Rapid and Sensitive Real-time PCR Quantitative Detection of *Listeria monocytogenes* without Enrichments in Artificially Contaminated Chilled Pork

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Abstract— The aim of this study was to develop a rapid real-time PCR method for detection of *Listeria monocytogenes* in artificially contaminated chilled pork, without the pre-enrichment steps. For specificity test, no amplification signals and no Tm at ~78.37℃ were retrieved from other spoilage and pathogenic bacteria strains. The standard curve was constructed by ten-fold serial dilutions of a suspension of pure *L. monocytogenes*, the R^2 and E were respectively 0.999 and 102.4%. The detection limits of *L. monocytogenes* were up to 10^0 cfu/ml for both pure culture and artificially contaminated chilled pork. This assay was then applied to enumerate *L. monocytogenes* in artificially contaminated chilled pork samples and the results were compared to those obtained by plating onto selective medium for *L. monocytogenes*. A comparison between two methods reported no log underestimation of the microbial loads. The real-time PCR is a useful tool for the screening of *L. monocytogenes* in chilled pork and was performed within 12 h, and also can accurately quantify *L. monocytogenes* in chilled pork samples.

Keywords— ***Listeria monocytogenes*; Real-time PCR; Chilled pork**

I. INTRODUCTION

Listeria monocytogenes, a human food-borne pathogen responsible for listeriosis, is widely distributed in the environment and therefore can be found in unprocessed foods of animal origin[1,2], such as raw meat, poultry, milk and etc. *L. monocytogenes* has been traditionally studied on the basis of pre-enrichment with subsequent plating on selective media, biochemical reactions, the CAMP test, and serological tests[3].

Alternative molecular methods have become very important tools in the study of *L. monocytogenes*, because they are believed to overcome problems associated with labor-intensive, sensitivity-lacking and time-consuming procedures[4,5]. Of these alternative methods, those based on real-time PCR have demonstrated great potential because of their high specificity and sensitivity[6]. Real-time PCR could offer significant advantages for enumeration of bacteria directly from food samples and it is widely used in food microbiology[7].

For recent research, some methods require enrichment procedures prior to real-time PCR in order to detect a small number of target cells[8]. However, methods such as culture-enrichment cannot be used prior to real-time PCR, since they influence the initial amount of target in an uncontrolled manner, and they only are qualitative.

In the present work our aim was to develop a new procedure to specifically isolate total target DNA of *L. monocytogenes*, and to establish a quantitative real-time PCR assay without pre-enrichments. This was achieved as follows: (a) evaluation of the performance of the assay (specificity, sensitivity, standard curve); (b) application of the assay for detection of *L. monocytogenes* in artificially contaminated chilled pork, comparing it with traditional microbiological culture methods.

II. MATERIALS AND METHODS

Bacterial strain

The *L. monocytogenes* CICC 21583 strain used in this study was obtained from China Center of Industrial Culture Collection, China. Viable counts were obtained by plating a dilution made in buffered peptone water onto selective culture (Land Bridge, Beijing, China), and incubated at 37°C for 48 h.

DNA extraction

Bacterial DNA was extracted using the TIANamp Bacteria DNA Kit (Tiangen Biotech Beijing Co., Ltd., China) according to the manufacturer's instruction

with some modifications. Finally, DNA was eluted with TE buffer (Tiangen) and stored at -20°C.

Optimization of real-time PCR condition

Real-time PCR was optimized using SYBR Premix Ex Taq™ Ⅱ Kit (TaKaRa Biotechnology Dalian Co., Ltd., China). Primers *Hly*-f (ACTTCGGCGCAATCAGTGA) and *Hly*-r (TTGCAACTGCTCTTTAGTAACAGCTT) used in this study were based on previous work[9], using the *L. monocytogenes* specific *hly* gene encoding listeriolysin. Reactions were carried out using a ABI Prism 7500 (Applied Biosystems, USA). PCR was performed in a final volume of 20 μL including 2 μL of template DNA, 10 μL of SYBR Premix Ex Taq™ Ⅱ, 0.4 μL of ROX Reference Dye Ⅱ, 0.4 μL (10 mM concentration) of each primer, and RNase Free dH_2O (TAKARA). The cycling parameters consisted of: 95°C for 30 s, followed by 45 cycles of 95°C for 5 s, 60°C for 34 s, and a dissociation stage. A no-template negative control was included in each run.

