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Highly discriminatory typing method for *Listeria monocytogenes* using polymorphic tandem repeat regions

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ABSTRACT

Tandem repeats (TR), which are repetitive nucleotide sequences in DNA, are polymorphic both in repeat number and sequence. In this study, we developed a new typing method, multilocus TR sequence analysis (MLTSA), for the foodborne pathogen *Listeria monocytogenes* using sequence polymorphisms in three tandem repeat regions. The obtained dendrogram clustered *L. monocytogenes* strains of lineage I and lineage II separately, and formed three groups within the lineage I cluster, each of which included one of the three major *L. monocytogenes* epidemic clones (ECI, ECIa, and ECII). These results were consistent with a previously established virulence-gene-based MLST method. In comparison, our method grouped some epidemiologically related isolates together, which virulence-gene-based MLST did not. Moreover, our method, using three tandem repeat regions, showed a higher discriminatory power than the MLST method, which uses six virulence gene regions. This MLTSA approach using sequence polymorphisms in TR regions could be a useful tool in the epidemiological study of *L. monocytogenes*.

1. Introduction

Tandem repeats (TR) are defined as two or more repeated nucleotide sequence units and are found in both prokaryotes and eukaryotes. Because DNA strand slippage occurs at high rates during DNA replication, such sequences are highly variable in repeat number, even within the same species (Schlötterer and Tautz, 1992). This polymorphism has been widely used for the discrimination and identification of bacterial pathogens such as *Escherichia coli* (Keys et al., 2005; Lindstedt et al., 2004b; Noller et al., 2003), *Bacillus anthracis* (Keim et al., 2000; Le Flèche et al., 2001; Ryu et al., 2005), *Haemophilus influenzae* (van Belkum et al., 1997), *Mycobacterium tuberculosis* (Frothingham and Meeker-O'Connell, 1998), *Neisseria meningitidis* (Yazdankhah et al., 2005), *Salmonella enterica* (Lindstedt et al., 2004a; Ramisse et al., 2004), *Vibrio parahaemolyticus* (Kimura et al., 2008), and *Yersinia pestis* (Klevytska et al., 2001). Strain discrimination based on tandem repeat polymorphisms could contribute to infection source identification and speculation of strain virulence.

In many cases, the tandem repeat units are composed of perfectly identical nucleotide arrays and the variable number of the repeat units in a given genetic region is used for typing bacterial isolates. Even when some of the repeat units have nucleotide sequence variability (van Belkum et al., 1998), results are not affected as long as the numbers of nucleotides in each repeat unit are stable and can be used for the typing analysis. On the other hand, a number of studies on *Mycobacterium* species have made use of sequence variations in these tandem repeat loci for differentiating bacterial isolates (Ablordey et al., 2005; Amonsin et al., 2004; Frothingham, 1995). These studies showed higher discriminatory ability of sequence analysis over fragment length polymorphism analysis on TR regions.

Listeria monocytogenes is a ubiquitous bacterium that can cause serious listeriosis infections in humans, with a high mortality rate of 20–30%. In order to identify the infection source, understand the genetic characteristics of isolates, and prevent future cases of infection, bacterial pathogens such as *L. monocytogenes* need to be characterized with an appropriate typing method. Pulsed-field gel electrophoresis (PFGE) has been widely used and is considered to be the gold standard for typing *L. monocytogenes* isolates. Although PFGE using ApaI and AscI enzymes has high discriminatory power, difficulty in interpreting the data and comparing data between laboratories is a limitation of fragment-based methods. Multilocus sequence typing (MLST), on the other hand, overcomes these drawbacks. Specifically, MLST using virulence genes has higher discriminatory power for *L. monocytogenes*.

