

Research Article

Application of Melting Temperature in Melting Curve of qPCR to Determine *Listeria monocytogenes* Presence in Golden Needle Mushroom

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This study developed a method to determine *Listeria monocytogenes* presence in golden needle mushrooms by melting temperature (T_m) in a melting curve of qPCR. For identical samples ($n = 35$), the results for *L. monocytogenes* presence determined by T_m values were compared with the results from a conventional detection method (culture-based procedures). The samples that showed the negative result in the conventional method were subsequently examined with the T_m value of qPCR. T_m values for *Escherichia coli* ($87.5 \pm 0.4^\circ\text{C}$), *Salmonella* ($87.6 \pm 0.1^\circ\text{C}$), *Staphylococcus aureus* ($79.2 \pm 0.0^\circ\text{C}$), *Listeria innocua* ($80.5 \pm 0.0^\circ\text{C}$), *Listeria ivanovii* ($79.0 \pm 0.4^\circ\text{C}$), *Listeria welshimeri* ($78.8 \pm 0.4^\circ\text{C}$), and *Listeria monocytogenes* ($83.7 \pm 0.2^\circ\text{C}$) were different, and thus, no similar T_m values of *L. monocytogenes* were observed with other bacteria. From 35 golden needle mushrooms, 26 samples (74.3%) were *L. monocytogenes* positive with T_m value of qPCR, but only 13 samples (37.1%) of 35 samples were *L. monocytogenes* positive using the conventional detection method. Of the samples that were positive with the T_m value of qPCR, but negative with the conventional detection method, 4 samples were selected randomly, and typical *L. monocytogenes* colonies were detected in CHROMagar. These results indicate that the T_m value in the melting curve of qPCR can be used to detect *L. monocytogenes* in golden needle mushrooms.

1. Introduction

Golden needle mushrooms are long thin white mushrooms. They are popular in East Asia and known as Enokitake or lily mushrooms. However, there were *Listeria monocytogenes* infections associated with the consumption of the golden needle mushrooms [1, 2]. In 2020, 36 people were infected with *L. monocytogenes* after eating golden needle mushrooms in the United States, and 31 people were hospitalized, and 4 died in California, Hawaii, and New Jersey [2].

L. monocytogenes is a Gram-positive zoonotic pathogen that causes listeriosis, which can be severe or fatal, in the immunocompromised, including pregnant women, newborns, and elderly [3–8]. The fatality rate of *L. monocytogenes* is higher than that of other foodborne

illnesses [9, 10]. Thus, the regulations in many countries have a zero-tolerance policy for *L. monocytogenes*.

The conventional *L. monocytogenes* detection method follows the sequence of pre-enrichment, selective enrichment, and isolation on selective media. This procedure may take 4–5 days to obtain results [11, 12]. Thus, more advanced, sensitive, and rapid microbial detection methods need to be developed to complement or replace the conventional culture-based procedures.

Molecular detection methods developed for *L. monocytogenes* include PCR, quantitative real-time PCR (qPCR), and multiplex qPCR [13–16]. These molecular detection methods can provide information about the composition of food products as well as pathogens in food [17, 18]. qPCR is such technology that may allow the rapid and quantitative detection of pathogens with high specificity

and sensitivity. Among the available chemistries for qPCR, intercalating dyes such as SYBR Green are mostly used due to universal applicability because of low cost. Lee et al. [19] established an *L. monocytogenes* detection method combining preenrichment and qPCR to improve a detection limit. However, it was challenging to get the C_T value for the golden needle mushroom because of the microflora and food matrix.

The presence of multiple DNA species in the same reaction can give rise to multiple peaks in the melting curve, typically indicating the presence of contaminating or off-target amplification products. Thus, when multiple species of DNA are amplified, it is difficult to distinguish which C_T value was the value we want. Melting curve analysis in qPCR was first introduced to characterize PCR products in 1997 and the amplicons of different species with different lengths and different guanine (G) and cytosine (C) contents melted at various temperatures [20]. Thus, the specificity of the reaction is determined by the melting temperature (T_m) of the amplicon. T_m is defined as the temperature at which 50% of the DNA amplicon is in a double-stranded configuration.

Therefore, this study developed the method to detect *L. monocytogenes* in golden needle mushrooms with T_m values in the melting curve of qPCR analysis.

