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Rapid Quantitative Detection of *Listeria monocytogenes* in Meat Products by Real-Time PCR

David Rodríguez-Lázaro,[1] Anna Jofré,[2] Teresa Aymerich,[2] Marta Hugas,[2]† and Maria Pla[1]*

Institute of Food and Agricultural Technology, University of Girona, Girona,[1] and Institute for Food and Agricultural Research and Technology, Meat Technology Center-IRTA, Monells,[2] Spain

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We describe a quick and simple method for the quantitative detection of *Listeria monocytogenes* in meat products. This method is based on filtration, Chelex-100-based DNA purification, and real-time PCR. It can detect as few as 100 CFU/g and quantify as few as 1,000 CFU/g, with excellent accuracy compared to that of the plate count method. Therefore, it is a promising alternative for the detection of *L. monocytogenes* in meat products.

Listeria monocytogenes is a human pathogen widely distributed in the environment (15, 16, 31). Meat products are a major source of *L. monocytogenes* (9, 21, 22, 24, 27, 29, 32). As clinical cases of listeriosis are usually associated with high loads of *L. monocytogenes* (10, 11) and as it is difficult to eradicate listeriae from the environment of the food processing plants (12), the International Commission on Microbiological Specification for Foods concluded that 100 CFU of *L. monocytogenes* per g of food at the time of consumption is acceptable for nonrisk consumers (14, 19).

Conventional testing methods for the detection of *L. monocytogenes* in food involve growth in preenrichment medium, followed by growth on selective medium and a battery of confirmatory biochemical and serological tests (11). These methods are labor-intensive and time-consuming, often taking up to 10 days. A rapid alternative method is real-time (RTi)-PCR, which allows an accurate and unambiguous identification and a precise quantification of nucleic acid sequences (17, 20). Furthermore, the lack of post-PCR steps reduces the risk of cross-contamination and allows high throughput and automation.

We present a rapid and sensitive assay for the reliable quantitative identification of *L. monocytogenes* organisms in meat products based on a simple and rapid sample handling and RTi-PCR.

Optimization of the assay. In two independent experiments (as recommended in International Organization for Standardization document ISO 16140 [6]), we artificially contaminated 25 g of cooked ham slices (7) containing 2% fat (4) with decreasing amounts of an overnight culture of *L. monocytogenes* CTC 1010 (100 μl of 10-fold dilutions in peptone water to reach from 10^6 to 10 CFU/g). Slices were vacuum packed to allow better distribution of the inoculum and immediately diluted (1:10) with 0.1% peptone–0.85% NaCl and homogenized for 1 min in stomacher bags (125-μm pore size; Biochek). *L. monocytogenes* was identified and quantified in all samples by both standard microbiological methods (according to document ISO 11290 [5]) and RTi-PCR-based methods performed at least in triplicate.

We compared three different pre-PCR filtration treatments: (i) no additional filtration, (ii) filtration through a 22- to 25-μm-pore-size filter (Miracloth filter; Calbiochem), and (iii) filtration through a nylon membrane with an 11-μm pore size (Millipore). In theory, *L. monocytogenes* should not be retained by either of these filters (30). We also tested the convenience of an additional DNA purification and concentration step. Two milliliters of each sample was centrifuged for 5 min at 10,000 × *g* and 4°C. The pellets were suspended in 100 μl of a suspension of 6% Chelex-100 resin (Bio-Rad) in water, incubated at 56°C for 20 min, vortexed, boiled for 8 min, vortexed again, and immediately chilled on ice. Finally, the sample was centrifuged for 5 min at 14,000 × *g*. Chelex-100 is an ion-exchange resin specifically designed for extraction of PCR-ready template DNA. The removal of PCR inhibitors is accomplished by scavenging of contaminating metal ions that catalyze the digestion of DNA. In addition, an improvement in the lysis of gram-positive bacteria has been reported (28). TaqMan-based RTi-PCR assays targeting the *hly* gene (25) were performed in parallel with 1 μl of either the initial filtrate (without nucleic acid isolation) or the Chelex-100 final supernatant. Bacterial concentrations were calculated by interpolation of the cycle threshold (C_T) values to a standard curve constructed with serial dilutions of an *L. monocytogenes* genomic DNA solution previously quantified with PicoGreen (Molecular Probes, Inc., Eugene, Oreg.) in an LS50B luminescence spectrometer (Perkin-Elmer Corp., Norwalk, Conn.).

