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Rapid detection of viable *Listeria monocytogenes* in chilled pork by real-time reverse-transcriptase PCR

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ABSTRACT

The objective of this study was to develop an RNA-dependent real-time reverse-transcriptase PCR (real-time RT-PCR) method for the detection of *Listeria monocytogenes* in chilled pork without the need for pre-enrichment steps, and the soundness of the method was simultaneously validated and evaluated by DNA-based real-time PCR and traditional culture methods. For specificity testing, a lack of amplification signals and no Tm peak at ~78.37 °C were obtained from any of 41 other bacterial strains associated with meat species under the conditions used. The R^2 and efficiency of standard curves constructed by ten-fold serial dilutions of pure *L. monocytogenes* were respectively 0.995 and 90.1%; lower than that of the DNA-based assay. The detection limit was up to 10^0 cfu/mL in both pure culture and in artificially contaminated chilled pork samples. Quantitative detection showed that the RNA-based assay obtained relatively accurate results when samples had undergone treatments (such as high pressure), but without treatment, the results showed a slight deviation compared with plate counts. The RNA-dependent real-time RT-PCR method developed in this study was found to be rapid and sensitive and should be useful for reliable detection of viable *L. monocytogenes* in chilled pork, especially for pre-treated samples. However, this method cannot be recommended to accurately quantify *L. monocytogenes*, but only to show the presence of live cells and approximately predict its level of contamination.

1. 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 (Berrada, Soriano, Picó, & Mañes, 2006; Oravcová, Trncíková, & Kaclíková, 2007), 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 (Rodríguez-Lázaro et al., 2007). Alternative molecular methods, independent of cultivation, 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 (D'Urso et al., 2009; Mar, Pierobon, Tafi, Signoretto, & Canepari, 2000).

Of these alternative methods, those based on real-time PCR have demonstrated great potential because of their high specificity and sensitivity (Nogva, Rudi, Naterstad, Holck, & Lillehaug, 2000; Rodríguez-Lázaro et al., 2004). The real-time PCR approach relies on the optical detection of amplification products by measuring the fluorescent signal generated by either intercalating dyes or specific dual-labeled probes. Further, most of real-time PCR methods use DNA extraction, followed by real-time PCR for rapid and sensitive detection, as well as for enumeration of *L. monocytogenes* in food (Berrada et al., 2006; Grady, Sedano-Balbás, Maher, Smithc, & Barrya, 2008; Navas et al., 2006; Oliveira, Ribeiro, Bergamini, & Martinis, 2010; Rodríguez-Lázaro, Jofré, Aymerich, Garriga, & Pla, 2004; Rodríguez-Lázaro, Jofré, Aymerich, Hugas, & Pla, 2005; Rossmanith, Krassnig, Wagner, & Hein, 2006; Traunšek et al., 2011; Vanegas, Vásquez, Martinez, & Rueda, 2009). Since DNA-based real-time PCR detects nucleic acids from cells that are both live and dead, such a procedure does not give any indication of the extent of viability (Wolffs, Norling, & Radstrom, 2005). Studies showed that DNA from dead bacterial cells can serve as templates for the PCR many days after cell viability has been lost (Allmann et al., 1995; Kobayashi et al., 2009; Rodríguez-Lázaro, Hernández, D'Agostino, & Cook, 2006), and thus will contribute to positive PCR signals. In the past, most PCR diagnostic methods for *L. monocytogenes* have included an initial culture-enrichment step

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to improve sensitivity whereby after enrichment, the amount of living cells considerably exceeds the amount of dead cells (Uyttendaele, Boxstael, & Debevere, 1999). However, methods such as culture-enrichment cannot be used prior to qPCR, since they influence the initial amount of target in an uncontrolled manner. Presently it has been reported that some pre-treatments prior to extraction of nucleic acids can circumvent this problem, such as the use of ethidium monoazide bromide (EMA) (Flekna et al., 2007; Lee & Levin, 2009; Minami, Yoshida, Soejima, Yaeshima, & Iwatsuki, 2010; Nogva, Dromtorp, Nissen, & Rudi, 2003; Rudi, Naterstad, Dromtorp, & Holo, 2005) or immuno-magnetic separation (IMS) (Akkaya, Atabay, Gok, & Kara1, 2011; Ayaz, Ayaz, Kaplan, Dogru, & Aksoy, 2009; Ormanci, Erol, Ayaz, Iseri, & Sariguzel, 2008; Yang, Qu, Wimbrow, Jiang, & Sun, 2007). However, it has been reported that EMA can also penetrate the membrane of viable bacterial cells, resulting in the loss of a percentage of the genomic DNA of viable cells and PCR inhibition (Hein, Flekna, Wagner, Nocker, & Camper, 2006; Nocker, Sossa-Fernandez, Burr, & Camper, 2007); Most of the IMS studies have lower capture efficiency, so need selective enrichment techniques to recover bacteria in food samples. Therefore these methods are not well suited for routine use.

