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A rapid typing method for *Listeria monocytogenes* based on high-throughput multilocus sequence typing (Hi-MLST)

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ABSTRACT

Listeria monocytogenes infects humans via food products, causing listeriosis. Consequently, food companies pay meticulous attention to the risk of contamination of their products by this bacterium. While fragment analysis methods such as pulsed-field gel electrophoresis (PFGE) are used to trace the sources of contamination for this bacterium, some drawbacks have been identified, namely the complexity of the methods and the difficulty of making data comparisons. As an alternative, multilocus sequence typing (MLST) is now seeing widespread use; however, owing to its cost, time, and labor requirements, its diffusion into the food industry has been slow. Thus, in the present study, a High-throughput MLST (Hi-MLST) method, which can rapidly, simply, and cheaply perform MLST analyses using a next-generation sequencer (NGS) that can analyze a large volume of base sequences at once was developed. Firstly, a multiplex PCR method designed to amplify seven genes for use in MLST was developed. The discriminatory potential of the developed method was confirmed *in silico*, and was verified that it has the same discriminatory potential as conventional methods. Next, MLST analysis using multiplex PCR and NGS was performed for 48 strains of *L. monocytogenes*. The sequences obtained from this analysis have sufficiently reliable quality for all of the genes from of all the strains. Thus, this method could classify the 48 strains into 39 sequence types (ST) with a Diversity index (DI) of 0.989. In summary, using the Hi-MLST method developed in the present study, which combined multiplex PCR and NGS, cut the costs to 1/6th and the time to 1/20th that of conventional MLST methods.

1. Introduction

Listeria monocytogenes are Gram-positive, facultatively anaerobic, non-sporulating, short bacilli that have flagella and are hence motile (Gray and Killinger, 1966; Ferreira et al., 2014). The bacteria exhibit a comparatively high tolerance to various environmental factors, due to their salt tolerance and ability to proliferate at low temperatures (Ferreira et al., 2014). Consequently, their distribution has been reported through a diverse range of environments, including soil (Soni et al., 2014), rivers (Lyautey et al., 2007), and food production sites (Eglezos and Dykes, 2011; Ohshima et al., 2014). In addition, they have been found in the intestinal tracts and on the body surface of animals (Gray and Killinger, 1966; Ferreira et al., 2014), and have been isolated from the feces of livestock and from farm drainage (Dreyer et al., 2015).

L. monocytogenes infects humans and livestock, causing listeriosis (Gray and Killinger, 1966; Ferreira et al., 2014). While the main symptoms of human listeriosis include fever, headaches, and vomiting, the infection is often asymptomatic in healthy adults (Farber and Peterkin, 1991). However, in pregnant women, newborn children, the elderly, and those with an underlying disease, the symptoms often become severe, and can cause meningitis, septicemia, or miscarriage (Farber and Peterkin, 1991). Due to high mortality rate, listeriosis is a significant problem in developed nations. In the United States, severe listeriosis occurs in approximately 1600 people every year, and it is estimated that around 260 of these people die (Scallan et al., 2011). As a consequence, the numbers of this bacterium in some ready-to-eat (RTE) foods have been regulated to lower than 100 cfu/g in Japan (Ministry of Health, Labour and Welfare, 2015).

The main route of infection of the bacterium in humans is thought to be the ingestion of contaminated food products (Farber and Peterkin, 1991; Scallan et al., 2011). A large number of reported food poisoning cases have been reported from dairy products such as cheese (Garedew et al., 2015; Rückerl et al., 2014), processed meats (Garedew et al., 2015; Martín et al., 2014), and ready-to-eat food products such as salads (Gombas et al., 2003; Garedew et al., 2015). Therefore, preventing the contamination of food products by *L. monocytogenes* is an important matter of concern for many food companies.

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To prevent the contamination of products with food poisoning-bacteria and spoilage bacteria, it is necessary to determine the routes of contamination and their place of manufacture (Wiedmann, 2002). Determining the route of infection requires the discrimination of the bacteria at the strain level, and molecular typing methods are therefore used in many cases. Fragment analysis methods such as ribotyping, pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP), are often used to discriminate between strains of L. *monocytogenes* (Barancelli et al., 2014; Lomonaco et al., 2011; Takahashi et al., 2007; Vongkamjan et al., 2013). However, due to subtle variations in the concentration and mobility of the bands in these methods, it is difficult to communicate the data at a global level (Gasanov et al., 2005). Additionally, these fragment analysis methods have the drawback (Jadhav et al., 2012). Consequently, strain discrimination methods using DNA sequencing techniques have become commonplace in recent years, and methods have been developed in which sequence analysis is performed on multiple regions of the genome, such that strains are discerned from comparison of the base sequences. Such methods include multilocus sequence typing (MLST) (Ragon et al., 2008; Zhang et al., 2013), and multilocus variable number of tandem repeat analysis (MLVA) (Chenal-Francisque et al., 2013; Miya et al., 2008, 2012).

MLST is a method that typically uses 5–7 housekeeping genes for base sequence analysis with allele numbers assigned from differences in the base sequence, which are used to group the bacteria. MLST is used for a tracking contamination source in the food industry and for epidemiological analysis (Ragon et al., 2008; Zhang et al., 2013). At present, MLST methods have been developed for a large number of bacteria, and both alleles and sequence types (ST) are being compiled into databases, making it possible to distribute data internationally. However, with current MLST methods, the costs and time involved are problematic (Tong et al., 2011), particularly at sites where many bacterial strains are isolated, such as food production sites, making the method impractical for this application. In the present study, a high-throughput MLST (Hi-MLST) method, using a next-generation sequencer (NGS), can simultaneously analyze the sequences of a large number of fragments, was developed. In addition, the developed method was used to evaluate 48 strains of *L. monocytogenes* isolated of different origins.

