Short Report

Development and In-House Validation of a Real-Time Polymerase Chain Reaction for the Detection of *Listeria monocytogenes* in Meat

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Abstract

Listeriosis is a foodborne disease caused by *Listeria monocytogenes*. The aims of this work were to develop and validate an in-house real-time polymerase chain reaction (RT-PCR) for the detection of *L. monocytogenes*, and to determine its prevalence in raw ground beef samples from 53 butcheries that also sell ready-to-eat foods. One set of primers and one hydrolysis probe were designed for *hly* gene detection and then challenged with pure strains. The detection was successful for all *L. monocytogenes* strains analyzed and negative for all non-*L. monocytogenes* strains (detection limit, 10 colony forming unit [CFU]/mL). Inclusivity, exclusivity, and analytical accuracy were 100%. *L. monocytogenes* was detected in 41.5% of raw ground beef samples from the 53 butcheries analyzed. This RT-PCR may be a valuable method for rapid detection of *L. monocytogenes* in meat.

Keywords: *Listeria*, detection, RT-PCR, validation, meat

THE DISEASE CAUSED BY *Listeria monocytogenes* can range from a mild febrile illness to a systemic (invasive) listeriosis. Raw meat can be a source for *L. monocytogenes* dissemination in the environment of retail markets or consumers' kitchens, and can cause cross-contamination with ready-to-eat (RTE) products (Luo *et al.*, 2017). Traditional methodologies to search for *L. monocytogenes* in food are sensitive and remain the "gold standard" as compared with other methods (Gasanov *et al.*, 2005). However, real-time polymerase chain reaction (RT-PCR) is being increasingly applied for the detection of *L. monocytogenes* in food (Junge *et al.*, 2012). The aims of this work were, therefore, (1) to develop and validate an in-house RT-PCR to detect *L. monocytogenes* and (2) to determine *L. monocytogenes* prevalence with the RT-PCR developed in raw ground beef samples from 53 butcheries that also sell RTE foods.

One set of primers and one hydrolysis probe were designed for *hly* gene detection (encoding the pore-forming cytolysin listeriolysin). The DNA sequences of *hly* from *L. monocytogenes* strains were recovered from GenBank and aligned using PriSM software (Broad Institute, Cambridge, MA). For the design, a conserved sequence region was selected, based on multiple alignments of the genes using Primer Express 3.0 software. The primer and hydrolysis probe sequences designed were (5′–3′): Forward CACAAGTGGTAAGTTCCGGTCA, Reverse TTGCCAGGTAACGCGAGAAA, and Probe FAM-CCGTTCTCCACCATTCCCAAGC-3IABkFQ. All primers and probes were purchased from Integrated DNA Technologies (IDT, Coralville, IA).

The RT-PCR designed was challenged with 12 *L. monocytogenes* strains, 3 *Listeria seeligeri*, 3 *Listeria welshimeri*, and 3 *Listeria innocua* strains. The detection was successful for all *L. monocytogenes* strains analyzed and negative for all non-*L. monocytogenes* strains. To define the limit of detection (LOD), DNA of 12 *L. monocytogenes* strains at 10^1 to 10^4 colony forming unit (CFU) was analyzed. LOD was 10 CFU/mL for all strains, consistent with a previous report (Gattuso *et al.*, 2014). Robustness was determined by the amplification of DNA extracts from tubes containing 10^2 CFU/mL of 12 *L. monocytogenes* strains, in three alternate days and by two operators with different training level. The same results were obtained when the previously mentioned variables were introduced.

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TABLE 1. STRAINS USED FOR EXCLUSIVITY ASSAY OF REAL-TIME POLYMERASE CHAIN REACTION

Bacterial species	Bacterial species
Pseudomona aeruginosa	Proteus mirabilis
Yersinia enterocolitica	S. flexneri
Shigella sonnei	Proteus vulgaris
Salmonella enterica serovar Enteritidis	E. coli (enteropathogenic)
Shigella flexneri	Shigella dysenteriae
Edwardsiella tarda	Morganella morgnaii
Salmonella enterica serovar Dublin	E. coli (enterotoxigenic)
Salmonella enterica serovar Meleagridis	Salmonella enterica serovar Senftenberg
Escherichia coli	Salmonella enterica serovar Typhimurium
S. flexneri	Klebsiella pneumoniae
Salmonella enterica serovar Derby	E. coli
E. coli (enteroaggregative)	Hafnia alvei
P. aeruginosa	Enterobacter cloacae
E. coli (enteroinvasive)	Shigella dysenteriae
S. flexneri	E. coli (enterohemorrhagic)

These strains were used to determine the ability to not detect *Listeria monocytogenes*.

For RT-PCR validation, 43 strains of *L. monocytogenes* and 30 strains of non-*L monocytogenes* (Table 1) belonging to the collection of IGEVET (Instituto de Genética Veterinaria "Ing. Fernando Noel Dulout"; UNLP-CONICET) were used. They were stored at −70°C and grown in 4 mL brain heart infusion broth (Biokar, Zac de Ther, France) at 37 ± 1°C for 18–24 h. Serial strain dilutions were performed in buffered peptone water (Biokar) and the inoculum level was confirmed by plating on plate count agar (Britania, BA, Argentina). Plates were incubated at 37°C for 18–24 h. DNA extraction was made according to Leotta *et al.* (2005). The reaction mixture contained 10 μL of Master mix qPCR Probe (PB-L, Buenos Aires, Argentina), 0.4 μL of 10 μM of each forward and reverse primers, 0.2 μL of 100 μM probe, and 4 μL of DNA in a final volume of 20 μL. Strain ATCC 19115 and molecular biology grade water were included as external positive and no-template control in each run. Cycling used was initial hot-start step at 95°C for 3 min, followed by 40 cycles of a denaturation step at 95°C for 15 s and an annealing/extension step at 60°C for 30 s. Fluorescence was recorded only at the end of the annealing/extension step. The cycle threshold value was <34.