To circumvent this problem, determination of RNA has been proposed as a promising indicator of cell viability (Rodríguez-Lázaro, Hernández, D'Agostino, & Cook, 2006) since RNA has a shorter half-life than DNA in dead cells. Therefore this could provide a direct indication of viability based on the presence of RNA. Recently studies have been carried out to determine the suitability of using RNA to quantify pathogens by real-time reverse-transcriptase PCR (real-time RT-PCR) (McCabe et al., 2011a, 2011b; Miller, Davidson, & D'Souza, 2011, Miller, Draughon, & D'Souza, 2010). However most of these methods required one- or two-step enrichments prior to real-time RT-PCR in order to detect a small number of target cells (Fujikawa & Shimojima, 2008). Until now, rapid detection of viable *L. monocytogenes* without enrichments in chilled pork by real-time RT-PCR has not been reported.

In the present work our aim was to develop a new procedure to specifically isolate total target RNA of *L. monocytogenes* from other nucleic acids and interfering compounds in chilled pork, and to establish a quantitative real-time RT-PCR assay without pre-enrichment that would eliminate dead and severely damaged *L. monocytogenes*. This was achieved as follows: (a) evaluation of the performance of the assay (specificity, sensitivity, standard curve) in comparison with DNA-based real-time PCR methods; (b) application of the assay for the detection of *L. monocytogenes* in artificially contaminated chilled pork, and comparing it with traditional microbiological culture methods.

2. Material and methods

2.1. Bacterial strain

The *L. monocytogenes* CICC 21583 strain used in this study was obtained from China Center of Industrial Culture Collection, Beijing, China. Viable counts were obtained by plating a dilution made in buffered peptone water onto selective agar (PALCAM agar base with selective supplements) (Land Bridge, Beijing, China), and incubated at 37 °C for 72 h.

2.2. RNA extraction and cDNA synthesis

A 1 mL aliquot of RNAiso™ Plus reagent (TaKaRa Biotechnology Dalian Co., Ltd., China) was added to the tubes containing the bacterial cells and mixed repeatedly before incubating for 5 min at room temperature. Then 200 μL of chloroform was added and mixed well. Immediately after extraction for 5 min the tubes were centrifuged at 12,000 g for 15 min (4 °C). The supernatant was transferred into a new sterilized 1.5 mL tube and 400 μL of isopropanol was added, incubated at room temperature for 10 min, and then centrifuged at 12,000 g for 10 min (4 °C). The supernatant was removed and 1 mL 75% ethanol was added and the tube inverted several times and centrifuged at 12,000 g for 5 min (4 °C). The supernatant was removed using a transferpettor. RNAs were suspended with RNase Free dH_2O (TaKaRa), and 1 μL RNase Free DNase I (5 U/μL, TaKaRa) was added and incubated at 37 °C for 20–30 min to digest DNA. Purified RNAs were resuspended with 50 μL RNase Free dH_2O. The quantity of extracted RNA was determined spectro-photometrically (NanoDrop 2000 spectrophotometer, Thermo Scientific, Wilmington, DE, USA), and the integrity of RNA was confirmed by gel electrophoresis.

RNA amplification was performed using PrimeScript RT Master Mix (Perfect Real Time) (TaKaRa Biotechnology Dalian Co., Ltd., China) according to the manufacturer's instructions with some modifications. The cDNA was synthesized with 2 μL total RNA (100–300 ng). The primer used was Random 6 mers and Oligo dT Primer. Reactions were carried out in a Mastercycler ep cycler (Eppendorf, Germany). PCR conditions: 37 °C for 15 min, 85 °C for 5 s and cooling to 4 °C. The synthesized cDNAs were stored at −20 °C.

