



REAL-TIME PCR DETECTION OF *LISTERIA MONOCYTOGENES* IN FOOD SAMPLES OF ANIMAL ORIGIN

Jaroslav Pochop*¹, Miroslava Kačániová¹, Lukáš Hleba¹, Jana Petrová¹,
Lubomír Lopašovský², Adriana Pavelková³, Alica Bobková²

Address: ¹ Department of Microbiology, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Trieda Andreja Hlinku 2, 949 76 Nitra, Slovakia;

² Department of Food Hygiene and Safety, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Trieda Andreja Hlinku 2, 949 76 Nitra, Slovakia;

³ Department of Animal Products Evaluation and Processing, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Trieda Andreja Hlinku 2, 949 76 Nitra, Slovakia.

*Corresponding author: pochop.jaroslav@gmail.com

ABSTRACT

The aim of this study was to follow the contamination of food with *Listeria monocytogenes* by using Step One real time polymerase chain reaction (PCR). We used the PrepSEQ Rapid Spin Sample Preparation Kit for isolation of DNA and SensiFAST SYBR Hi-ROX Kit for the real-time PCR performance. In 24 samples of food of animal origin without incubation were detected strains of *Listeria monocytogenes* in 15 samples (swabs). Nine samples were negative. Our results indicated that the real-time PCR assay developed in this study could sensitively detect *Listeria monocytogenes* in food of animal origin without incubation. This could prevent infection caused by *Listeria monocytogenes*, and also could benefit food manufacturing companies by extending their product's shelf-life as well as saving the cost of warehousing their food products while awaiting pathogen testing results. The rapid real-time PCR-based method performed very well compared to the conventional method. It is a fast, simple, specific and sensitive way to detect nucleic acids, which could be used in clinical diagnostic tests in the future.

Keywords: Real-time PCR, *Listeria monocytogenes*, detection kit, ready-to-eat food

INTRODUCTION

Classical microbiological methods for detection of food-borne bacteria involve the use of pre-enrichment and/or specific enrichment, followed by the isolation of the bacteria in solid media and a final confirmation by biochemical and/or serological tests (Reed *et al.*, 2000). Determining bacterial viability is a key issue for the application of food risk management; thus a rational approach for detecting only viable bacterial cells by using molecular-based methods is necessary. However, the PCR does not distinguish among viable and dead bacterial cells. DNA from bacterial dead cells can serve as a template for the PCR many days after cell viability has been lost (Allmann *et al.*, 1995; Rodríguez-Lázaro *et al.*, 2007). All over the world, public health agencies are concerned with food safety assurance due to globalization of food markets, growing demand for minimally processed ready-to-eat (RTE) foods and increasing numbers of meals served outside home (Reed *et al.*, 2000) *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella* spp. are among the most commonly studied food-borne pathogens and are of major concern because of their association with foods such as poultry, ready-to-eat products, dairy products, fruits and vegetables (Allmann *et al.*, 1995). High populations of *L. monocytogenes* have been reported for some RTE products, including outbreak samples, but the growth rate of *L. monocytogenes* in vegetables is generally lower compared to milk, dairy and processed meat products (Chen *et al.*, 2006). It is also important to consider that minimally processed vegetables are often used as ingredients to pre-prepare salads, which may have longer refrigerated shelf lives, representing a risk of increasing of *L. monocytogenes* population (Aguado *et al.*, 2004). Most cases of listeriosis are caused by the ingestion of *L. monocytogenes* - contaminated RTE foods that do not require heating prior to consumption (Gasnov *et al.*, 2005). Diagnosis of listeriosis and detection of *L. monocytogenes* is commonly based on classical culturing and serological identification methods that are laborious and time-consuming, requiring up to ten days for completion and often suffer from variability due to their dependence on the phenotypic characteristics of the bacteria. (Liu *et al.*, 2009). Different PCR-based diagnostics for detection of *L. monocytogenes* have been increasingly applied, however, complex sample preparation methods and especially the use of gel electrophoresis endpoint detection have

