Variant Detection of Variable Number Tandem Repeat Loci of *Listeria innocua* by High Resolution DNA Melting Analysis

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Abstract

Microbial contamination caused by *Listeria* organism becomes a major problem in chilled and frozen food processing plants. *Listeria innocua* is the most frequently found species in finished products and plant facilities such as equipments and plant environments. High Resolution DNA Melting Analysis (HRMA) is a highly potential method for variant detection of Tandem Repeat (TR) loci of *L. innocua* which can be applied for tracking sources of *L. innocua* contamination in food processing plants. Variant scanning by HRMA is an effective method for revealing the differences of selected TR loci of *L. innocua*. Five TR loci of *L. innocua* were identified and characterized by Unipro UGENE program. All primers were designed and analyzed using Primer3 program. Ninety-four isolates of *L. innocua* from our *L. innocua* collections were used in this study. All Five TR loci were successfully amplified. The TR ampilcons were scanned for variants by HRMA using Lightcycler⁹480 system. Only 3 TR loci were found discriminatory in this data set. These ninety-four isolates of *L. innocua* could be subtyped by HRMA giving Hunter and Gaston discrimination indices (D-value) of 0.45. Hence, the High Resolution DNA Melting Analysis (HRMA) could be a highly potential method for variant detection of TR loci of *L. innocua.*

Keywords: *Listeria innocua*, Variant Scanning, Variable Number Tandem Repeat, Tandem Repeat, High Resolution DNA Melting Analysis

1. Introduction

Listeria spp. is a gram-positive, mesophillic bacterium which has ability to survive and grow at refrigerated temperature. Moreover, it has ability to form biofilm in machine or on wet surface which is difficult to eliminate from food processing plant and causing the contamination in finished products continuously. From all 8 species of *Listeria* spp., *L. monocytogenes* is the species which is most widely associated with human disease, listeriosis. Therefore, regulatory commissions of Department of Livestock Development of Thailand and various countries such as The Food and Drug Adminis-tration (FDA) of United States require zero-tolerance

(negative in 25 g samples) for *L. monocytogenes* (1). However, many food importers require that all species of *Listeria*, not only *L. monocytogenes*, are not to be found in food products. It has been reported that *L. innocua* is the most frequently found species in finished products and plant facilities such as equipments and plant environments (2-6). In order to meet customer requirements, controlling and preventing *L. innocua* contamination in food processing plants are necessary. Therefore, tracking possible sources of *L. innocua* contamination in food processing plants is mandatory for achieving this goal.

Molecular subtyping of bacteria is a beneficial method for tracking sources of contamination because it has ability to discriminate among different strains of bacteria and also infers an intimate relationship of bacterial strains between products and environments. Variant detection of TR of bacteria is a powerful molecular subtyping method for tracking sources of bacterial contamination because TR is highly specific element for each strain and can be used as markers for subtyping. High Resolution DNA Melting Analysis (HRMA) is a method for detection DNA sequence variations by amplification of the interested region in the present fluorescent dye, follow by denaturation of the amplicons by increasing the temperature in small increments where the fluorescence is monitored continuously and the melting profiles are obtained. HRMA is a simple, rapid, and sensitive closed-tube single step method which can reduce the risk of contamination (7). So variant scanning of TR loci by HRMA is a potential method for subtyping bacterial strains. Hence, this study was conducted to develop HRMA method for variant detection of TR loci for *L. innocua* strain differentiation. The method in turn can help food industry in managing the risk of *L. innocua* contamination in food industry by tracking *L. innocua* sources of contamination.

2. Materials and Methods

2.1   Bacterial strains

Ninety-four isolates of *L. innocua* obtained from our *L. innocua* collections were used in this study. All samples were identified as *L. innocua* by biochemical method and were stored in 50% glycerol as cell stock at -20°C.

2.2   Isolation of DNA

Cell stocks of *L. innocua* were activated by culturing on Tryptone Soy Agar (TSA) supplement with 0.6% yeast extract (YE) at 37°C for 24 h. A single colony was inoculated into Tryptone Soy Broth (TSB) supplement with 0.6% YE incubated at 37°C for 24h. Cell pellets were recovered from 3 mL TSB culture by centrifugation at 12,000 rpm for 10 minutes.

Supernatant was discarded and cell pellets were stored at -20°C.

