 11 basic Analytical mill (IKA); of which, 25 grams were used for isolation of *L. monocytogenes*.

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Isolation of *L. monocytogenes*

Isolation of *L. monocytogenes* from food samples was carried out using RAPID[®]*L.mono* Medium (BIO-RAD) according to the manufacturer's specifications. Firstly, enrichment was performed by homogenizing 25 grams of samples in 225 mL of Half Fraser broth (BIO-RAD) using a Stomacher 400 Circulator (Seward, England) and incubated at 30 °C for 24 hours. Thereafter, enriched cultures were spread on RAPID[®]*L.mono* Medium (BIO-RAD) and incubated at 37 °C for another 24 hours. Suspected *L. monocytogenes* colonies (blue without yellow halo) were then streaked onto RAPID[®]*L.mono* Medium before being purified twice by streaking onto Tryptone Soya Yeast Extract Agar (TSYEA). Colonies were confirmed by PCR using primers (F: 5'-CGGAGGTTCCGCAAAAGATG-3'; R: 5'-CCTCCAGAGTGATCGATGTT-3')

targeting *hlyA* gene of the bacterium (Furrer et al., 1991). Positive isolates were then grown in BHI Broth (Oxoid) and stored in 50% glycerol at -80 °C.

DNA extraction

DNA of *L. monocytogenes* was extracted using GeneJET Genomic DNA Purification Kit (ThermoFisher Scientific, USA) according to the manufacturer's instructions. Briefly, isolates were streaked from glycerol stocks onto TSYEA, confirmed by PCR as described above and grown overnight in BHI broth. Subsequently, cell pellets (from 5 mL of BHI culture) were homogenized in 180 µL of Gram-positive bacteria lysis buffer (20 mM Tris HCl, pH 8.0; 2 mM EDTA; 1.2 % Triton X-100; 20 mg/ml lysozyme) and incubated at 37 °C for 30 minutes. To release DNA from the cells, 200 µL of Lysis Solution and 20 µL of Proteinase K were added, followed by incubation at 56 °C for 30 minutes. Subsequently, 20 µL of RNase A solution was added and the mixture was incubated for 10 minutes at room temperature. After adding 400 µL of 50 % ethanol, the mixture was loaded onto the spin column and subjected to centrifugation at 6000×g (1 minute). The flow-through was discarded and the column was washed with 500 µL of Wash Buffer I and II respectively. After elution with 200 µL of Elution Buffer, DNA concentration and quality were assessed by measuring absorbances at 260 nm, 280 nm and 230 nm on a Nanodrop 2000 (ThermoFisher Scientific, USA).

Multi-locus sequencing typing and phylogenetic analysis

Multi-locus sequencing typing of 47 *L. monocytogenes* isolates was performed following the scheme available at https://bigsdbs.pasteur.fr/listeria/primers_use_d.html. Specifically, internal fragments of seven house-keeping genes *abcZ* (ABC transporter), *bglA* (beta glucosidase), *cat* (catalase), *dapE* (succinyl diaminopimelate desuccinylase), *dat* (D-amino acid aminotransferase), *ldh* (L-lactate dehydrogenase), and *lhkA* (histidine kinase) were amplified and sequenced. PCR amplifications were carried out using published primers, and the following PCR program was used: 94°C (4 mins); 35 cycles of 94°C (30s), 52°C (30s), 72°C (2 mins); and 72°C (10 mins). PCR reactions were prepared in a final volume of 50 µL, containing 25 µL of GoTaq® G2 Hot Start Colorless Master Mix 2X (Promega, USA), 0.4 pmol/µL of each primer, and 10 ng of DNA template. PCR products were purified using GeneJET PCR Purification (ThermoFisher Scientific) following manufacturer's instructions and verified on

1.5% agarose gel before being sent to Macrogen (Seoul, Korea) for sequencing. All sequences have been submitted to GenBank under accession numbers MN460372-MN460653.

Allele numbers of the obtained sequences were determined by querying against *L. monocytogenes* MLST database at <https://bigsdbs.pasteur.fr/listeria/listeria.html>. Allelic profile of seven house-keeping genes were then used to define the sequence type (ST) and clonal complex (CC) of each isolate (Ragon et al., 2008). BioNumerics software (temporary evaluation license, Applied Maths, Belgium) was used to construct a minimum spanning tree (MST), showing phylogenetic relationships between isolates. Permission to publish these results was obtained from Applied Maths.