hampered the transition from research to routine use in food microbiology laboratories (Liu et al., 2009; Churchill et al., 2006; Levin, 2003). Application of real-time PCR surmounts these shortcomings by removing the manipulation of the PCR products after amplification, thus reducing the risk of false-positive results. Furthermore, results with better sensitivity and specificity can be obtained in an hour (Norton, 2002). Considerable reagent cost is one of the key factors, beside standardization and validation issues (Raymaekers et al., 2009), that influence wider routine adoption of real-time PCR-based tests in diagnostic laboratories. In a high-throughput setting a reduction in the reagent volume used in each reaction significantly reduces the cost of diagnosis.

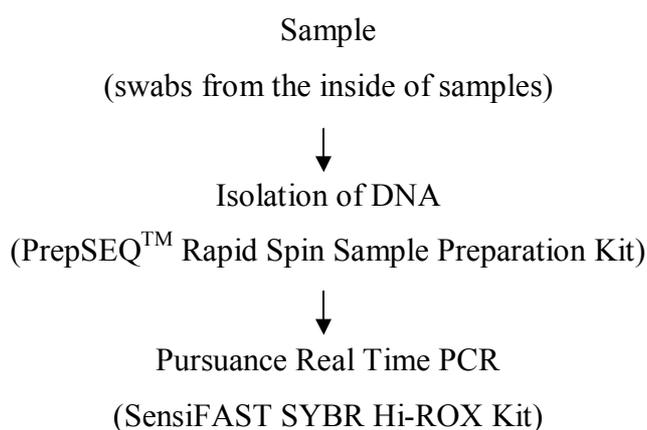
The aim of this study was to follow the contamination of food of animal origin with *Listeria monocytogenes* by Step One real-time PCR.

MATERIAL AND METHODS

Food Samples

A total of 24 food samples were used in this study. The samples were obtained by taking swabs from the inside of food of animal origin (fish). After the taking of the samples, we were advanced as shown in the Scheme 1.

Scheme 1 Progress after taking the samples



General Sample Preparation Protocol

Load 750 μL of sample onto the spin column and cap the column. Microcentrifuge for 3 min at maximum speed. Aspirate, then discard the supernatant. Add 50 μL of Lysis Buffer to the pellet. Resuspend by pipetting up and down, or vortex until the pellet is resuspended. Cap the tube, then incubate at 95 $^{\circ}\text{C}$ for 10 min. Allow the sample to cool for 2 min at room temp. Microcentrifuge for 1 min at maximum speed. Add 250 μL of water. Microcentrifuge for 1 min at maximum speed. Proceed with PCR, or store the tube at -20°C .

Measuring the concentration of DNA-UV-spectrophotometric quantification of DNA

The absorbance at a wavelength of 260 nm was measured and DNA concentration was calculated on base of the observation that double stranded DNA solution with a concentration of 5 $\mu\text{g.mL}^{-1}$ has a density about 0.1. To determine the contamination of the protein preparation the additional measuring absorbance at 280 nm was used. Preparation is considered as uncontaminated if proteins A260/A280 are between 1.8 and 2.0. Spectrophotometric measurements were done using UV 1101 photometer (Biotech, UK).

Thermal cycling conditions were as follows: 3 minutes of incubation at 95 $^{\circ}\text{C}$, followed by 40 cycles of 5 sec. denaturation at 95 $^{\circ}\text{C}$ and 15 sec. annealing and elongation at 60 $^{\circ}\text{C}$. Data were collected during each elongation step.

Reaction mix composition

Sensi Fast sybr Hi-ROX Mix	10 μL
Forward Primer	0.8 μL
Reverse Primer	0.8 μL
H ₂ O	9.4 μL
Template	4 μL

Listeria monocytogenes. Primer name:

- Division I: Primer D1 5' CGA TAT TTT ATC TAC TTT GTC A 3' and D1 5' TTG CTC CAA AGC AGG GCA T 3'.

