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MLVA subtyping of *Listeria monocytogenes* isolates from meat products and meat processing plants

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ABSTRACT

Listeria monocytogenes is widely distributed in meat products and the meat-processing industry thus posing a risk to consumers. The aim of this study was to evaluate the suitability of the multilocus variable-number tandem-repeat analysis (MLVA) for use as a *L. monocytogenes* subtyping technique for surveillance and routine control in meat products and meat processing plants. A collection of 113 isolates (including control strains and isolates from meat products and meat processing plants) were subject to MLVA analysis using two different platforms for fragment sizing: 1.) ABI 3730xl DNA analyzer (Life Technologies) as the reference method and 2.) The QIAxcel Advanced System (Qiagen). Although discrepancies in fragment sizing were observed it was possible to standardize results in order to assign the same allele for a given fragment independently of the platform used for fragment sizing. A total of 27 different MLVA profiles were obtained considering all the isolates ($N = 113$), 24 of them corresponding to the meat industry isolates ($N = 106$). MLVA and multilocus sequence typing (MLST) results were compared and yielded Simpson's diversity indices of 0.907 and 0.872, respectively. The congruence between both typing methods was measured with the adjusted Wallace coefficient (AW). Using MLVA as the primary method, AW = 0.946 suggested that MLVA can predict the sequence type with high accuracy. Given its discriminatory power and high throughput, MLVA could be considered a rapid, reliable, and high-throughput alternative to existing subtyping methods for surveillance and control of *L. monocytogenes* in the meat-processing industry.

1. Introduction

Listeria monocytogenes is a ubiquitous bacterium that is known as the causative agent of human listeriosis, an important foodborne disease with a high fatality rate particularly in high-risk population such as the elderly, immunocompromised patients, pregnant woman and newborn infants. In the European Union, an increasing trend of listeriosis has been registered from 2008 to 2015. A total of 1524 confirmed human listeriosis cases were reported in 2015 with a fatality rate of 17.7% (EFSA-ECDC, 2016).

L. monocytogenes is widely distributed in food-processing environments (Ferreira, Wiedmann, Teixeira, & Stasiewicz, 2014; Martin et al., 2014; Paoli, Bhunia, & Bayles, 2005; Tompkin, 2002) thus posing a risk of contamination of food products. Contaminated foods are considered the main vehicle for listeriosis (Scallan et al., 2011) particularly ready-to-eat (RTE) foods, which are intended to be consumed without further processing. Outbreaks and sporadic cases of listeriosis are generally associated to the consumption of RTE foods such as soft cheese, smoked fish, vegetables and meat and meat products (Buchanan, Gorris, Hayman, Jackson, & Whiting, 2017; EFSA-ECDC, 2016).

Characterization of *L. monocytogenes* strains is needed in order to determine its virulence potential, for surveillance purposes and epidemiological tracking (Kathariou, 2002; Swaminathan & Gerner-Smidt, 2007). Among the 13 described serotypes of *L. monocytogenes*, serotypes 1/2a, 1/2b and 4b are implicated in most cases of human listeriosis and outbreaks (Clark et al., 2010; Doumith, Buchrieser, Glaser, Jacquet, & Martin, 2004; EFSA-ECDC, 2015; EFSA-ECDC, 2016; Pagotto, Ng, Clark, & Farber, 2006). On the other hand, serotype 1/2c are commonly described in food-processing environments and food products (Gelbicova & Karpiskova, 2009; Martin et al., 2014; Thévenot et al., 2006) but it has rarely been implicated in human listeriosis cases (Orsi, den Bakker, & Wiedmann, 2011).