Genomic DNA was extracted from cell pellets using Genomic DNA extraction kit (RBC bioscience, Taiwan) according to the manufacturer's protocol for Gram-positive bacteria. The purified DNA was stored at -20°C until use.

2.3   TR selection and primer design

Complete genome sequence of *L. innocua* CLIP 11262 was retrieved from NCBI database (NCBI Reference Sequence: NC_003212.1). The retrieved sequence was submitted into UGENE (version 1.11.4) program in order to identify tandem repeat (TR) loci in genome sequence. The TR containing at least 5 nucleotides were chosen. The sequence of each TR loci was individually submitted to Primer 3 (version 0.4.0) program (bioinfo.ut.ee/primer3-0.4.0/) to design specific TR primers for amplification. The details of selected TR loci and primer sequences which used in this study are listed in Table 1

Table1.   Details of TR loci presented in *L. innocua* CLIP 11262 and the primers used to amplify the TR loci.

TR locus	Repeat sequence	Location	Primer sequence (5′→ 3′)
TR1	ACAAAT	117923	TCGGTCGATTAAAGTCGAAA CCACCTTCATTTGCGTTACC
TR2	AAAAT	678153	CGATAAACAGCAGGTCCATT GGCACAAAGCATGCTAGAAA
TR3	CGGTAGACC	1124253	GACAAAAGTAAGTCATGCGGGTATTT TAGCTACAATCGGATTAACGG
TR4	TTTTA	2471516	AAAAACTGTCACCATTCGATGT ACTTAATCAATCAGTTCCAAATGC
TR5	TTTACTGGG	2741368	TTGATGTGCTGGATCTGC TAAATCCAGTAAACCCGGTAAATCC

2.4   TR-HRMA development

Ninety-four *L. innocua* isolates were amplified using selected TR primers from Table 1 by LightCycler480® instrument (Roche, Germany). The amplification of TR loci were performed in total volume of 25 uL contain 50 ng *L. innocua* genomic DNA, 1x PCR reaction buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTP, 0.2uM of forward and reverse primer, 0.5 U Taq DNA polymerase and 1x Light Cycler®480 ResoLight dye. The amplification condition was 1 cycle of 5 minutes at 94°C; 30 cycles of 30 seconds at 94°C; 40 seconds at 60°C for TR1, TR2, TR3 and TR4 and 40 seconds at 55°C for TR5; 1 minutes at 72°C followed by HRMA ramping from 60°C to 99°C with 50 acquisitions per 1°C where the melting curves were obtained.

Variant scanning of selected TR loci was carried out on LightCycler®480 Gene Scanning software (Roche, Germany) comprised 3 steps: normalization of melting curves, which involves equaling to 100% of the initial fluorescence and to 0% of the fluorescence remnant after DNA dissociation; shifting of the temperature axis of the normalized melting curve to the point where the entire DNA is completely denatured; and finally, an analysis of melting profiles. Samples generating different curve shapes and melting temperature in different graphs were classified as different TR-HRM genotypes.

2.5   Clustering of TR-HRMA scanning and D-value evaluation

The TR-HRMA genotypes of each locus of 94 isolates were coded with different numbers. The coded numbers of different TR loci of each isolate were combined for assigning TR patterns. The TR patterns were submitted to goeBURST program (global optimal eBURST) which Minimum Spanning Tree (MST) was generated. The MST was used to infer the relationship between isolates and contaminated locations. The discriminatory index (D-value) described by Hunter and Gaston (8) of individual and combined TR loci was also evaluated by this equation:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} nj(nj-1)$$

When   N = number of isolates tested

S = number of different genotypes

nj = number of isolates belonging to genotype j.

2. Results and discussions

Several research from various countries have demonstrated that *L. innocua* was the *Listeria* species most commonly found in fresh foods, ready to eat foods, plant facilities and plant environment. However, the bacteria which found on those environments might not be the bacteria that contaminated in the products since the conventional identification method used today could not differentiate different strains of the same bacterial species. On the other hand, the molecular methods have ability to differentiate amongst different strains of bacteria, making it possible to determine the real source of contamination (6). Thus, development of molecular subtyping method is necessary for monitoring and tracking the sources of contaminated bacteria for food safety improvement (9). Detection of TR loci by HRMA is a molecular method based on amplification of TRs in bacterial genome and has been adopted in molecular epidemiology investigations due to its relatively high discriminatory power and convenience to perform which successfully used for the identification of TR loci in *Yersinai pertis* (7), *Mycobacterium tuberculosis* (10) and *Bacillus licheniformis* (11). In this study, variants detection of TR loci of *L. innocua* using HRMA was developed.