2.3. 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.

2.4. Establishment of the real-time PCR system of pure bacterial cultures

A 1 mL aliquot of a 10^3 cfu/mL of *L. monocytogenes* strain CICC 21583 was added to 9 mL of Trypticase Soy Yeast Extract Broth (TSB-YE) (Land Bridge, Beijing) and incubated at 37 °C for 20 h to obtain the concentration of freshly grown *L. monocytogenes* cells desired for each experiment.

2.4.1. Optimization of real-time PCR condition

Real-time PCR was optimized by hot-start condition using SYBR Premix Ex Taq™ II Kit (TaKaRa Biotechnology Dalian Co., Ltd., China). Primers *Hly*-f (ACT TCG GCG CAA TCA GTG A) and *Hly*-r (TTG CAA CTG CTC TTT AGT AAC AGC TT) used in this study were based on previous work of Amagliani, Omiccioli, Brandi, Bruce, and Magnani (2010), using the *L. monocytogenes*-specific *hly* gene encoding listeriolysin. SYBR Green I and ROX were used as reporter and passive reference dyes respectively. Reactions were carried out in MicroAmp optical eight-tube strips using a ABI Prism 7500 (Applied Biosystems, USA) sequence detection system. PCR was performed in a final volume of 20 μL including 5 μL of template DNA or cDNA, 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. Fluorescent signals were collected at the extension step. A no-template negative control was included in each run.

2.4.2. Specificity

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

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

Species	Origin[a]	Strain[b]	Real-time PCR results
Listeria monocytogenes	CICC	CICC 21583	+
Enterococcus faecium	CICC	ATCC19434	–
Enterococcus faecalis	CICC	ATCC13433	–
Leuconostoc mesenteroides	Ham pork	This laboratory	–
Weissella cibaria	Ham pork	This laboratory	–
Salmonella typhimurium	Jiangsu Provincial Center for Disease Prevention and Control	CICC 50013	–
E. coli O157:H7	CICC	CICC 21530	–
Enterobacter aerogenes	CICC	ATCC13048	–
Enterobacter cloacae	CICC	ATCC 13047	–
Yersinia enterocolitica	CICC	CICC 21565	–
Yersinia enterocolitica	Jiangsu Provincial Center for Disease Prevention and Control	UU	–
Listeria innocua	Jiangsu Exit–Entry Inspection and Quarantine Bureau	UU	–
Pseudomonas aeruginosa	Chicken meat	This laboratory	–
Citrobacter breakii	Chicken meat	This laboratory	–
Enterobacter sakazakii	Jiangsu Provincial Center for Disease Prevention and Control	UU	–
Escherichia coli	Chicken meat	This laboratory	–
Escherichia coli	Pork sausage	This laboratory	–
Corynebacterium variabilis	Pork sausage	This laboratory	–
Staphylococcus xylosus	Pork sausage	This laboratory	–
Staphylococcus sciuri	Pork sausage	This laboratory	–
Lactobacillus plantarum	Pork sausage	This laboratory	–
Tetragenococcus halophilus	Pork sausage	This laboratory	–
Staphylococcus saprophyticus	Pork sausage	This laboratory	–
Staphylococcus xylosus	Pork sausage	This laboratory	–
Staphylococcus epidermidis	Pork sausage	This laboratory	–
Klebsiella oxytoca	Pork sausage	This laboratory	–
Citrobacter sp.	Chilled pork	This laboratory	–
Acinetobacter sp.	Chilled pork	This laboratory	–
Serratia spp.	Chilled pork	This laboratory	–
Aeromonas sp.	Chilled pork	This laboratory	–
Pseudomonas sp.	Chilled pork	This laboratory	–
Hafnia alvei	Chilled pork	This laboratory	–
Enterobacter aerogenes	Chilled pork	This laboratory	–
Staphylococcus sp.	Chilled pork	This laboratory	–
Weissella hellenica	Chilled pork	This laboratory	–
Staphylococcus sp.	Chilled pork	This laboratory	–
Staphylococcus sp.	Chilled pork	This laboratory	–
Pseudomonas putida	Chilled pork	This laboratory	–
Pantoea sp.	Chilled pork	This laboratory	–
Acinetobacter sp.	Chilled pork	This laboratory	–
Pseudomonas sp.	Chilled pork	This laboratory	–
Pseudomonas sp.	Chilled pork	This laboratory	–

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

PCR product, which was obtained after completion of the PCR cycles, using an additional thermal step (95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, and 60 °C for 15 s). The Tm peak of the PCR product was calculated based on the initial fluorescence curve by plotting the negative derivative of the fluorescent signal over temperature versus temperature.