Statistical analysis

Occurrence of *L. monocytogenes* was calculated for individual food categories (sausage and sliced meat). Chi Square test was performed to compare the occurrence and compute P-values. Mann-Kendall test was used to test for consistently increasing or decreasing trends (monotonic) in occurrence of *L. monocytogenes* over time (Pohlert, 2015). All statistical analyses were done using Excel add-in XlStat v2019.1.1 (Addinsoft, Boston, USA).

Results

Occurrence and seasonality of *L. monocytogenes* in RTE foods

Occurrence of *L. monocytogenes* in sausages and sliced meats ranged from 8.2 % to 20.0% and 26.8 % to 35.4 % between 2013 and 2015 respectively (Table 1). Combining data from all three years, contamination of *L. monocytogenes* was significantly more frequent in sliced meat than in sausage (31.0 % vs. 14.7 %, $\chi^2=9.691$, $P=0.002$). Notably, there was a drop in the occurrence of *L. monocytogenes* in sausages in 2015 (8.2 % vs. 17.5 % in 2013 and 20.0 % in 2014); however, there was no significant upward or downward trend in *L. monocytogenes* rate over the years (Mann-Kendall test, $P>0.05$). Similarly, no significant monotonic trend was found for *L. monocytogenes* rate in sliced meats. Regarding the occurrence of *L. monocytogenes* in both food types in relation to sampling season, significant differences were found ($\chi^2=12.083$, $P=0.007$), with the peak being in summer (37.5 %) and the lowest occurrence being in winter (12.5%) (Table 2). Further studies are required to validate the correlation between environmental temperature and occurrence of *L. monocytogenes* in RTE foods in Vietnam.

Table 1. Occurrence of *L. monocytogenes* in RTE sausages and sliced meats collected between 2013 and 2015.

| Year | Sample type | Number of test sample | Positive samples (%) |
|-------|-------------|-----------------------|----------------------|
| 2013 | Sausage | 40 | 7 (17.5) |
| | Sliced meat | 40 | 12 (30.0) |
| 2014 | Sausage | 40 | 8 (20.0) |
| | Sliced meat | 48 | 17 (35.4) |
| 2015 | Sausage | 49 | 4 (8.2) |
| | Sliced meat | 41 | 11 (26.8) |
| Total | Sausage | 129 | 19 (14.7) |
| | Sliced meat | 129 | 40 (31.0) |

Table 2. Occurrence of *L. monocytogenes* in RTE sausages and sliced meats in relation to sampling season.

| Sampling season | Number of test sample | Positive samples (%) |
|-----------------|-----------------------|----------------------|
| Spring | 64 | 14 (21.9) |
| Summer | 64 | 24 (37.5) |
| Autumn | 66 | 13 (19.7) |
| Winter | 64 | 8 (12.5) |
| Total | 258 | 59 (22.9) |

Multi-locus sequence typing of *L. monocytogenes* isolates

A minimum spanning tree was constructed based on MLST data to show the phylogenetic relatedness between 47 *L. monocytogenes* isolates (12/59 isolates could not be recovered from glycerol stocks). These isolates were segregated into 15 sequence types (ST), with five of which being novel (assigned as ST1585, 1586, 1587, 1588, and 1589) (Figure 1). Irrespective of the food source, ST2 was the most prevalent (17/47), followed by ST3 (12/47). On the other hand, the other STs comprised of only one or two sequences. All sequences could be attributed to existing clonal complexes (CC): CC1, CC2, CC3, CC8, CC87, CC121, CC155, CC199, and CC224. Among these, CC2, CC3, CC8, and CC155 were represented by two or three STs while the other five CCs were represented by only one ST. Of note, 34/47 sequences belonged to hypervirulent CC1, CC2, and CC3 (Figure 1). In relation to the time frame of sampling, ST2 was isolated throughout the years (except in winter 2013, autumn 2015, and winter 2015), with the peak being in summer (9/17). Of note, ST2 was repeatedly collected from sliced meat products of the manufacturer A during the period 2014-2015 (summer 2014, n=2; autumn 2014, n=1; and summer 2015, n=1). For ST3, this genotype was intermittently isolated during the study period. Most ST3 isolates were detected in spring (7/12), followed by autumn (3/12). Similar to ST2, ST3 was repeatedly isolated from sliced meat products of the manufacturer B (autumn 2013, n=3; and spring 2014, n=4) and the manufacturer C (spring 2015, n=3; and summer 2015 n=1). These results suggest that ST2 and ST3 could persist in sliced meat processing plants during a relatively long time period.