PCR products were detected by monitoring the increase in fluorescence of the reporter dye at each PCR cycle. Applied Biosystems software plots the normalized reporter signal, ΔR_n , (reporter signal minus background) against the number of amplification cycles and also determines the threshold cycle (Ct) value i.e. the PCR cycle number at which fluorescence increases above a defined threshold level.

RESULTS AND DISCUSSION

The most sensitive detection of *Listeria monocytogenes* was obtained using PrepSEQTM Rapid Spin Sample Preparation Kit and SensiFAST SYBR Hi-ROX Kit A detection kit compatible with StepOneTM Systems was less time-consuming than the other methods and relatively easy to use. Thus, the PCR-based detection of bacteria depends on the efficiency of DNA extraction procedure used to prepare the template DNA. In our samples without incubation we could detect strain of *Listeria monocytogenes* in 15 out of 24 samples (swabs), as it is shown in Figure 1. Nine samples were negative.

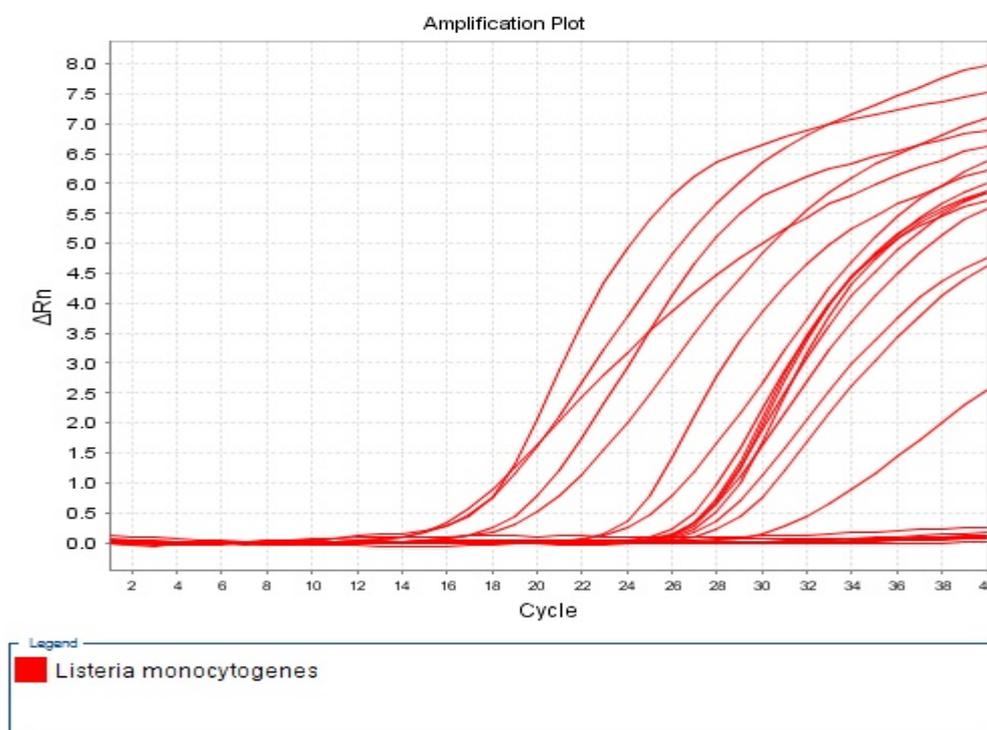


Figure 1 Real-time PCR detection of *Listeria monocytogenes* without incubation

The (Ct) value of positive listeria samples was on average 20.95, whereby the lowest value of positive listeria samples was found at 7.7 and the highest value was at 27.73. The