2.4.3. Sensitivity

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 RNA and DNA were extracted and subsequently subjected to real-time PCR or real-time RT-PCR. At the same time, an aliquot of 100 μL was plated to evaluate the CFU of each dilution.

2.4.4. Standard curve

To construct standard curves, RNA or 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. A linear relationship was produced by plotting the Log copy number against the C_T value. 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. Correlation coefficients (R^2) and efficiencies of amplification were calculated.

2.4.5. Quantitative detection of pre-treated L. monocytogenes culture

A bacterial culture of *L. monocytogenes* (approximately 3.6×10^8 cfu/mL) was prepared and separated into three identical aliquots, and then subjected to one of each of the following procedures: heat-treated at 90 °C for 30 min (non-viable cells) (D'Urso et al., 2009); exposure to ultraviolet light for 5 min; and

treatment with high pressure (200 MPa, 5 min at 10 °C). Bacterial RNA and DNA were extracted from aliquots of treated cultures and subsequently subjected to real-time RT-PCR or real-time PCR. Simultaneously, three aliquots were plated to evaluate the effect of this molecular method.

2.5. Artificial contamination of pork for RT-PCR

2.5.1. Contamination of chilled 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, direct extraction of bacterial nucleic acids was undertaken as follows: 20 g each sample was homogenized in 80 mL of saline peptone water and shaken for 20 min at room temperature. Then two 2 mL aliquots were transferred into two 2 mL sterile tubes, one for DNA and the other for RNA extraction. Each of the two tubes was centrifuged (Avanti J-E, Beckman Coulter, American) at 200 g (4 °C) for 1 min. The supernatant (1 mL) was aseptically transferred into a 1.5 mL sterile centrifuge tube and a further centrifugation was carried out at 12,000 g for 2 min (4 °C). The pellet was stored at −80 °C until required for extraction of nucleic acids.

2.5.2. Chilled pork samples for sensitivity testing

The sensitivity of artificially contaminated samples was tested on the influence of constituents of pork. The concentrations of *L. monocytogenes* inoculated on chilled pork ranged from approximately from 10^6 to 10^{-1} cfu/mL. The artificially contaminated samples were extracted for RNA and DNA and then subjected to real-time RT-PCR or real-time PCR. Viable counts were obtained simultaneously by plating a dilution made in buffered peptone water onto selective culture media and then incubating at 37 °C for 72 h.

2.5.3. Chilled pork samples for quantitative testing

RNA and DNA were each extracted from *L. monocytogenes* obtained from artificially contaminated pork samples and then subjected to real-time RT-PCR or real-time PCR. Quantitative results for each were obtained from standard curves. 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.

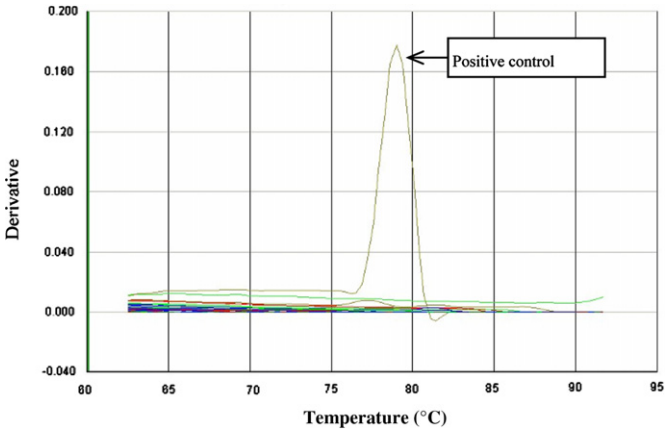

Fig. 1. Melting curve analysis of the amplification products of *L. monocytogenes*-specific *hly* gene sequence in specificity test.