2. Materials and Methods

2.1. Inoculum Preparation. Isolated colonies of *Escherichia coli* NCCP14038, *Salmonella* Typhimurium NCCP11116, and *Staphylococcus aureus* ATCC27664 were inoculated into 10 mL tryptic soy broth (TSB; Becton, Dickinson and Company, Sparks, MD, USA) and incubated at 37°C for 24 h. After that, 0.1-mL aliquots of the cultures were inoculated into 10 mL fresh TSB, followed by incubation at 37°C for 24 h. *Listeria monocytogenes* strains IL1-1, HL1-1, PL1-1, D2L3-1, and BL5-1 isolated from mushrooms, *Listeria ivanovii* ATCC19119, *Listeria innocua* ATCC33090, and *Listeria welshimeri* strains BKY11 from raw beef, and *L. welshimeri* ATCC35897 from decaying plant material were inoculated into 10 mL tryptic soy broth with 0.6% yeast extract (TSBYE; Becton, Dickinson and Company, and incubated at 30°C for 24 h. Aliquots (0.1 mL) of the cultures were inoculated into 10 mL fresh TSBYE, followed by incubation at 30°C for 24 h. The subcultures were harvested by centrifugation at $1,912 \times g$ and 4°C for 15 min. The resulting cell pellets were washed twice with phosphate-buffered saline (PBS; pH 7.4, 8.0 g NaCl, 1.5 g NaHPO₄, 0.2 g KH₂PO₄, and 0.2 g KCl in 1 L distilled water). Pellet suspensions were diluted with PBS to 7 log CFU/mL, and 1 mL of the inoculum was used for DNA extraction.

2.2. DNA Extraction. According to the manufacturer's protocol, the DNA of the bacteria was extracted using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). In brief, the cell pellets were resuspended in 180 μ L enzymatic lysis buffer in a microcentrifuge tube and incubated at 37°C for 30 min. Twenty-five microliters of proteinase K and 200 μ L Buffer AL were added into the mixture and incubated

at 56°C for 30 min. Two hundred microliters of ethanol (96–100%) were added to the mixture, and it was mixed thoroughly by vortexing. The mixture that flowed through the DNeasy Mini spin column was placed in a 2-mL collection tube, and the collection tube was centrifuged at $6,000 \times g$ for 1 min. The flow-through and collection tubes were then discarded. Five hundred microliters of buffer AW1 were added to the DNeasy Mini spin column and centrifuged at $6,000 \times g$ for 1 min. Flow-through and collection tubes were then discarded. The DNeasy Mini spin column was placed in a new 2-mL collection tube, and 500 μ L buffer AW2 was added to the DNeasy Mini spin column, followed by centrifugation for 3 min at $20,000 \times g$ to dry the DNeasy membrane. Flow-through and collection tubes were discarded, the DNeasy Mini spin column was placed in a microcentrifuge tube, and 200 μ L Buffer AE was added directly onto the DNeasy membrane. After incubating it at room temperature for 1 min, the DNeasy Mini spin column was centrifuged at $6,000 \times g$ for 1 min for the elution. The eluent was then used as template DNA for qPCR analysis.

2.3. Determination of T_m Value Using qPCR. Primers developed by Lee et al. [19] were used. The *iapF* (5'-TGG GAT TGC GGT AAC AGC AT-3') and *iapR* (5'-TTA TCA ACA CCA GCG CCA CT-3') were designed according to the *iap* gene sequence. A 25 μ L reaction mixture was as follows: 1 μ L template DNA, 12.5 μ L 2 \times Rotor-Gene SYBR Green PCR master mix, 6.5 μ L RNase-free water, 2.5 μ L forward primer, and 2.5 μ L reverse primer. The amplification conditions were as follows: initial denaturation at 95°C for 300 s, 35 cycles of denaturation at 95°C for 5 s, and annealing and extension at 56°C for 10 s. To measure the T_m value, melt curve analysis was carried out after amplification by slow heating from 72°C to 95°C, with fluorescence acquisition at 1°C interval and a 5 s hold at each increment. Melting curve analysis tools of the Rotor-Gene Q Series Software (Qiagen, Germany) were used to identify specific T_m values of the amplified region of the template DNA.

2.4. Detection of *L. monocytogenes* in Golden Needle Mushroom

2.4.1. Sample Preparation. From August to October 2019, 35 golden needle mushroom samples were purchased in local supermarkets. The edible area of the sample was cut with a flame-sterilized knife. It was mixed with sterilized tweezers to gather the inside and outside of the edible part evenly. One golden needle mushroom sample was divided into two sample bags. One sample bag was analyzed by the conventional *L. monocytogenes* detection method, and the other was analyzed for *L. monocytogenes* presence with a T_m value of qPCR.