The inclusion of a Chelex-100-based DNA purification step prior to RTi-PCR considerably increased the sensitivity of the method (Table 1); i.e., detection was consistent down to 10^3 CFU/g and organisms could be detected in at least 50% of the replicates containing 10^2 CFU/g of cooked ham. According to our Chelex-100-based pre-PCR protocols, 10^3 CFU/g renders theoretically 2 genome equivalents per RTi-PCR. Thus, inoculum levels below this one should produce inconsistent RTi-PCR results (i.e., 10^2 CFU/g renders 0.2 genome equivalents per reaction, or 1 genome equivalent with a probability of

* Corresponding author. Mailing address: Institute of Food and Agricultural Technology, University of Girona, Campus Montilivi s/n, E-17071 Girona, Spain. Phone: 34-972 419852. Fax: 34-972 418399. E-mail: maria.pla@udg.es.

† Present address: European Food Safety Authority, B-1140 Brussels, Belgium.

TABLE 1. RTi-PCR-based detection of *L. monocytogenes* with three filtration strategies and with and without Chelex-100-based DNA purification[a]

Approx no. of CFU/g	Signal ratio with indicated treatment					
	Chelex-100			No Chelex-100		
	F1	F2	F3	F1	F2	F3
10^6	+	+	+	+	+	+
10^5	+	+	+	3/6	+	+
10^4	+	+	+	4/6	+	+
10^3	+	+	+	1/6	3/6	3/6
10^2	6/8	4/8	5/8	0/8	1/8	0/8
10^1	0/8	0/8	0/8	0/8	0/8	0/8

[a] F1, F2, and F3 correspond to alternative pre-PCR filtration steps. F1 indicates no filtration, F2 indicates filtration with 22- to 25-μm-pore-size filters, and F3 indicates filtration with an 11-μm-pore-size filter. The approximate numbers of CFU per gram are the sizes of the initial nocula. The signal ratio is the number of positive reactions versus the total number of reactions. + indicates that *L. monocytogenes* DNA was amplified in all six replicates performed in two independent experiments.

20%). Moreover, this result was independent of the filtration conditions. In contrast, RTi-PCR analyses performed directly after filtration were only capable of consistently detecting 10^4 CFU/g. We therefore concluded that a Chelex-100-based DNA purification step is essential to attain a detection limit compatible with the current recommended levels for *L. monocytogenes* (19).

We then evaluated the ability of the method to quantify *L. monocytogenes* organisms in cooked ham. We constructed regression curves of the C_T values obtained from cooked ham samples and the corresponding *L. monocytogenes* inocula. The coefficient of correlations (R^2) (Table 2) demonstrated that the quantification method was linear over a range of four logs, from 10^6 to 10^3 CFU/g. The efficiency of the reaction (18), the optimal value of which is 1 (i.e., with the slope of the regression curve being −3.32 [13]), was determined (Table 2) and indicated that the RTi-PCR performance was excellent (above 0.83) with all of the pre-PCR filtration treatments. Moreover, these values were similar to those obtained when DNA or bacterial pure cultures were analyzed (25). In conclusion, the RTi-PCR assays worked well for samples of cooked ham treated as described above.

One parameter that is critical for the validation of an alternative method is the relative accuracy, i.e., the closeness of agreement between a test result and the accepted reference value (documents ISO 3534–1 [4a] and ISO 16140 [6]). A relative accuracy of 100% indicates total agreement between the alternative method and the reference method. In two independent experiments, we artificially contaminated samples of cooked ham with 100 μl of serial 10-fold dilutions of an overnight *L. monocytogenes* culture (from approximately 10^6 to 10^3 CFU/g). The mean C_T values determined were extrapolated to the corresponding standard regression curve, previously calculated experimentally, and the resulting theoretical CFU numbers were compared to those obtained by the standard *L. monocytogenes* enumeration method (document ISO 11290 [5]). The relative accuracy of the RTi-PCR-based method with respect to the reference plate count method (5) varied from 89.12 to 116.28% (Table 3), which fits with document ISO 16140 recommendations (6). Interestingly, all three filtration strategies produced similar results, indicating that they can all be used.

TABLE 2. Linearity of RTi-PCR C_T values versus numbers of CFU of *L. monocytogenes* organisms per gram of cooked ham[a]

Expt	Treatment	R^2	E
1	F1	0.987	1.09
	F2	0.984	1.02
	F3	0.994	0.83
2	F1	0.967	1.21
	F2	0.998	1.02
	F3	0.994	1.01

[a] Linearity persisted along 4 logarithmic units. R^2 indicates the regression coefficient. PCR efficiency (E) is obtained from the formula $E = 10^{-1/s} - 1$, where S is the slope (13, 18). Experiments 1 and 2 correspond to two independent experiments. F1, F2, and F3 indicate the three different pre-PCR filtration steps: no filtration (F1), filtration through 22- to 25-μm-pore-size filters (F2), and filtration through 11-μm-pore-size filters (F3).