2.6. Statistical analysis

Statistical analysis was performed by using the General ANOVA procedure of SPSS 18.0 to determine the statistical differences between the plate counting and the RNA- and DNA-based detection methods.

3. Results

3.1. Validation of the RNA-based detection protocols

A 137 bp fragment of the *hly* gene, one of the specific virulence genes of *L. monocytogenes*, was chosen as a target for detection of *L. monocytogenes* 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).

In order to quantify *L. monocytogenes* cells on chilled pork, standard curves were created using serial dilutions of *L. monocytogenes* CICC 21583 culture in buffered peptone water.

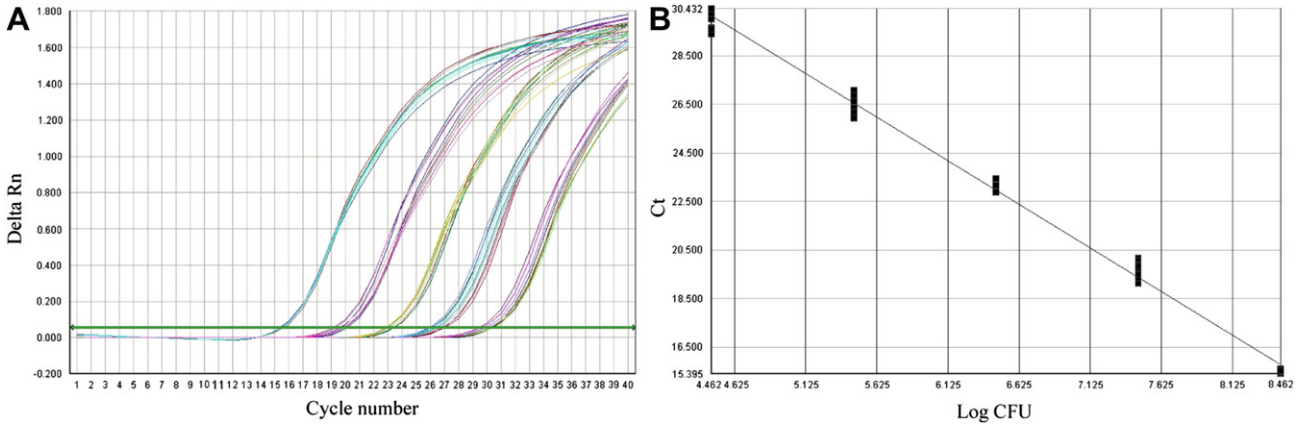

Fig. 2. Standard curve generated by RNA-based real-time RT-PCR amplification of serial dilutions of *L. monocytogenes* cells in buffered peptone water. The PCR reactions for each dilution are indicated by the dots ($n = 3 \times 3$). The straight line calculated by linear regression was $y = -3.586x + 46.141$ with a square regression coefficient of $R^2 = 0.995$. Colony forming units of *L. monocytogenes* of amplification curves (from left to right over the baseline) (A) were 1.6×10^7, 1.6×10^6, 1.6×10^5, 1.6×10^4, 1.6×10^3 cfu/mL respectively.