A total of 118 portions of 10 g raw ground beef were obtained from a commercial retail or store, in the same day and confirmed to be *L. monocytogenes*-free according to ISO 11290-1:1996/Amd.1:2004(E). Serial dilutions of 43 *L. monocytogenes* strains and 30 non-*L. monocytogenes* strains (Table 1) were done as described previously. Meat samples were inoculated with 10 (*n* = 43) and 100 (*n* = 43) CFU of *L. monocytogenes* per gram, and 30 meat samples were inoculated with 10³ CFU of non-*L. monocytogenes* per gram. Two samples were mock-inoculated with 1 mL of sterile Half Fraser Broth (HFB) (Becton Dickinson, Le Pont de Claix, France). Then, each sample was pre-enriched in 90 mL HFB for 24 h at 30°C and subsequently analyzed according to the reference culture method ISO 11290-1:1996/Amd.1:2004(E) and the in-house developed RT-PCR. DNA extraction was performed by foodproof® Short Prep II (Biotecon Diagnostics, Postdam, Germany) after the pre-enrichment step. Then the PCR analysis was done following the protocol already described. Inclusivity, exclusivity, and analytical accuracy were 100%. These results are in line with other RT-PCR methods for *L. monocytogenes* detection validated in meat products (Garrido *et al.*, 2012; Heo *et al.*, 2014).

L. monocytogenes can multiply at refrigeration temperatures, form biofilms on different materials and under various conditions, and resist a range of environmental stresses (Law *et al.*, 2015). Thus, the presence of this bacterium in retail shop environments is a risk due to the possibility of cross-contamination with food products (Leotta *et al.*, 2016). We, therefore, conducted a field study to apply and validate the RT-PCR developed. Raw ground beef samples from 53 butcheries that also sell RTE foods including cooked products, cheeses, vegetables, and/or cold meats were analyzed. Raw ground beef samples were analyzed according to ISO 11290-1:1996/Amd.1:2004(E) and the RT-PCR developed. *L. monocytogenes* was simultaneously detected by RT-PCR and isolated from raw ground beef in 22 of the 53 butcheries analyzed (41.5%). The prevalence of *L. monocytogenes* obtained in this study was higher than that reported in Algeria (8.9%) and Malaysia (9.0%), but lower than that obtained in Turkey (45.0%) and Brazil (48.7%) (Du *et al.*, 2017).

The RT-PCR developed showed adequate performance with pure strains, artificially contaminated raw ground beef samples, and naturally contaminated samples. This screening could allow a rapid intervention on contaminated food and the environment where it is produced. Moreover, the short time required to obtain a negative result could allow a fast verification on the procedures of meat and meat products or a fast commercialization of ground beef. In the future, the evaluation of this assay on other food matrices may also extend its application in food safety analyses.

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Disclosure Statement

No competing financial interests exist.

References

Du X, Zhang X, Wang X, Su Y, Li P, Wang S. Isolation and characterization of *Listeria monocytogenes* in Chinese food obtained from the central area of China. Food Control 2017;74:9–16.

Garrido A, Chapela MJ, Román B, Ferreira M, Lago J, Vieites JM, Cabado AG. Development of a multiplex real-time PCR method for simultaneous detection of *Salmonella enterica*, *Shigella flexneri* and *Listeria monocytogenes* in processed food samples. Eur Food Res Technol 2012;234:571–580.

Gasanov U, Hughes D, Hansbro PM. Methods for the isolation and identification of *Listeria* spp. and *Listeria monocytogenes*: A review. FEMS Microbiol Rev 2005;29:851–875.

Gattuso A, Gianfranceschi MV, Sonnessa M, Delibato E, Marchesan M, Hernandez M, De Medici D, Rodriguez-Lazaro D. Optimization of a Real Time PCR based method for the detection of *Listeria monocytogenes* in pork meat. Int J Food Microbiol 2014;184:106–108.

Heo EJ, Song BR, Park HJ, Kim YJ, Moon JS, Wee SH, Kim JS, Yoon Y. Rapid detection of *Listeria monocytogenes* by real-time PCR in processed meat and dairy products. J Food Prot 2014;77:453–458.

Junge B, Grönewald C, Berghof-Jäger K. BIOTECON diagnostics foodproof *Listeria monocytogenes* Detection Kit, 5′ nuclease in combination with the foodproof ShortPrep II Kit. J AOAC Int 2012;95:92–99.

Law JW, Ab Mutalib NS, Chan KG, Lee LH. An insight into the isolation, enumeration, and molecular detection of *Listeria monocytogenes* in food. Front Microbiol 2015;6:1227.

Leotta GA, Brusa V, Galli L, Adriani C, Linares L, Etcheverría A, Sanz M, Sucari A, Peral García P, Signorini M. Comprehensive evaluation and implementation of improvement actions in butcher shops. PLoS One 2016;11:e0162635.

Leotta GA, Chinen I, Epszteyn S, Miliwebsky E, Melamed IC, Motter M, Ferrer M, Marey E, Rivas M. Validación de una técnica de PCR múltiple para la detección de *Escherichia coli* productor de toxin shiga. Rev Arg Microbiol 2005;37:1–10.

Luo L, Zhang Z, Wang H, Wang P, Lan R, Deng J, Miao Y, Wang Y, Wang Y, Xu J, Zhang L, Sun S, Liu X, Zhou Y, Chen X, Li Q, Ye C. A 12-month longitudinal study of *Listeria monocytogenes* contamination and persistence in pork retail markets in China. Food Control 2017;76:66–73.

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