We previously proposed a fragment-size-based multilocus variable-number of tandem repeat typing analysis method (MLVA) for *L. monocytogenes* (Miya et al., 2008). This method is fragment-based; however, the number of fragments is limited to three and the sizes are reported as integers, allowing for ease of interpretation. This MLVA method focused on *L. monocytogenes* serotype 4b strains because of their epidemiological importance. Strains of this serotype

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have been responsible for most human listeriosis epidemics as well as the majority of human sporadic cases in many parts of the world (Farber and Peterkin, 1991; Schuchat et al., 1991). Even though serotype 4b strains are genetically homogeneous compared to strains of other serotypes (Graves et al., 1994; Mereghetti et al., 2002; O'Donoghue et al., 1995; Ridley, 1995), the newly developed MLVA was more discriminative for *L. monocytogenes* strains of this important serotype than PFGE and MLST. Meanwhile, other serotypes, such as 1/2a and 1/2b, are also of epidemiological importance as the causative agents of outbreaks and sporadic listeriosis cases (McLauchlin, 1990).

In this study, therefore, we developed a typing method for *L. monocytogenes*, multilocus TR sequence analysis (MLTSA), which is based on nucleotide sequences of tandem repeat regions. This method, using only three regions and is applicable to all serotypes of this pathogen, has high discriminatory power comparable to MLST, which uses six highly polymorphic virulence and virulence-associated genes (Zhang et al., 2004). To our knowledge, there is no previous report on a typing method for *L. monocytogenes* using sequence polymorphisms in TR regions.

2. Materials and methods

2.1. Bacterial strains

A total of 70 *L. monocytogenes* strains were used in this study (Table 1). The strains were composed of 21 serotype 1/2a, 8 serotype 3a, 8 serotype 1/2b, 4 serotype 3b, 28 serotype 4b, and one serotype 1/2c. By origin, 36 were isolated from raw ready-to-eat seafood (Handa et al., 2005; Handa-Miya et al., 2007; Miya et al., 2010), 10 were pork isolates obtained in Japan (Takahashi et al., 2007), 19 were American clinical isolates kindly provided by Dr. Martin Wiedmann (Cornell University, Ithaca, NY), and the remaining five strains were purchased from culture collections, such as American Type Culture Collection (ATCC; Manassas, VA, USA), National Collection of Type Cultures (NCTC; London, United Kingdom), and Collection de l'Institut Pasteur (CIP; Paris, France). The strains were serotyped using commercial *Listeria* antiserum (Denka Seiken, Tokyo, Japan).

2.2. Tandem repeat regions

The three tandem repeat (TR) regions used in this study (TR1 to TR3) were described in our previous study (Miya et al., 2008). Details of these regions, such as locations of the tandem repeat regions in *L. monocytogenes* F2365 (serotype 4b; GenBank accession no. AE017262) (Nelson et al., 2004) and EGDe (serotype 1/2a; GenBank accession no. AL591824) (Glaser et al., 2001), and protein annotations are shown in Table 2.

2.3. MLTSA (multilocus TR sequence analysis)

Amplification of tandem repeat regions was performed in 20-μl mixtures containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 20 pmol of each primer, 0.2 mM of each dNTP, 10 ng of template DNA and 0.5 U of Takara Taq DNA polymerase (Takara Bio, Otsu, Japan) using a Gene Amp 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). PCR conditions, amplification and sequencing primers for TR2 and TR3 were described previously (Miya et al., 2008). Amplification and sequencing primers for TR1 were described by Murphy et al. (2007) as LM-TR-3 region primers. Obtained sequences were deposited in the DNA Data Bank of Japan (DDBJ; National Institute of Genetics, Shizuoka, Japan) under accession numbers AB699358 through AB699567.

Table 1
The 70 *L. monocytogenes* strains used in this study.