2. Materials and methods

2.1. Hi-MLST experimental design

2.1.1. Bacterial strains

A total of ten *L. monocytogenes* strains with different serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, and 4e, accordingly) were used for designing the Hi-MLST experiments (Table 1). These bacterial strains were kept at −80 °C until use. The bacteria were cultured in trypticase soy broth (TSB; Becton Dickinson and Company, Franklin Lakes, NJ, US) at 37 °C, for 18–24 h. Bacterial cells were harvested by centrifugation at 8000 × *g* for 3 min, and the supernatant discarded.

Among the clinical isolates, the ILSI Food Safety Laboratory's collection (FSL) strains had been kindly provided by Dr. Martin Wiedmann (Cornell University, NY, US) and the others had been purchased from the American Type Culture Collection (ATCC; Manassas, Va), Collection del'Institut Pasteur (CIP: Paris, France), and National Collection of Type Cultures (NCTC; London, United Kingdom). Ethical approval was not required for the study since there was no direct patient involvement and only bacterial strains were retrospectively studied.

The genomic DNA was extracted using NucleoSpin Tissue Kit (Macherey-Nagel GmbH, Düren, Germany), according to the manufacturer's instructions.

2.1.2. Primer design for Hi-MLST

abcA (encoding ABC transporter), *bglA* (encoding beta glucosidase), *cat* (encoding catalase), *dapE* (encoding succinyl-diaminopimelate desuccinylase), *dat* (encoding D-amino acid aminotransferase), *ldh* (encoding L-lactate dehydrogenase), and *lhkA* (encoding histidine kinase) were chosen as targets. The next-generation sequencer used in this study (454 GS Junior; F. Hoffmann-La Roche Ltd., Basel, Switzerland) is suitable for sequencing 400–500 bp fragments, and the analyzed amplicon sizes could not exceed this range. New primer sets were therefore designed to shorten sequence regions. Firstly, the sequences of the targeted regions of 22 *L. monocytogenes* strains were downloaded from GenBank. The sequences were aligned using GENETYX ver.11 (GENETYX co., Tokyo, Japan), and compared. Each primer set was designed in highly conserved sequence regions, to amplify fragments with sizes between 342 and 442 bp (Table 2). The primer sets were then analyzed for specificity and annealing temperatures using Primer Express Software (Thermo Fisher Scientific Inc., Waltham, MA, US). In addition, 10-bp 5′ terminal universal tails were introduced into the primers, to be used in the second reaction of the two-step PCR method for NGS sequencing developed by Boers et al. (Fig. 1) (Boers et al., 2012). For the second PCR reaction, the designed primers included 454 sequencing-specific adapters as isolated-specific multiplex identifiers (MIDs) (Supplementary Table 1). These primers targeted the universal tail regions attached during the first PCR reaction.

2.1.3. Comparison of the discriminatory power of Hi-MLST and conventional MLST

Sequence data analyzed by Ragon et al. were used to compare the outputs from the MLST method developed in this study and the conventional method. These sequences (Accession numbers FM180227-FM180445) were downloaded from GenBank. Cluster analysis was performed with BioNumerics v.4.0 software (Applied Maths, Sint-Martens-Latem, Belgium), and STs were determined as with the conventional MLST method. For the newly developed MLST method, the downloaded sequences were shortened to correspond to the amplified regions. Next, the sequences were aligned using the ClustalW program in GENETYX v.11 software, and allele numbers were assigned. STs were then determined based on allele numbers, with BioNumerics v.4.0 software. Discriminatory power (Diversity index: DI) was calculated using Simpson's index of diversity and was compared (Hunter and Gaston, 1988).

2.1.4. Multiplex amplification of target gene

The first PCR reaction, amplifying the MLST gene targets, comprised two multiplexed PCR mixtures, called Set1 (for genes *abcZ*, *bglA*, *cat*, and *dapE*) and Set2 (for *dat*, *ldh*, and *lhkA*), respectively. The PCR mixtures contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.25 mM of each primer, 25 ng template DNA, 0.5 U Ex Taq DNA polymerase (Takara Bio, Otsu, Japan), with a final volume of 50 μL. Amplifications were performed using the GeneAmp PCR System 9700 Thermal cycler (Thermo Fisher Scientific Inc., MA, US), followed by the following conditions: 94 °C for 5 min; then: 45 cycles of 94 °C for 30 s, 64 °C for 40 s, 72 °C for 1 min; and finally, 72 °C for 10 min. After amplification, PCR product sizes were analyzed by capillary electrophoresis (QIAxcel; Qiagen GmbH, Hilden, Germany). The QIAxcel DNA High Resolution Kit (Qiagen GmbH) was used. The PCR products were purified with Agencourt AMPure XP kit (Beckman Coulter, Inc., Brea, *CA*, USA), according to the manufacturer's instructions, with the following modification: the PCR products were purified with the bead solution diluted to 75% with 1 × Tris-EDTA.

The second PCR reaction was performed to attach the 454 sequencing-specific adapters and MIDs to the purified products from the first PCR reaction. The PCR mix contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.25 mM of each primer (Supplementary Table 1), 25 ng of the first PCR product as template, and 0.5 U Ex Taq DNA polymerase (Takara Bio). The final volume of the PCR mixture was 50 μL. The PCR was performed using the GeneAmp PCR System 9700 Thermal cycler (Thermo fisher scientific Inc.), and the following conditions: 94 °C for 5 min; followed by 35 cycles of 94 °C for