Discussion

Listeria monocytogenes contamination in RTE foods is a well-documented food-safety problem. Despite strict international regulations and control, outbreaks and precautionary food recall events associated with *L. monocytogenes* contamination are still frequently notified (CDC, 2019). The persistence of *L. monocytogenes* in RTE foods could be explained by its ubiquitousness in diverse environments, its ability to form biofilms and grow at refrigerated temperatures (Kocot and Olszewska, 2017; Walker *et al.*, 1990). Indeed, *L. monocytogenes* has been isolated from soil, water, feed, and especially food

processing facilities (Alonso *et al.*, 2014; Berrang *et al.* 2010; Linke *et al.*, 2014). Furthermore, biofilm formation allows the bacterium to resist extreme conditions and persist for long periods on both nutritive and non-nutritive surfaces, especially in areas that are difficult to sanitize (Alonso *et al.*, 2014; Kocot and Olszewska, 2017). Consequently, end products could be cross-contaminated through handling processes. Management of hazards associated with *L. monocytogenes* contamination in RTE foods requires extensive knowledge of the bacterium's occurrence in the products and its temporal fluctuation, in order to set up proper monitoring systems and control measures. Additionally, understanding the molecular characteristics of *L. monocytogenes* isolates is of paramount importance to identify the contamination source and elaborate prevention strategies (Wu *et al.*, 2016).

In this study, the occurrence and molecular epidemiology of *L. monocytogenes* were investigated in RTE sausages and sliced meats commercialized in local stores and supermarkets in Hanoi, Vietnam during the period of 2013-2015. The occurrence of *L. monocytogenes* ranged from 8.2 to 20.0 % in sausages and 26.8 to 35.4 % in sliced meats during the study period. According to Vietnam Ministry of Health's Decision No 46/2007/QĐ-BYT on Promulgation Regulation of Maximum Level of Biological and Chemical Pollution in Food, Vietnam has a *zero tolerance* regarding *L. monocytogenes* in RTE meats. Therefore, positive samples identified in this study are not compliant to the Vietnamese

laws. Higher occurrences of *L. monocytogenes* in RTE sliced meats could be due to the fact that the manufacturing process of this product category in Vietnam is less automated than that of RTE sausages. Specifically, slicing and packaging steps of sliced meat products are performed manually in most food manufacturing plants in Vietnam. Therefore, cross-contamination of sliced meat products with persistent strains could occur during these steps. The presence of ST2 or ST3 in multiple sliced meat samples from the same manufacturers in this study strongly supports this speculation.

According to previous reports, the occurrence of *L. monocytogenes* in RTE meats varies between countries and regions. In Americas, estimates of *L. monocytogenes* occurrence in deli meats (sliced meat) and sausages were reported at approximately 0.2 % for the United States (Luchansky *et al.*, 2017), whereas in Brazil, *L. monocytogenes* contamination in sausages could be as high as 39.8 % (Asturiano Ristori *et al.*, 2014). Similarly, highly divergent values were reported for European countries (EFSA, 2018). Occurrence of *L. monocytogenes* in heat-treated/cooked meat products (or sausages) in Sweden, United Kingdom, Poland, and Italy were found to be between 1.20 and 2.38% (Iannetti *et al.*, 2016; Kurpas *et al.*, 2018); whereas slightly higher values were found for Austria, Spain, and Turkey, between 4.5 and 6.4% (Kurpas *et al.*, 2018). However, the occurrence of *L. monocytogenes* in RTE sausages could be as high as 54 % in Slovakia (Kačániová *et al.*, 2015). For Asian countries, a recent study in China suggested that *L. monocytogenes*

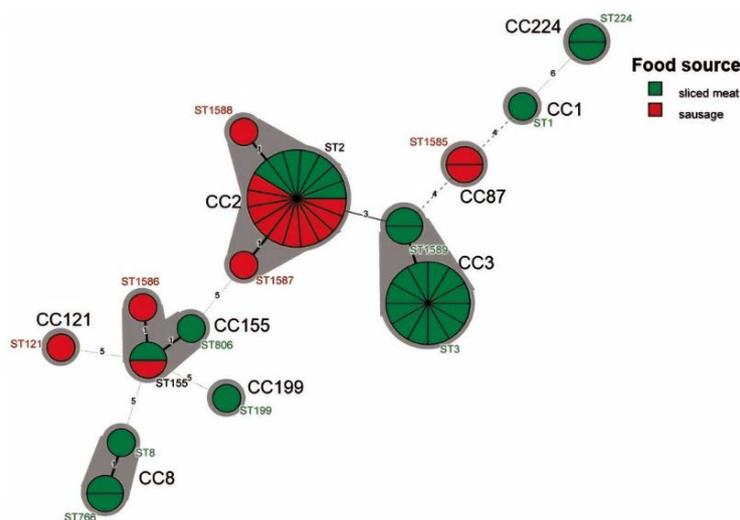


Figure 1. Minimum spanning tree showing genetic relatedness between 47 *Listeria monocytogenes* isolated from ready-to-eat meats in Hanoi, Vietnam. Each circle represents one sequence type (ST) and their sizes are proportional to the number of strains. The circles were subdivided into one pie slice per isolate. Links between circles represent the number of allelic mismatches between STs, as indicated. Gray zones around STs represent clonal complex (CC), as indicated.