minimum level of detection was 2.5 genome equivalents (GE) per reaction (positive amplification in all replicates) with the CT value of 36.6 ± 1.6 . The real-time PCR assay we developed was approximately 1000-fold more sensitive than conventional PCR assays (gel electrophoresis after PCR) for detection of *L. monocytogenes*. At lower detection limit, CT values were 37.3, 38.5, and 39.3 for apple, watermelon, and grape juices, respectively (**Hye-Jin et al., 2010**). **Berrada et al. (2006)** performed a study on the presence or absence of *L. monocytogenes*, in 77 different types of salads served in restaurants in Valencia (Spain), by real-time PCR and conventional methods. **Rantsiou et al., (2008)** also used the technique of real-time PCR to quantify *L. monocytogenes* in 66 different samples of foods. They detected four positive samples without pre-enrichment and, nine positive samples after 24–37 hours enrichment. With careful target selection and construction of primers and probe with optimization of the assay it is possible to develop the real-time PCR assay that is conducted in the 5 μ L volume. Developed assays for quantitative detection and identification of *L. monocytogenes* have the potential to meet all these criteria as reliable, fast, sensitive and specific method. The reduction of total reaction volume significantly reduces the cost of diagnosis and increases potential for automation allowing increased samples throughput for analytical laboratories (**Rantsiou et al., 2008**). Exogenous internal controls can also be designed to be co-amplified with the pathogen specific primers but have an altered nucleic acid sequence internal to the primers (i.e. a small deletion, insertion or mutation) allowing their differentiation from the pathogen amplicon (**Stocher et al., 2003**). Loss of sensitivity or inhibition can cause false negative PCR results, the reporting of which may lead to contaminated foods being made available for human consumption (**Nogva et al., 2000**). The increase of listeriosis cases in the European Union may not only be seen as the cause for needing and developing rapid and reliable methods for the food industry and official control bodies. This increase may also have several other reasons such as changes of surveillance systems, improved rates of reportings, changes of consumption behaviour and growing life expectancy (**Warriner and Namvar, 2009**).

CONCLUSION

The rapid real-time PCR based method performed very well compared to the conventional method. It is a fast, reproducible, simple, specific and sensitive way to detect nucleic acids, which could be used in clinical diagnostic tests in the future. Conventional PCR methods need amplification product separation by gel electrophoresis followed by

hybridization with a probe. These time-consuming protocols are now gradually being replaced by more convenient and rapid real-time PCR assays. The decision to find “the most suitable” method for the detection and tracing of *Listeria monocytogenes* in food processing is difficult as information should not only relate to the presence of the pathogen, but also to useful information about transmission routes and types of strains correlating to raw-materials, mid-products, end-products and product environment. This will further contribute to better understanding the contamination and transmission routes of food-borne pathogens. The application of molecular methods can result in a better comprehension of the spread of a specific pathogen in a processing plant, thereby allowing the implementation of corrective actions to eliminate or decrease the risk associated with its presence in the final product. Our results indicate that the Step One real-time PCR assay developed in this study could sensitively detect *Listeria monocytogenes* in food of animal origin. The rapid real-time PCR-based method performed very well compared to the conventional method. It is a fast, simple, specific and sensitive way to detect nucleic acids, which could be used in clinical diagnostic tests in the future.

Acknowledgments: This study was supported by KEGA Cultural and Educational Grant Agency no. 013SPU-4/2012.

REFERENCES

- AGUADO, V. – VITAS, A. I. – GARCIA-JALON, I. 2004. Characterization of *Listeria monocytogenes* and *Listeria innocua* from a vegetable processing plant by RAPD and REA. In *International Journal of Food Microbiology*, vol. 90, 2004, p. 341–347.
- ALLMANN, M. – HOFLEIN, C. – KOPPEL, E. – LUTHY, J. – MEYER, R. – NIEDERHAUSER, C. – WEGMULLER, B. – CANDRIAN, U. 1995. Polymerase chain reaction (PCR) for detection of pathogenic microorganisms in bacteriological monitoring of dairy products. In *Research of Microbiology*, vol. 146, 1995, p. 85-97.
- BERRADA, H. – SORIANO, J. M. – PIC ´ O, Y. – MANES, J. 2006. Quantification of *Listeria monocytogenes* in salads by real time quantitative PCR. In *International Journal of Food Microbiology*, vol. 107, 2006, p. 202–206.
- CHEN, Y. – ROSS, W.H. – GRAY, M.J. – WIEDMANN, M. – WHITING, R.C. – SCOTT, V.N. 2006. Attributing risk to *Listeria monocytogenes* subgroups: dose response in relation to genetic lineages. In *Journal of Food Protection*, vol. 69, 2006, p. 335–344.