Isolate	Serotype	Lineage	Origin	Sampling date	Store no.
FSL C1-117	1/2a	II	Human sporadic		
FSL J1-101	1/2a	II	Human sporadic		
FSL R2-499	1/2a	II	Human epidemic		
6-9	1/2a	II	Minced tuna	15-Dec-02	3
13-20	1/2a	II	Minced tuna	5-Nov-03	5
22-13-3	1/2a	II	Minced tuna	16-Nov-04	7
22-18-5	1/2a	II	Minced tuna	16-Nov-04	12
25-8-1	1/2a	II	Minced tuna	9-Dec-04	12
26-1-2	1/2a	II	Minced tuna	13-Jan-05	7
26-16-2	1/2a	II	Minced tuna	13-Jan-05	12
30-11-1	1/2a	II	Minced tuna	17-Mar-05	2
37-1-1	1/2a	II	Minced tuna	9-Jun-05	17
38-16-3	1/2a	II	Minced tuna	16-Jun-05	15
26-26-2	1/2a	II	Salmon roe	13-Jan-05	9
28-9-1	1/2a	II	Salmon roe	3-Feb-05	11
30-8-1	1/2a	II	Salmon roe	17-Mar-05	10
39-17-1	1/2a	II	Salmon roe	21-Jul-05	13
5-2	1/2a	II	Cod roe	10-Dec-02	2
20-7-1	1/2a	II	Cod roe	28-Oct-04	6
29-13-2	1/2a	II	Cod roe	17-Feb-05	14
40-4-1	1/2a	II	Cod roe	26-Jul-05	17
25-5-1	3a	II	Cod roe	9-Dec-04	12
25-6-1	3a	II	Cod roe	9-Dec-04	12
26-2-3B	3a	II	Cod roe	13-Jan-05	7
26-19-2	3a	II	Cod roe	13-Jan-05	12
26-29-2	3a	II	Cod roe	13-Jan-05	9
34-9-1	3a	II	Cod roe	28-Apr-05	16
39-9-1	3a	II	Cod roe	21-Jul-05	17
40-4-4	3a	II	Cod roe	26-Jul-05	17
FSL J1-038	1/2b	I	Human sporadic		
FSL J1-177	1/2b	I	Human sporadic		
29-10-1	1/2b	I	Minced tuna	17-Feb-05	13
23-4-1	1/2b	I	Salmon roe	25-Nov-04	8
25-4-3	1/2b	I	Salmon roe	9-Dec-04	10
26-22-1B	1/2b	I	Salmon roe	13-Jan-05	10
40-5-1	1/2b	I	Salmon roe	26-Jul-05	17
13-19	1/2b	I	Cod roe	5-Nov-03	5
FSL J1-169	3b	I	Human sporadic		
9-17	3b	I	Salmon roe	2-Feb-03	4
39-8-1	3b	I	Salmon roe	21-Jul-05	17
Lmc11	3b	I	Pork		
ATCC19115	4b	I	Human		
CIP101821	4b	I	Human		
CIP102551	4b	I	Human		
NCTC9863	4b	I	Human		
FSL F2-658	4b	I	Human		
FSL F2-637	4b	I	Human sporadic		
FSL F2-642	4b	I	Human sporadic		
FSL F2-672	4b	I	Human sporadic		
FSL F2-689	4b	I	Human sporadic		
FSL M2-042	4b	I	Human sporadic		
FSL J1-012	4b	I	Human epidemic		
FSL J1-119	4b	I	Human epidemic		
FSL J1-220	4b	I	Human epidemic		
FSL N1-225	4b	I	Human epidemic		
CIP103575	4b	I	Milk (epidmic related)		
FSL J1-110	4b	I	Cheese (epidemic related)		
FSL N3-013	4b	I	Food (epidemic related)		
20-5-1	4b	I	Cod roe	28-Oct-04	1
34-18-2	4b	I	Cod roe	28-Apr-05	18
Lma5	4b	I	Pork		
Lma7	4b	I	Pork		
Lmb15	4b	I	Pork		
Lmb17	4b	I	Pork		
Lmb20	4b	I	Pork		
Lmc1	4b	I	Pork		
Lmc26	4b	I	Pork		
Lmc32	4b	I	Pork		
Lmc39	4b	I	Pork		
FSL J1-094	1/2c	II	Human sporadic		