Evaluation of the detection of the protocols

The specificity of the primers was tested using *L. monocytogenes* CICC 21583 strain as a positive control and 41 bacterial strains (Table 1) as negative controls, representing the main spoilage and pathogenic bacteria found in chilled pork. Besides amplification curves, the specificity of real-time PCR was evaluated using the melting temperature (Tm) calculated from the melting curve of the PCR product.

Tabel 1 List of target and non-target bacterial species used for specificity tests.

Species	Strain[a]
Listeria monocytogenes	CICC 21583
Enterococcus faecium	ATCC19434
Enterococcus faecalis	ATCC13433
Leuconostoc mesenteriodes	This laboratory
Weissella cibaria	This laboratory
Salmonella typhimurium	CICC 50013
E.coli O157:H7	CICC 21530
Enterobacter aerogenes	ATCC13048
Enterobacter cloacae	ATCC 13047
Yersinia enterocolitica	CICC 21565
Yersinia enterocolitica	UU
Listeria innocua	UU
Pseudomonas aeruginosa	This laboratory
Citrobacter breakii	This laboratory
Enterobacter sakazakii	UU
Escherichia coli	This laboratory
Escherichia coli	This laboratory
Corynebacterium variabilis	This laboratory
Staphylococcus xylosus	This laboratory
Staphylococcus sciuri	This laboratory
Lactobacillus plantarum	This laboratory
Tetragenococcus halophilus	This laboratory
Staphylococcus saprophyticus	This laboratory
Staphylococcus xylosus	This laboratory
Staphylococcus epidermidis	This laboratory
Klebsiella oxytoca	This laboratory
Citrobacter sp.	This laboratory
Acinetobacter sp.	This laboratory
Serratia spp.	This laboratory
Aeromonas sp.	This laboratory
Pseudomonas sp.	This laboratory
Hafnia alvei	This laboratory
Enterobacter aerogenes	This laboratory
Staphylococcus sp.	This laboratory
Weissella hellenica	This laboratory
Staphylococcus sp.	This laboratory
Staphylococcus sp.	This laboratory
Pseudomonas putida	This laboratory
Pantoea sp.	This laboratory
Acinetobacter sp.	This laboratory
Pseudomonas sp.	This laboratory
Pseudomonas sp.	This laboratory

[a]: CICC—China Center of Industrial Culture Collection; ATCC--American Type Culture Collection; ATCC—American Type Culture Collection; UU—Unknown.

The detection and quantification limits of the real-time PCR assays were determined by using cultures of *L. monocytogenes* strain CICC 21583. Ten-fold dilutions of original cultures were performed, and the bacterial DNA were extracted and subsequently subjected to real-time PCR. At the same time, an aliquot of 100 μL was plated to evaluate the CFU of each dilution.

To construct standard curves, DNA was isolated from ten-fold serial dilutions of suspensions of pure *L. monocytogenes* culture (approximately 1.6×10^7 cfu/mL), and was subsequently amplified as described above. The final concentrations of *L. monocytogenes* in cultures ranged from 1.6×10^7 to 1.6×10^3 cfu/mL. In parallel, the same sample was counted for determination of viable cell counts on selective agar. For each dilution, nine C_T -values were generated (DNA extraction per sample in triplicate and real-time PCR per DNA extraction in triplicate) and were included when calculating the standard curve.