TABLE 3. Relative accuracy of RTi-PCR assay with Chelex-100-based purification for quantification of *L. monocytogenes* organisms[a]

Approx no. of CFU/g	Relative accuracy					
	Expt 1			Expt 2		
	F1	F2	F3	F1	F2	F3
10^6	101.90	103.09	104.87	92.61	92.81	93.38
10^5	103.82	105.28	104.61	89.12	90.30	91.32
10^4	104.36	102.81	106.64	100.12	94.94	101.12
10^3	116.28	105.07	107.96	115.50	103.59	108.35

[a] Experiments 1 and 2 correspond to two independent experiments. F1, F2, and F3 correspond to alternate pre-PCR filtration steps. F1 indicates no filtration, F2 indicates filtration with 22- to 25-μm-pore-size filters, and F3 indicates filtration with an 11-μm-pore-size filter. The approximate numbers of CFU per gram are the sizes of the initial inocula. Relative accuracy was calculated as a percentage of log numbers of CFU per gram, obtained by the RTi-PCR-based method and the *L. monocytogenes* standard enumeration method (5). The three filtration strategies did not generate statistically different results ($P > 0.05$).

We previously demonstrated that the RTi-PCR assay used in this work was appropriate for the precise quantification of an *L. monocytogenes* strain regardless of its genetic background by extensive evaluation of the assay using a large panel of isolates of serovars of this bacterium. The homogeneous results obtained for isolates representative of the three phylogenetic divisions of the species indicated that this assay can be applied to the entire species *L. monocytogenes* (25). Therefore, it is likely that the methods here developed are suitable for the detection and quantification of *L. monocytogenes* in cooked ham regardless of the serovar.

Application to other meat products. We assessed the applicability of our method to different meat products (according to the recommendations of document ISO 16140 [6]): raw pork (4.99% fat), Frankfurter sausages (19.8% fat), and fermented sausages (38.2% fat). In three independent experiments, slices of each meat product were artificially contaminated with decreasing amounts of an *L. monocytogenes* CTC 1010 overnight culture and analyzed by both standard microbiological and RTi-PCR-based methods. The sensitivity and quantification capacity of the RTi-PCR method were consistently as good as for cooked ham. With the 11-μm-pore-size filtration strategy, consistent detection was achieved down to 10^2 CFU/g (Table 4) and excellent relative accuracy values were obtained for the

TABLE 4. Accuracy of the quantification of *L. monocytogenes* organisms by RTi-PCR in three different food matrices[a]

Approx no. of CFU/g	Relative accuracy		
	Raw pork	Frankfurter sausages	Fermented sausages
10^6	101.12 ± 0.30	99.77 ± 0.52	98.62 ± 1.30
10^5	97.99 ± 1.37	100.60 ± 0.41	101.77 ± 3.85
10^4	100.16 ± 4.11	99.48 ± 2.56	103.80 ± 1.45
10^3	100.88 ± 2.89	100.12 ± 2.15	94.98 ± 2.53
10^2	BQ	BQ	BQ

[a] Samples were filtered through nylon membranes with 11-μm pores. Approximate numbers of CFU per gram are initial sizes of the inocula. Relative accuracy was calculated as a percentage of log numbers of CFU per gram obtained by the RTi-PCR-based method and the *L. monocytogenes* standard method of enumeration (5). Values are expressed as means ± standard deviations of the results obtained in three independent experiments. All samples tested positive ($n = 9$). BQ, below the range of quantification.

three matrices down to 10^3 CFU/g (Table 4). Thus, the method of choice combined pre-PCR filtration through nylon membrane with 11-μm pores and Chelex-100-based purification. This method can be used to analyze a spectrum of meat products that differ in the ways they are processed, fat contents, or accompanying microbiotas; it is quick and easy to perform, which is especially relevant for protocols for routine use in food microbiology laboratories.

As foods are complex matrices, several publications report on filtration or Chelex-100-based protocols for PCR detection of various pathogenic species (reviewed in references 23 and 28); however, to our knowledge they have never been used with quantitative purposes. Most available detection systems require selective enrichment steps to overcome the problem of potential PCR inhibitors, especially for low pathogen concentrations (1, 2, 3, 8, 26). Remarkably, our method does not require any culture steps, meaning that results can be obtained considerably quicker. Moreover, it is compatible with the ISO methods for detection and for enumeration of *L. monocytogenes*. The RTi-PCR assay (25) could be used in combination with our pre-PCR strategy (single filtration step and Chelex DNA purification) as a complementary routine technique for the quick quantification of *L. monocytogenes* down to 1,000 CFU/g and detection of down to 100 CFU/g in meat products.

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