Five TR loci within *L. innocua* CLIP 11262 genome were identified by Unipro UGENE program and designated TR1 to TR5. The details of the selected TR and primers are shown in Table 1. All five TR loci could be successfully amplified without non-specific amplicons (Figure 1).

Variant scanning of TR amplicons by HRMA was analyzed by LightCycler®480 Gene Scanning software. Three out of 5 TR loci (TR1, TR3 and TR5) showed different HRMA melting profiles (Figure2).The difference curves of HRMA analysis of these 3 loci demonstrated that different strains could be distinguished from each other with by the difference in curve shapes and melting
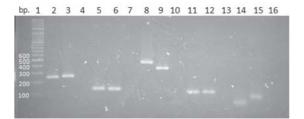

Figure1. Agarose gel electrophoresis of the PCR amplicons of *L. innocua* (isolate A and isolate B). Lane 1: Marker; Lane 2-3: TR1 loci, isolate A and B, respectively. Lane 5-6: TR2 loci (isolate A and B, respectively), Lane 8-9: TR3 loci (isolate A and B, respectively), Lane 11-12: TR4 loci (isolate A and B, respectively), Lane 14-15: TR5 loci (isolate A and B, respectively), and Lane 4, 7, 10, 13 and 16: negative control (TR1, TR2, TR3, TR4 and TR5, respectively).

temperature. For TR2 and TR4 loci, there were no significantly differences on curves among 94 *L. innocua* isolates (data not shown). It was possible that these loci had no variation of TR sizes among tested isolates. The variants obtained from TR1, TR3 and TR5 loci by HRMA were 2, 6 and 4, respectively. All 94 isolates of *L. innocua* were clustered into 8 genotypes by the goeBURST algorithm. Eight genotype groups were identified by TR-HRMA based on clustering of single locus variants (SLVs). Size of the circle correlate with the number of isolates belongs to the genotype. TR-HRMA genotype relationships are shown in a minimum spanning tree (MST) (Figure 3). The MST shows that: 1) *L. innocua* isolates in genotype 1, 5 and 8 are found in finished products, plant environment and worker's hands; 2) *L. innocua* isolates in genotype 2, 3 and 7 are found on machine, boots and weighing machine, respectively; 3) *L. innocua* isolates in genotype 4 and 6 are found only on floor. Discriminatory index (D-value) described by Hunter and Gaston were evaluated for individual and combined TR loci. Individually, D-value for TR1, TR3 and TR5 were 0.38, 0.20 and 0.06,

respectively. While combining 3 TR loci led to D-value of 0.45. Hunter and Gaston recommended an index of greater than 0.9 for good differentiation. The D-value of TR-HRMA method on this study was quite low. This could partly due to the similar locations of the samples which would be expected to reduce diversity. However, these results could infer genetic relatedness between *L. innocua* isolates and contaminated locations.

In conclusion, variant detection of TR loci by HRMA in this study was a rapid and easy-to-perform method which successfully discriminated among different strains of *L. innocua*. Therefore, this method could be a potential method for subtyping of *L. innocua* that can be applied in food industry.
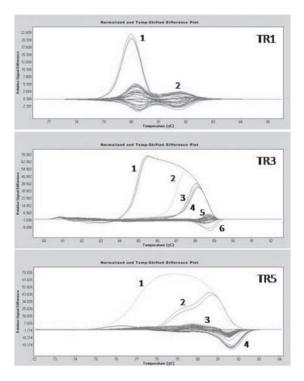

Figure3. Cluster analysis of 8 TR-HRMA patterns of 94 isolates of *L. innocua* at a single locus variant level. Each node in a tree represents a different TR-HRMA pattern, numbered for reference. The size of the circle correlates with the number of isolates in each pattern.

5. Acknowledgement

6. References

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Figure2. Difference curves derived from HRMA of TR1, TR3 and TR5 loci from 94 *L. innocua* isolates. Similar curves are similarly colored for ease of identification.
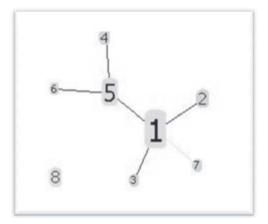
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