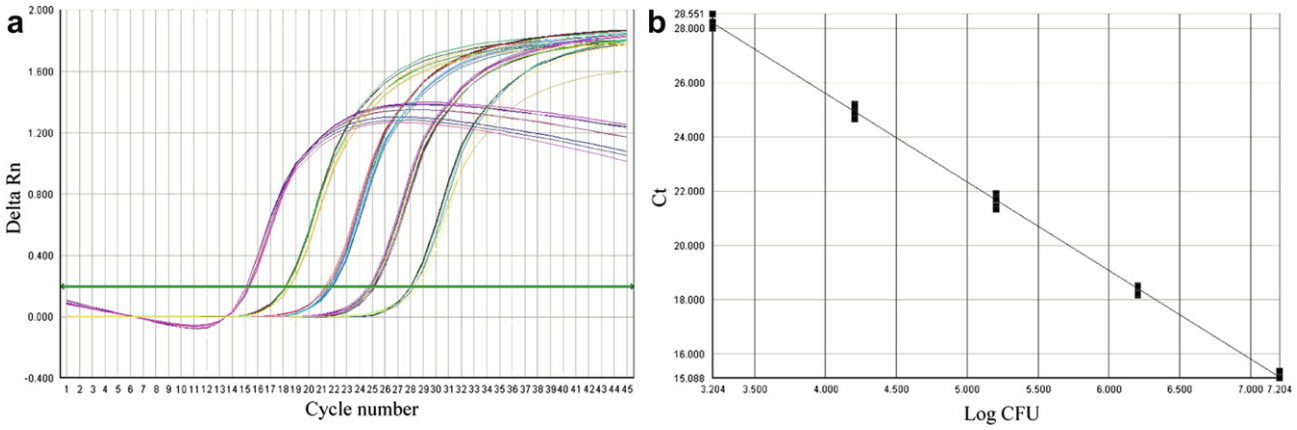

Fig. 3. Standard curve generated by DNA-based real-time RT-PCR amplification of serial dilutions of *L. monocytogenes* cells in buffered peptone water. The PCR reactions for each dilution are indicated by the dots ($n = 3 \times 3$). The straight line calculated by linear regression was $y = -3.265x + 38.664$ with a square regression coefficient of $R^2 = 0.999$. Colony forming units of *L. monocytogenes* of amplification curves (from left to right over the baseline) (A) were 1.6×10^7, 1.6×10^6, 1.6×10^5, 1.6×10^4, 1.6×10^3 cfu/mL respectively.

Both RNA and DNA were extracted and subjected to real-time PCR and real-time RT-PCR, in order to construct the standard curves. The linearity range of each standard curve was from 1.6×10^7 cfu/mL to 1.6×10^3 cfu/mL, covering 5 orders of magnitude. At the RNA level, the equation of the Log cfu/mL versus the threshold cycle (C_T) values obtained was $y = -3.586x + 46.141$ with a R^2 of 0.995; the reaction efficiency ($E = 10^{-1/\text{slope}} - 1$) (Pfaffl, 2001) was 90.1% (see Fig. 2). For DNA-based real-time PCR, the efficiency and correlation coefficient (R^2) was 102.4% and 0.999 respectively (see Fig. 3). Based on the information as described above, the R^2 and E value were acceptable in each of the two methods. However, when comparing the RNA standard curve with the DNA standard curve, the efficiency and R^2 value of standard curve was lower for RNA than for DNA. 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, and subsequently assessed as described above. Results indicated the minimum level of RNA-based detection was 10^0 cfu/mL, the same log as that of DNA-based detection (data not shown).

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

In order to assess the applicability of real-time RT-PCR for direct detection and quantification of *L. monocytogenes*, artificially contaminated chilled pork samples were investigated. In the first case, the probability of *L. monocytogenes* detection by real-time RT-PCR was determined over the range 10^6 to 10^{-1} cfu/mL. The actual detection limit of artificially contaminated chilled pork was up to 10^0 cfu/mL, and was equal to the DNA-based detection.

For detection of artificially contaminated chilled pork, CFUs were obtained by the real-time RT-PCR (or real-time PCR) assays using calibration curves of *L. monocytogenes* RNA (or DNA) standards. Table 2 shows that the calculated CFUs obtained by RNA-based real-time RT-PCR were generally one logarithmic unit higher than that of the traditional plating method, but the CFUs were the same for both DNA-based real-time PCR and plate count methods. The statistical analysis performed by the application of General ANOVA procedure corrected by plate count, showed that RNA-based detection and DNA-based detection methods revealed 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, but significant differences ($P < 0.01$) were observed for RNA-based detection compared with those obtained by plate counts (Table 2). However, for detection of pre-treated samples, such as those heated or, subjected to UV and high pressure processing (HPP), the results obtained from RNA-based real-time RT-PCR were not significantly different compared with results from plate counts. However, the results obtained from the DNA-based method were about 10^7-10^8 cfu/mL, the same level as found for samples before treatments (see Table 3).