Quantitive testing artificial contamination of pork

Chilled pork samples (20 g) were aseptically weighed into sterile plastic styrofoam trays. The tray-packaged pieces of pork were placed under ultraviolet light for 20 min, and then inoculated with 200 μL of different concentrations of *L. monocytogenes*, and placed at room temperature. For inoculated chilled pork, extraction of bacterial DNA was undertaken as follows: each sample was homogenized in 80 mL of saline peptone water and shaken for 20 min at room temperature. The aliquot was centrifuged (Avanti J-E,

Beckman Coulter, American) at 200 g for 1 min. The supernatant (1 mL) was aseptically transferred into a sterile centrifuge tube and a further centrifugation was carried out at 12,000 g for 2 min. DNA were each extracted from *L. monocytogenes* obtained from artificially contaminated pork samples and then subjected to real-time PCR. Simultaneously, traditional plating methods were performed for bacterial enumeration. By comparing the PCR results with the traditional counts we were able to evaluate the detection accuracy of this method.

III. RESULTS

Validation of the DNA-based detection protocols

A 137 bp fragment of the *hly* gene, was chosen as a target by real-time PCR. The specificity test of this method was assessed with the *L. monocytogenes* CICC 21583 and a collection of 41 other bacteria commonly found in meat (Table 1). The *L. monocytogenes* tested was correctly amplified, while the other bacterial strains gave significantly lower fluorescence signals than the threshold (data not shown). Also the Tm analysis showed peaks of the positive control (*L. monocytogenes*) at ~78.37°C, while negative controls did not show any Tm at ~78.37°C (see Fig. 1).

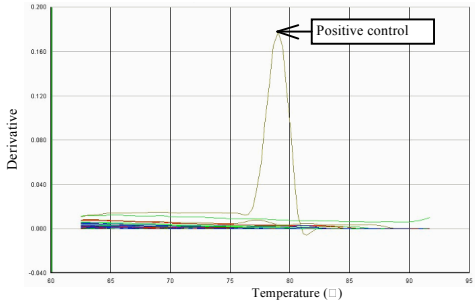

Figure 1. Melting curve analysis of the real-time PCR amplification products of *L. monocytogenes*-specific *hly* gene sequence in specificity test.

In order to quantify *L. monocytogenes* cells on chilled pork, standard curves were created using serial dilutions of *L. monocytogenes* culture in buffered peptone water. The linearity range of each standard curve was from 1.6×10^7 cfu/mL to 1.6×10^3 cfu/mL. The equation of the Log cfu/mL versus the threshold cycle (C_T) values obtained was y= −3.265x+38.664 with a R^2 of 0.999; the efficiency ($E = 10^{-1/slope} - 1$) was 102.4% (see Fig. 2). The R^2 and E value were acceptable in this method. For the detection limit of *L. monocytogenes* culture, a series of ten-fold dilutions was measured, covering eight orders of magnitude ranging from 10^6 to 10^{-1} cfu/mL Results indicated the minimum level of DNA-based detection was 10^0 cfu/mL, the same log as that of detection of artificial contaminated chilled pork samples (data not shown).

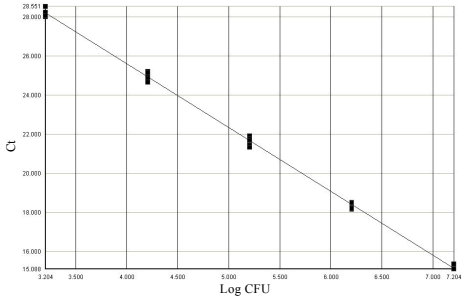

Figure 2. Standard curve generated by DNA-based real-time PCR amplification of serial dilutions of *L. monocytogenes* cells in buffered peptone water.

3.3 Quantitative detection of *L. monocytogenes* in artificially contaminated pork samples

In order to assess the applicability of real-time PCR for direct detection and quantification of *L. monocytogenes*, artificially contaminated chilled pork samples were investigated. CFUs were obtained by the real-time PCR assays using calibration curves of *L. monocytogenes* DNA standards. Table 2 shows that the calculated CFUs obtained by DNA-based real-time PCR were the same Log as that of traditional method.

Table 2. Comparison of the quantificative detection of DNA-based *L. monocytogenes* real-time PCR assays with the traditional plating methods in artificially contaminated chilled pork samples.