occurred in 13.5 % of RTE pork (Wang *et al.*, 2018). In Malaysia, approximately 13.2 % of RTE chicken and 6.7 % of RTE beef products were contaminated with *L. monocytogenes* (Jamali *et al.*, 2013). Significantly lower contamination rates were found in RTE meats in Thailand and Japan, at 3.3 % in ham salad and 0.8 % in heat-treated RTE meats respectively (Shimajima *et al.*, 2016; Stonsaovapak and Boonyaratanakornkit, 2010). In Vietnam, Thuc *et al.* (2008) revealed that the occurrence of *L. monocytogenes* in RTE sausages in Hanoi was 21.7 %, which is comparable to our results. Overall, the occurrence of *L. monocytogenes* in RTE meats in Hanoi, Vietnam, could be considered high among Asian countries.

The seasonal occurrence of *L. monocytogenes* in RTE sausage and sliced meat was found to be the highest in summer, which is in line with studies by Rivoal *et al.* (2010) and Sauders *et al.* (2012). Nonetheless, data on seasonal variation of *L. monocytogenes* from different investigators are not in total agreement, with the peak of occurrence being in spring (Dalzini *et al.*, 2016), autumn (Elmali *et al.*, 2015), winter (Wang *et al.*, 2018) or no clear pattern of seasonality at all (Mohammed *et al.*, 2010). The increase of *L. monocytogenes* occurrence during summer months could be due to higher ambient temperature that supports *L. monocytogenes* proliferation in raw materials. As a result, heat treatment procedures were not able to completely eradicate its contamination in the final products. Furthermore, higher ambient temperature could also accelerate the growth of the remaining bacteria during storage. Indeed, increases in growth rate were observed in studies by Uhlich *et al.* (2006) and Szczawiński *et al.* (2017) when different strains of *L. monocytogenes* were inoculated into food samples and kept at higher temperatures. Nevertheless, further studies are required to confirm these speculations.

In our study, the predominant clonal complexes identified were CC2 and CC3, which have been shown to be among the most globally prevalent isolates (Zhang *et al.*, 2019). Additionally, these clonal complexes are hypervirulent and have been reported to cause food outbreaks (Maury *et al.*, 2016). Clonal complex 2 was responsible for outbreaks in USA (pasteurized milk, 1983), United Kingdom and Ireland (pâté, 1987-1988), and Italy (corn and tuna salad, 1997) (Aureli *et al.*, 2000; Cantinelli *et al.*, 2013), whereas CC3 is prevalent in cooked products and includes a strain that caused 1994 Illinois chocolate milk outbreak (Cantinelli *et al.*, 2013; Wang *et al.*, 2018). Notably, hypervirulent CC2 and CC3 harbor

three out of five novel STs identified in this study. Another hypervirulent clone (CC1) (Maury *et al.*, 2016) was also detected, although, only in one sample. On the other hand, CC9 or CC121, which were reported in several foods or processing plants (Martin *et al.*, 2014; Maury *et al.*, 2016), were not common in Vietnam, as only one sausage sample was found to be contaminated with *L. monocytogenes* CC121. Altogether, these results revealed the pervasiveness of hypervirulent strains in RTE meats in Vietnam. This represents an increased risk to vulnerable populations, such as the elderly or pregnant women. Strikingly, in this study, ST2 and ST3 were isolated multiple times from sliced meat products of the same manufacturers during a relatively long time period. Previous studies have evidenced that ST2 and ST3 could persist in food processing facilities despite regular sanitization (Chen *et al.*, 2019; Martin *et al.*, 2014; Oxaran *et al.*, 2017; Věghová *et al.*, 2017). Therefore, the high occurrence of these strains in RTE meats in Vietnam is likely related to their persistence in food processing plants.

Conclusions

In conclusion, this is the first study to report the occurrence and molecular characteristics of *L. monocytogenes* in RTE meats in Vietnam. These data indicate the necessity to establish a monitoring system and control measures for *L. monocytogenes* at both production and retail levels in Vietnam.

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