4. Discussion

In recent years there has been an increase in the number of real-time PCR assays which are widely used for the detection of pathogenic bacteria in food (Berrada et al., 2006; Grady et al., 2008, 2009; Kim & Cho, 2010; Vanegas et al., 2009), including PCR assays for the detection of *L. monocytogenes,* where specific DNA

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

Sample	Approx cfu[a]	RNA-based detection		DNA-based detection	
	Mean ± SD (cfu)	Mean ± SD (C_T)	Mean ± SD[b] (cfu)	Mean ± SD (C_T)	Mean ± SD[c] (cfu)
1	$(3.33 \pm 0.61) \times 10^5{}_d$	27.88 ± 0.22	$(8.52 \pm 1.30) \times 10^5{}_e$	19.66 ± 0.12	$(6.66 \pm 0.58) \times 10^5{}_d$
2	$(2.30 \pm 0.20) \times 10^4{}_d$	30.60 ± 0.39	$(1.29 \pm 0.36) \times 10^5{}_e$	23.12 ± 0.14	$(5.78 \pm 0.59) \times 10^4{}_d$
3	$(1.83 \pm 0.01) \times 10^3{}_d$	33.58 ± 0.33	$(1.57 \pm 0.35) \times 10^4{}_e$	26.89 ± 0.08	$(4.04 \pm 0.24) \times 10^3{}_d$
4	$(2.43 \pm 0.15) \times 10^2{}_d$	36.52 ± 0.23	$(1.98 \pm 0.32) \times 10^3{}_e$	29.20 ± 0.47	$(8.20 \pm 2.56) \times 10^2{}_f$

d–f For plate counting, RNA-based detection and DNA-based detection, different superscripts in the row indicate significant differences (d,e and e,f, $P < 0.01$; d–f, $P < 0.05$).
[a] Colony forming units obtained by plating on PALAMN plates for *L. monocytogenes*.
[b] The value obtained by RNA-based real-time RT-PCR assays using *L. monocytogenes* RNA standards.
[c] The value obtained by DNA-based real-time PCR assays using *L. monocytogenes* DNA standards.

Table 3
Quantitative detection of *L. monocytogenes* with different treatments using three methods (plate counts, RNA-based detection and DNA-based detection methods).

Sample[a]	Mean ± SD (cfu/mL)		
	Plating counts	RNA-based detection	DNA-based detection
Heating-treated	ND[b]$_x$	ND$_x$	$(7.66 \pm 0.29) \times 10^7{}_y$
UV-treated	$(1.53 \pm 0.32) \times 10^5{}_x$	$(3.80 \pm 0.77) \times 10^5{}_x$	$(1.09 \pm 0.04) \times 10^8{}_y$
HPP-treated	$(7.27 \pm 0.21) \times 10^5{}_x$	$(1.32 \pm 0.61) \times 10^6{}_x$	$(1.18 \pm 0.04) \times 10^8{}_y$

x, y Significant differences in the row in three detection methods ($P < 0.01$).
[a] Samples were treated as follows: heat-treated at 90 °C for 30 min; exposure to ultraviolet light for 5 min; subjected to high pressure treatment (200 MPa 5 min 10 °C).
[b] Not determined.

target sequences are used as standards (Martinis, Duvall, & Hitchins, 2007; Oravcová, Trncíková et al., 2007; Rodríguez-Lázaro & Hernandez, 2006). However, the major drawback inherent in these methods is their inability to distinguish live and dead bacteria, because the presence of DNA is not indicative of bacterial viability (Keer & Birch, 2003; Kobayashi et al., 2009). However, since RNA has a shorter half-life than DNA in dead cells, the object of this study was to establish an RNA-dependent SYBR Green I real-time RT-PCR assay for the detection of *L. monocytogenes* without pre-enrichment, and to validate using a DNA-based technique.

