DETECTION OF THE BACTERIAL SPECIES *LISTERIA MONOCYTOGENES* IN FOODS OF ANIMAL ORIGIN BY STEP ONE REAL-TIME PCR

Jaroslav Pochop, Miroslava Kačániová, Lukáš Hleba, Simona Kunová

ABSTRACT

The aim of this study was to follow the contamination of sheep cheese with *Listeria monocytogenes* by using the real-time PCR. Classical microbiological methods for detection of food-borne bacteria involve the use of pre-enrichment and/or specific enrichment, followed by the isolation of bacteria in solid media and the final confirmation by biochemical and/or serological tests. We used the PrepSEQ Rapid Spin Sample Preparation Kit for isolation of DNA and MicroSEQ® *Listeria monocytogenes* Detection Kit for pursuance the Step One real-time PCR (Applied Biosystems). In the investigated samples without incubation we could detect strain of *Listeria monocytogenes* in two positive controls and five samples were negative, as well as internal positive control (IPC), which was positive in all samples. This Step One real-time PCR assay is extremely useful for any laboratory equipped by Step One real-time PCR. It is fast, reproducible, specific and sensitive way to detect nucleic acids, which could be used in clinical diagnostic tests in the future. Our results indicated that the real-time PCR assay developed in this study could sensitively detect *Listeria monocytogenes* in sheep cheese.

Keywords: real-time PCR, *Listeria monocytogenes*, food, detection

INTRODUCTION

Facultative anaerobic Gram-positive *Listeria monocytogenes* is a food-borne pathogen widely distributed in nature. It is commonly found in decaying vegetation, soil and water. Various dairy products, meat products, and types of seafood have been reported to be contaminated with this pathogen and are implicated in sporadic as well as epidemic cases of listeriosis **(Danielsson-Tham et al., 2004)**.

Rapid, cost-effective and automated food-borne pathogen detection and identification continues to be of major concern to the food industry and public health laboratories worldwide **(Malorny et al., 2004)**.

PCR and more recently real-time PCR technologies have become powerful diagnostic tools for the analysis of microorganisms in food and can potentially fulfil the requirements of the industry. Validation of PCR and real-time PCR based methods for pathogen detection in food is essential if such new technologies are to be adopted by the food testing industry on a large scale **(Malorny et al., 2003)**.

Traditional methods for detecting *L. monocytogenes* rely on selective enrichment and subsequent culturing on a selective medium, followed by isolation, biochemical identification, and, sometimes, serological confirmation **(Donnelly, 2002)**.

These methods are laborious and usually take several days to give a positive result. Recently, the rapid detection of *L. monocytogenes* in food system by real-time PCR has been reported **(Nogva et al., 2000; Hein et al., 2001)**.

The traditional method comprises selective enrichments (24–48 h) and isolation on selective media (48 h), followed by serological and/or biochemical species–specific identification. The recommended standard methods, for isolation of *L. monocytogenes* take five days to confirm a negative result and up to 10 days to confirm a positive result **(Grady et al., 2008)**.

In recent years, PCR-based methods have been reported as a rapid, specific and sensitive alternative, and have been increasingly used to identify several microbial species from food **(Mareček, 2005)** and clinical samples **(Hoorfar et al., 2003).**

Listeria monocytogenes is a ubiquitous food-borne pathogen responsible for causing Listeriosis a fatal disease of public health concern. *L. monocytogenes* infections are particularly dangerous to certain risk groups, including pregnant women, the elderly, newborns and immunocompromised patients **(Liu, 2006)**.

L. monocytogenes may enter the food chain through carrier animals that shed the organism in the milk and feces due to the microorganism resistance to adverse environmental conditions **(Chan et al., 2007)**.

Several large outbreaks of listeriosis have been associated with contaminated food such as vegetables, dairy products as soft cheeses, pasteurized milk and meat products, on which *L. monocytogenes* can multiply even at low temperatures **(Chaturongakul et al., 2006)**.

The aim of this study was to detect *Listeria monocytogenes* by Step One real-time PCR in sheep cheese.

MATERIAL AND METHODOLOGY

Food samples

Total of ten food samples, two positive controls and one negative control were used in this study. As the aim of investigation were observed milk products. Samples were obtained by taking swabs from the inside of the sheep cheese. After sampling, procedure shown in the Scheme 1 was used.