2.4.2. Conventional Detection Method. Twenty-five grams of each golden needle mushroom sample was placed into a bag containing 225 mL of *Listeria* enrichment broth (LEB;

Becton, Dickinson and Company) and homogenized with a pummeler, followed by incubation at 30°C for 48 h. Aliquots of the cultures were spread-plated on PALCAM agar (Oxoid), and the plates were incubated at 30°C for 48 h. The typical colonies of *L. monocytogenes* in the PALCAM agar were analyzed by 16s rRNA gene sequencing for *L. monocytogenes* identification as the following procedure. A single colony was suspended in 50 µL of a mixture of 0.25% SDS, 0.05 N NaOH, and sterile distilled water, and 100 µL of sterile distilled water was added. After heating the microcentrifuge tube containing the mixture at 99°C for 15 min, centrifugation of the microcentrifuge tube was performed at 20,000 × g and 4°C for 3 min. The supernatant was then used as a DNA template. PCR analysis for 16s rRNA gene sequences was performed using the primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT TC-3'). Sequencing was performed using the 3730xl DNA analyzer (Applied Biosystems™). The aligned 16s rRNA gene sequences were analyzed by National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) program using the GeneBank database.

2.4.3. Determination of *L. monocytogenes* Presence with *Tm* Value. Twenty grams of golden needle mushroom was placed into a bag containing 40 mL of LEB with 0.1% ferric citrate and homogenized with a pummeler, followed by incubation at 30°C for 3 h for enrichment [19, 21]. Three milliliter aliquots of the enriched samples were centrifuged at 6,000 × g and 4°C for 10 min, and supernatants were discarded. According to the manufacturer's protocol, DNA was extracted from the pellets using the DNeasy Blood and Tissue Kit. The qPCR conditions were the same as the conditions used in the 'Determination of *Tm* Value Using qPCR' section. *L. monocytogenes* IL1-1 DNA was used as a positive control for *Tm* value; the *Tm* values of mushroom samples were then compared with *Tm* value of this strain to determine positivity. In addition, this method was applied for the samples randomly selected from those showed negative results by the conventional detection method and were analyzed, and the enriched cultures of the samples were serially diluted with buffered peptone water (BPW; Dickinson and Company) and spread-plated on CHROMagar™ *Listeria* (CHROMagar, Paris, France).

3. Results

3.1. *Tm* Values for Bacteria. *Tm* values of *E. coli*, *S. Typhimurium*, *S. aureus*, *L. monocytogenes*, *L. ivanovii*, *L. innocua*, and *L. welshimeri* were identified. *Tm* value of *L. monocytogenes* was 83.7 ± 0.2°C. The *Tm* values of *L. innocua*, *L. welshimeri*, and *L. ivanovii* were 80.5 ± 0.0°C, 78.8 ± 0.4°C, and 79.0 ± 0.4°C, respectively. *Tm* values of *E. coli*, *S. aureus*, and *S. Typhimurium* were 87.5 ± 0.4°C, 79.2 ± 0.0°C, and 87.6 ± 0.1°C, respectively (Table 1). This result indicates that the *Tm* value of *L. monocytogenes* is 83.7 ± 0.2°C, which is different from those of the other bacteria.

3.2. *L. monocytogenes* Contamination in Golden Needle Mushroom. The qPCR method with the *Tm* value and the conventional detection method for *L. monocytogenes* were applied to golden needle mushrooms, and the detection results were compared. Of 35 samples, 13 samples (37.1%) showed the positive result for *L. monocytogenes* by the conventional detection method, but 26 samples (74.3%) showed the *L. monocytogenes* positive result with the *Tm* values of qPCR (Figure 1). In addition, the detection method using qPCR with *Tm* values detected *L. monocytogenes* in 75% samples from the samples that showed negative results in the conventional method, and *L. monocytogenes* colonies were observed in CHROMagar (Table 2). These results indicate that the presence of *L. monocytogenes* can be determined by the *Tm* value, and it is more accurate than the conventional detection method.

4. Discussion

Culture-based procedures and some conventional PCR detection methods make it difficult to identify *L. monocytogenes* in *Listeria* species such as *L. ivanovii*, *L. innocua*, and *L. welshimeri* because of the high genomic homology among *Listeria* species, displaying similar morphological, biochemical, serological, and molecular properties [22–27].