Molecular typing methods are crucial for the identification and monitoring of *L. monocytogenes* clonal groups along the food chain. There are many molecular methods currently available for typing *L. monocytogenes* isolates differing in discriminatory power and epidemiological concordance (EFSA BIOHAZ Panel, 2013; van Belkum et al., 2007). Pulsed-field gel electrophoresis (PFGE) has been long considered

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the "gold standard" method for *L. monocytogenes* subtyping because of its high discriminatory power, reproducibility and repeatability (Gerner-Smidt et al., 2006; Lukinmaa, Aarnisalo, Suihko, & Siitonen, 2004). However, PFGE is considered a laborious and time-consuming technique with limited data portability (EFSA BIOHAZ Panel, 2013; Heir, Lindstedt, Vardund, Wasteson, & Kapperud, 2000). Nowadays, sequence-based typing methods provide unambiguous and portable data that can be useful not only for typing purposes, but also for the study of the population structure and evolution of this pathogen (Nightingale, 2010). Multi-locus sequence typing (MLST) is a well-established sequenced-based typing method for studying population genetics of *L. monocytogenes* (Salcedo, Arreaza, Alcala, de la Fuente, & Vazquez, 2003) providing an easy and unambiguous inter-laboratory exchange of data through public databases (Nightingale, 2010). Multi-virulence-locus sequence typing (MVLST) has also been used as a sequence-based approach for *L. monocytogenes* genotyping showing an excellent epidemiological concordance (Chen, Zhang, & Knabel, 2007; Zhang, Jayarao, & Knabel, 2004). Nevertheless these techniques generally show a limited discriminatory power (den Bakker, Didelot, Fortes, Nightingale, & Wiedmann, 2008; EFSA BIOHAZ Panel, 2013; Ragon et al., 2008) for their use in the surveillance of *L. monocytogenes*. The advent of next generation sequencing technologies has dramatically reduced the cost of DNA sequencing making whole-genome sequencing (WGS) a convenient tool for molecular epidemiology and foodborne outbreak investigations (Datta, Laksanalamai, & Solomotis, 2013); thus, it is rapidly becoming the method of choice for *L. monocytogenes* genotyping in national reference laboratories. On the other hand, WGS is still prohibitive for most routine laboratories and generates massive amount of data requiring intensive bioinformatic analysis, especially for testing a large number of isolates. Recently, multiple-locus variable number of tandem repeat analysis (MLVA) has emerged as a powerful method to subtype foodborne pathogens such as *Salmonella enterica* serotypes Typhimurium and Enteritidis (Heck, 2009; Lindstedt, Heir, Gjernes, & Kapperud, 2003), *Escherichia coli* O157:H7 (Cooley et al., 2007) and *L. monocytogenes* (Lindstedt et al., 2008; Miya et al., 2008; Murphy et al., 2007; Sperry, Kathariou, Edwards, & Wolf, 2008). The approach is based on the detection of the number of tandem repeats at multiple variable-number tandem repeat loci distributed along the genome. Typically, multiplex PCR is used to amplify the tandem repeats and flanking regions and the amplification products are sized using capillary electrophoresis. MLVA is considered an easy and low-cost method which provides rapid and portable results with a high discriminatory power (Lindstedt et al., 2008; Sperry et al., 2008).

In this study, we have applied MLVA to subtype a collection of *L. monocytogenes* strains isolated from meat-processing plants using both an automatic sequencer and/or capillary electrophoresis for amplicon sizing. MLVA results were compared to those previously obtained with MLST to evaluate the discriminatory power of the technique for its implementation in systematic environmental and product monitoring in meat processing plants.

2. Material and methods

2.1. L. monocytogenes isolates

A total of 106 isolates of *L. monocytogenes* were obtained from meat products and meat processing plants. Ninety-six isolates were collected from 18 meat processing plants, including 53 isolates from RTE meat products (fermented sausages, dry ham, blood sausages and other cured pork products), 10 isolates from raw meat products (beef and pork) and 33 isolates from food contact surfaces. In addition, 10 isolates from the IRTA collection (recovered from meat products) were also analyzed. Seven control strains of *L. monocytogenes*, EGDe, ScottA, ATCC 35152 (equivalent to CIP104794), ATCC 19112 (equivalent to SLCC2372), ATCC 19114 (equivalent to SLCC2374), ATCC 19117 and CECT4032 (equivalent to F646/86) were used to calibrate the MLVA method.