- CHURCHILL, R. L. T. – LEE, H. – HALL, J. C. 2006. Detection of *Listeria monocytogenes* and the toxin listeriolysin O in food. In *Journal of Microbiology Methods* vol. 64, 2006, n. 2, p. 141–170.
- GASANOV, U. – HUGHES, D. – HANSBRO, P. M. 2005. Methods for the isolation and identification of *Listeria* spp. and *Listeria monocytogenes*: a review. In *FEMS Microbiological Review*, vol. 29, 2005, p. 851–875.
- HYE-JIN, K. – JAE-CHANG, CH. 2010. Simple and rapid detection of *Listeria monocytogenes* in fruit juice by real-time PCR without enrichment culture. In *Food Control*, vol. 21, 2010, p. 1419–1423.
- LEVIN, R. E. 2003. Application of the polymerase chain reaction for detection of *Listeria monocytogenes* in foods: a review of methodology. In *Food Biotechnology*, vol. 17, 2003, p. 99-116.
- LIU, D. – BUSSE, H. J. 2009. *Listeria*. In *Molecular Detection of Foodborne Pathogens*, Liu, D., Ed.; Taylor & Francis Group, CRC Press: Boca Raton, FL, 2009, p. 207–220.
- NOGVA, H. K. – RUDI, K. – NATERSTAD, K. – HOLCK, A. – LILLEHAUG, D. 2000. Application of 5'-nuclease PCR for quantitative detection of *Listeria monocytogenes* in pure cultures, water, skim milk, and unpasteurized whole milk. In *Applied and Environmental Microbiology*, vol. 66, 2000, p. 4266-4271.
- NORTON, D. M. 2002. Polymerase chain reaction-based methods for detection of *Listeria monocytogenes*: toward real-time screening for food and environmental samples. In *Journal of AOAC International*, vol. 85, 2002, p. 505–515.
- RANTSIOU, K. – ALESSANDRIA, V. – URSO, R. – DOLCI, P. – COCOLIN, L. 2008. Detection, quantification and vitality of *Listeria monocytogenes* in food as determined by quantitative PCR. In *International Journal of Food Microbiology*, vol. 121, 2008, p. 99–105.
- RAYMAEKERS, M. – SMETS, R. – MAES, B. – CARTUYVELS, R. 2009. Checklist for optimization and validation of real-time PCR assays. In *Journal of clinical Laboratory Analysis*, vol. 23, 2009, p. 145–151.
- REED, B.A. – GRIVETTI, L.E. 2000. Controlling on-farm inventories of bulk-tank raw milk: An opportunity to protect public health. In *Journal of Dairy Sciences*, vol. 83, 2000, p. 2988-2991.
- RODRÍGUEZ-LÁZARO, D. – LOMBARD, B. – SMITH, H. – RZEZUTKA, A. – D'AGOSTINO, M. – HELMUTH, R. – SCHROETER, A. – MALORNY, B. – MIKO, A. – GUERRA, B. – DAVISON, J. – KOBILINSKY, A. – HERNÁNDEZ, M. – BERTHEAU, Y. – COOK, N. 2007. Trends in analytical methodology in food safety and quality: monitoring

microorganisms and genetically modified organisms. In *Trends of Food Sciences and Technology*, vol. 18, 2007, p. 306-319.

STOCHER, M. – LEB, V. – BERG, J. 2003. A convenient approach to the generation of multiple internal control DNA for a panel of real-time PCR assays. In *Journal of Virological Methods*, vol. 108, 2003, p. 1-8.

WARRINER, K. – NAMVAR, A. 2009. What is the hysteria with Listeria? In *Trends in Food Science and Technology*, vol. 20, 2009, p. 245–254.