In our previous study [19], the *L. monocytogenes* detection system was established by preenrichment and qPCR using SYBR Green. SYBR Green is the most common intercalating dye and emits a fluorescent signal when intercalating with newly synthesized DNA. The more DNA generated in the qPCR reaction, the more fluorescence is detected. Melting curve analysis analyzes the change in fluorescence observed when double-stranded DNA incorporating dye molecules dissociates into single-stranded DNA as the reaction of temperature increases. When double-stranded DNA bound with SYBR Green I dye is heated, a sudden decrease in fluorescence is detected at *Tm*, due to dissociation of the DNA strands and subsequent release of the dye.

In this way, melting curve analysis can determine the specific amplification of PCR products in qPCR by using the difference in *Tm* values of the desired amplification products and other competitive products. Multiple peaks in the melting curve can indicate the presence of contaminated or off-target amplification products when multiple DNA species are present in the same reaction. The detection method using the *Tm* value has been used in several studies. Mokhtari et al. [28] described a qPCR to identify *Shigella* in food and stool sample using melting curve analysis. Singh et al. [29] introduced the detection of *L. monocytogenes* and *Salmonella* spp. in dairy products using qPCR-melt curve analysis. Li et al. [30] described a hexaplex qPCR to identify eleven species by melting curve analysis in commercial meat products. Li et al. [31] developed a dual SYBR Green qPCR method for the detection of impurities in beef products based on bovine-specific and general-purpose primers for vertebrates. Therefore, these studies and our results show

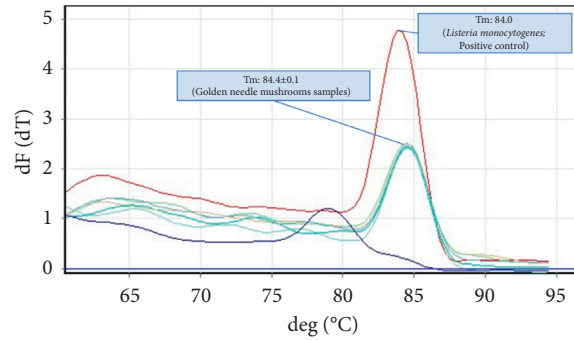


FIGURE 1: Comparison of T_m values in melting curve between *Listeria monocytogenes* in tryptic soy broth with 0.6% yeast extract media (positive control) and *L. monocytogenes* in golden needle mushroom samples.

TABLE 1: Comparison of T_m values for the bacteria in melting curves for the bacteria.

Bacteria	Strains	T_m (°C)
<i>Listeria monocytogenes</i>	IL1-1, HL1-1, PL1-1, D2L3-1 and BL5-1 isolated from mushrooms	83.7 ± 0.2
<i>Listeria ivanovii</i>	ATCC19119	79.0 ± 0.4
<i>Listeria innocua</i>	ATCC33090	80.5 ± 0.0
<i>Listeria welshimeri</i>	BKY11 isolated from raw beef, ATCC35897	78.8 ± 0.4
<i>Escherichia coli</i>	NCCP14038	87.5 ± 0.4
<i>Salmonella</i> Typhimurium	NCCP11116	87.6 ± 0.1
<i>Staphylococcus aureus</i>	ATCC27664	79.2 ± 0.0

TABLE 2: Detection of the *Listeria monocytogenes* in the samples, which showed a negative or positive results in the conventional method T_m value.

Sample	Conventional method	Detection by T_m (°C)	Observation by plating after 3 h enrichment
A	– ⁽¹⁾	79.0	–
B	–	84.3	+ ⁽²⁾
C	–	84.5	+
D	–	84.5	+

⁽¹⁾–, negative; ⁽²⁾+, positive.

that melting curve analysis can be applied to detect pathogens in food and impurities or ingredients in food.

In this study, the reliable qPCR method for identifying *L. monocytogenes* using the T_m value was developed, and it showed specific differences among *Listeria* species and even other bacteria. The culture-based conventional method needs more than 6 days to detect and identify *L. monocytogenes*. However, the method using the enrichment medium and the T_m value of qPCR took 6 h to detect *L. monocytogenes* in mushrooms, and the detection rate of *L. monocytogenes* was higher than that of the conventional detection method.

5. Conclusions

In conclusion, this study developed a method to determine *L. monocytogenes* presence in golden needle mushrooms by the T_m value in the melting curve of qPCR. This method can decrease the detection times, compared to culture-based procedure, and replace the method using the C_T value, which was influenced by food matrix and microflora in the food. Therefore, the detection method developed in this study can improve the safety for *L. monocytogenes* in golden needle

mushrooms with decreasing detection time and increasing specificity.

Data Availability

The data used to support the findings of this study are available from the first author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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