	Approx cfu[a]	DNA-based detection	
	Mean ± SD (cfu)	Mean ± SD (C_T)	Mean ± SD[b] (cfu)
1	$(3.33 \pm 0.61) \times 10^5{}_c$	19.66 ± 0.12	$(6.66 \pm 0.58) \times 10^5{}_c$
2	$(2.30 \pm 0.20) \times 10^4{}_c$	23.12 ± 0.14	$(5.78 \pm 0.59) \times 10^4{}_c$
3	$(1.83 \pm 0.01) \times 10^3{}_c$	26.89 ± 0.08	$(4.04 \pm 0.24) \times 10^3{}_c$
4	$(2.43 \pm 0.15) \times 10^2{}_c$	29.20 ± 0.47	$(8.20 \pm 2.56) \times 10^2{}_d$

[a] Colony forming units obtained by plating on PALAMN plates for *L. monocytogenes*;

[b] The value obtained by DNA-based real-time PCR assays using *L. monocytogenes* DNA standards;

[c-d] For plating counting, and DNA-based detection, different superscripts in the row indicate significant differences (c-d, $P < 0.05$).

The statistical analysis was performed by the application of General ANOVA procedure. Table 2

showed that only in bacterial Sample 4 the CFUs were significantly different($P < 0.05$) between plate counts and DNA based detection, while in all the other samples, the values obtained by DNA-based detection were not different. So the results of this DNA-based real-time PCR method were believable.

IV. DISCUSSION

In recent years there has been an increase in the number of molecular method for rapid and sensitive detection of pathogenic bacteria in food[10], and real-time PCR is a useful tool for detection of *L. monocytogenes*[11,12]. However, the major drawback inherent in these methods is their accurate quantitation dependent of the pre-enrichments. The pre-enrichments influence the initial amount of target in an uncontrolled manner. So the object of this study was to establish a rapid and sensitive DNA-dependent SYBR Green □ real-time PCR assay for the detection of *L. monocytogenes* without pre-enrichments.

The target gene *hly* encoding listeriolysin used in this study has been previously used in Taqman probe real-time PCR assays for the detection of *L. monocytogenes*[9], but has not been used in SYBR Green □ real-time PCR analysis, which is less expensive than fluorescent probes[13]. For quantitative PCR assays, specificity, sensitivity and the linear range of quantification are important parameters[14]. The protocol, after optimization, was finished within 12 h , and found to be to be highly specific for *L. monocytogenes* only (see Fig. 1 and Table 1) since no amplification signal was obtained when DNA extracted from other bacteria strains was used in the qPCR protocol. Simultaneously, the Tm supported the specificity of this protocol. Another experimental step carried out here was the construction of standard curves. Results were shown in Fig. 2. Correlation coefficient ($R^2 = 0.999$) and amplification efficiency (E = 102.4%) of the DNA-based method showed good range of linearity and efficiency. For sensitivity testing, the detection method of pure culture had the same limit level (approximately 10^0 cfu/mL) as that of artificially contaminated chilled pork samples; such a limit would be very useful for the detection of *L. monocytogenes* even in chilled pork samples, where lower loads of this micro-organism would be expected. For the quantitative detection of *L. monocytogenes*, comparing with the results of traditional plate method, the values obtained from real-time PCR assay mostly has not significant difference with that from traditional plate method (see Table 2). This method shows advantages over other recently described real-time PCR methods that require an enrichment step [15-17] or higher initial cfu/mL[18].Consequently, In this situation, it has to be considered that these techniques this real-time PCR assay which is capable of detecting *L. monocytogenes,* is characterized by high specificity, a wide dynamic range of quantification and high sensitivity, especially without pre-enrichment.

V. CONCLUSIONS

In terms of practicability, the DNA-based method have potential to be used for the positive screening of *L. monocytogenes*, and the major advantage of the DNA-based method is that it can detect *L. monocytogenes* without enrichments and also can accurately quantify *L. monocytogenes* within 12 h in artificially contaminated chilled pork.

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