The serotype and sequence type (ST) of the 106 isolates were previously determined by us (Martin et al., 2014). For control strains, ST and serotype was obtained from the Listeria MLST Database hosted at the web site of the Institut Pasteur (http://bigsdb.pasteur.fr/listeria/) and from Chenal-Francisque et al. (Chenal-Francisque et al., 2013) and Cantinelli et al. (Cantinelli et al., 2013).

2.2. DNA extraction from L. monocytogenes isolates

Genomic DNA was extracted using DNeasy® blood and Tissue kit (Qiagen GmbH, Hilden, Germany) and the QIAcube® automated sample preparation system (Qiagen). Cultures were grown overnight at 37 °C in Triptic Soy Broth (BD, Sparks, MD, USA) and 1 ml was centrifuged at 9000 × *g* for 5 min; the pellets obtained were resuspended in 180 µl of lysis buffer (35 mg/ml lysozyme in 20 mM Tris-HCl pH 8.0, 2 mM EDTA, 1.2% Triton) and the enzymatic lysis step was programmed at 37 °C for 1 h. DNA was eluted in 150 µl of AE buffer (10 mM Tris-HCl, 0.5 mM EDTA pH 9.0), quantified using Quant-It™ high sensitivity DNA assay kit (Invitrogen, Merelbeke, Belgium) and adjusted to 20 ng/µl. The DNA was split into aliquots and stored at − 20 °C for further use in PCR.

2.3. MLVA

The MLVA procedure was conducted using the eight primer pairs proposed by Sperry et al. (Sperry et al., 2008; Sperry, Kathariou, Edwards, & Wolf, 2009) and two different multiplex-PCR protocols were performed. The first protocol consisted in two 4-plex PCR reactions as in the reference protocol but using different dyes to label forward primers (Table 1), the Type-it Microsatellite PCR Kit (Qiagen), 0.2 µM of each primer (all purchased at Life Technologies [Carlsbad, California, USA]) and 2 µl of DNA. After amplification, fluorescent PCR products were resolved by automated capillary electrophoresis on an ABI 3730xl DNA analyzer (Life Technologies) with GeneScan-500 LIZ size standard (Life Technologies) using commercial GeneScan service (Macrogen Inc., Seoul, Rep. of Korea). Fragment sizes obtained were then analyzed using the Peakscanner software version 1.0 (Thermo-Fisher Scientific, Waltham, Massachusetts, USA). The second protocol consisted in three different multiplex-PCR reactions of 25 µl final volume using also the Type-it Microsatellite PCR Kit and the same primers (0.2 µM each) but not labelled. PCR reaction one (PCR1) contained primers for amplification of locus Lm-2, Lm-23 and Lm-32, the second PCR (PCR2) contained primers for locus Lm-3 and Lm-5 and the third PCR (PCR3) contained primers for locus Lm-8, Lm-10 and Lm-11. Thermal cycling conditions consisted of a denaturation step at 95 °C for 5 min and 35 cycles of 30 s at 94 °C, 30 s at 50 °C and 30 s at 72 °C and a final extension step at 72 °C for 5 min. All amplification reactions were performed in a thermal cycler GeneAmp PCR System 2700. The high-resolution capillary electrophoresis device QIAxcel Advanced System (Qiagen) was used to determine the size of PCR products. After PCR

Table 1
Parameters for the calculation of VNTR loci copy numbers with the ABI 3730xl platform.

VNTR locus	Offset	Repeat size (bp[a])	Min value	Max Value	Tolerance	Reaction group/ fluorescent dye
Lm-2	291	6	11	20	3	R1/VIC
Lm-8	185	15	3	4	7	R1/NED
Lm-10	316	6	3	9	3	R1/6-FAM
Lm-11	100	12	1	6	6	R1/6-FAM
Lm-3	200	9	1	9	4	R2/VIC
Lm-15	318	12	1	7	6	R2/6-FAM
Lm-23	169	3	15	42	1.5	R2/NED
Lm-32	83	6	10	21	3	R2/6-FAM

[a] bp, base pairs.