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* (Amagliani et al., 2010), but has not been used in SYBR Green I real-time PCR analysis, which is less expensive than fluorescent probes (Miller et al., 2010). Studies revealed that the relative expression level of the *hly* gene, which was normalized to the relative levels of the control gene 16S rRNA, was not different in the growth phase (up to 8.5 log cfu/mL) (Werbrouck, Botteldoorn, Uyttendaele, Herman, & Coillie, 2007). Therefore this study used the *hly* gene as a target for the RNA-based assay. For quantitative PCR assays, specificity, sensitivity and the linear range of quantification are important parameters (Hein et al., 2001). The protocol, after optimization, was 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 or RNA extracted from other bacteria strains was used in the qPCR protocol. Simultaneously, the melt temperature (Tm) supported the specificity of this protocol. Another experimental step carried out here was the construction of standard curves for RNA and DNA methods which were applied in parallel. Results are shown in Figs. 2 and 3. Correlation coefficient ($R^2 = 0.995$) and amplification efficiency ($E = 90.1\%$) of the RNA-based method showed good range of linearity and efficiency, but they were both somewhat lower than that of the DNA-based method ($R^2 = 0.999$ and $E = 102.4\%$). For sensitivity testing, the RNA-based method had the same limit level (approximately 10^0 cfu/mL) as the DNA-based method; 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. Further, in this situation, the RNA-based real-time RT-PCR assay which is capable of detecting *L. monocytogenes,* is characterized by high specificity, a wide dynamic range of quantification and high sensitivity. This method shows advantages over other recently described real-time PCR methods that require an enrichment step (Grady et al., 2009; Oravcová, Kuchta, & Kaclíková, 2007; Rossmanith et al., 2006; Vanegas et al., 2009) or higher initial cfu/mL (Kim & Cho, 2010).

For the quantitative detection of *L. monocytogenes*, the results showed that the values obtained from two molecular methods were higher than those from plate counts, particularly the RNA-based real-time RT-PCR assay (see Table 2). Consequently, it has to be considered that these techniques are influenced by a number of factors which do not necessarily affect plate counts. The micro-organisms are viable but are unable to be cultured, even in the selective culture medium. But with PCR which targeted RNA or DNA, it was possible to detected *L. monocytogenes* whether the cells were able to be cultured or not. Statistical findings indicated that, compared with the traditional culture method, the values obtained from RNA-dependent real-time RT-PCR method were significantly different ($P < 0.01$), and therefore this RNA-based assay did not accurately reflect the actual situation, but the results of the two molecular methods were both higher than traditional plate method. However, when the samples were treated by heating, exposure to UV or high pressure, the RNA-based method appeared more suitable than the DNA-based method (see Table 3). All of the treated bacteria could be detected at about 10^7–10^8 cfu/mL by the DNA-based methods, which was similar to the samples at untreated level. Perhaps the genes of the injured bacteria were unable to transcribe and express, despite the fact that they could repair and grow in culture. This would explain the difference in the quantitative results in treated samples between the RNA-based method and the traditional culture method were lower than that of quantitative results in untreated samples.

Studies have been carried out to determine the suitability of using RNA to quantify pathogens by real-time RT-PCR. However, in previously published studies associated with real-time RT-PCR, most of the methods have required one- or two-step enrichment procedures. For example, researchers have quantified *Salmonella enterica* by the RNA-based real-time RT-PCR which consisted of enrichments, and revealed that the RNA-based assay had the potential to detect viable *Salmonella enterica* in ready-to-eat products (McCabe et al., 2011a, 2011b). Also, Miller et al. (2011, 2010) used real-time RT-PCR with a short pre-enrichment (6 h) to detect *Salmonella enterica* in Serrano peppers, Jalapeno, lettuce and tomatoes, and they considered that this method rapidly and successfully detected *Salmonella* in food. Others studied real-time reverse transcription-multiplex PCR for simultaneous and specific detection of *rfbE* and *eae* genes of *E. coli* O157:H7, and deemed that rRT-MPCR could provide important information on the viability of EHEC O157:H7 in feces (Sharma, 2006). For *L. monocytogenes*, Werbrouck et al. (2007) optimized real-time RT-PCR method which this study was based on, and considered many factors could influence the outcome of the real-time RT-PCR data. Another study found that the real-time RT-PCR method containing an enrichment step allowed a better and faster estimation of viable cells from cells following bacteriocin injury in salad (Molinos, Abriouel, Omar, Martinez-Canamero, & Galvez, 2010). Furthermore quantification by real-time RT-PCR depends on how to improve the efficiency and accuracy of quantitative detection.

5. Conclusion

In terms of practicability, the RNA- and DNA-based method have potential to be used for the positive screening of *L. monocytogenes*, and the major advantage of the RNA-based method over the DNA-based method is that it can detect viable *L. monocytogenes* without

enrichments in treated chilled pork (such as vacuum-packaged and high pressure treated). However, it does need more steps to perform and only approximately predicts the level of contamination in chilled pork.

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