Scheme 1 Progress after sampling

Sample
(Swabs from the inside of samples)
↓
Isolation of DNA
(PrepSEQ™ Rapid Spin Sample Preparation Kit)
↓
Pursuance Real Time PCR
(MicroSEQ *Listeria monocytogenes* Detection Kit)

Bacterial Strains and DNA Extraction

As a pre-preparation step for the Step One real-time PCR, DNA extraction was performed using DNA extraction method: PrepSEQ Rapid Spin Sample Preparation Kit (Applied Biosystems, USA).

General Sample Preparation Protocol

Sample of 750 µL was loaded onto the spin column and microcentrifuged for 3 minutes at maximum speed. Supernatant was discarded and to the pellet was added 50 µL of Lysis Buffer. Samples were incubated for 10 minutes at 95°C.

MicroSEQ® *Listeria monocytogenes* Detection Kit

Assay to amplify the polymerase chain reaction (PCR) a unique microorganism specific DNA target sequence and a TaqMan® probe to detect the amplified sequence were used. 8-tube strips containing assay beads compatible with StepOne™ Systems were used. Samples of 30 µL to the lyophilized beads were loaded. MicroAmp® 48-Well Base and the MicroAmp® Cap Installing Tool to the tubes were used. MicroAmp® Fast 48-Well Tray on the sample block of the StepOne System was used.

Real-time PCR

Thermal cycling conditions were as follows: 2 minutes of incubation at 95°C, followed by 40 cycles of 1 sec. denaturation at 95°C and 20 sec. annealing and elongation at 60°C. Data were collected during each elongation step. 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, ΔRn, (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 were used.

RESULTS AND DISCUSION

The most sensitive detection of *Listeria monocytogenes* was obtained using PrepSEQ™ Rapid Spin Sample Preparation Kit and MicroSEQ® Listeria monocytogenes Detection Kit compatible with StepOne™ Systems, which was also less time-consuming than the other methods and was relatively easy to use. Thus, the PCR-based detection of bacteria depends on the efficiency of the DNA extraction procedure used to prepare the template DNA. In the investigated samples without incubation we could detect strain of *Listeria monocytogenes* in two positive controls, five samples were negative and the negative control was negative also, as well as internal positive control (IPC), which was positive in all samples, as shown in Table 1 and Figure 1. The threshold value was 0.86 by the investigated samples and 0.42 by internal positive control (IPC). The (Ct) values of the investigated samples were on average 16.61 and IPC (Ct) values were 31.49 on average 20.67, whereby the lowest value of the investigated samples was reached at 16.23 and the highest value was 16,98 and the lowest IPC value was reached at 25.12 and the highest accomplish value was 32.97.

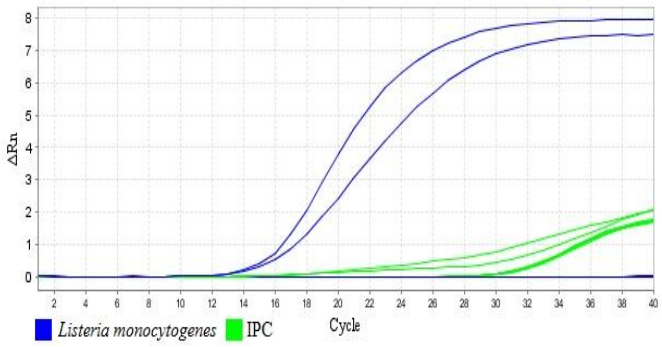
Table 1 Results of the investigated samples

Ct	*Listeria monocytogenes*	IPC
Positive control	16.23	29.69
Positive control # 2	16.98	25.12
Sample 1	negative	32.88
Sample 2	negative	32.80
Sample 3	negative	32.88
Sample 4	negative	32.97
Sample 5	negative	32.86
Negative control	negative	32.74

Recent reports have stated that the use of RT-PCR over the use of a traditional method has given an increased number of positive samples for *L. monocytogenes* **(Rodriguez-Lazaro et al., 2005)**.

We have fabricated and tested an integrated microchipbased DNA purification and real-time PCR system for bacterial detection. Although current PCR-based methods can be used to identify *L. monocytogenes* and other bacterial pathogens, most systems require manual nucleic acid extraction and sample preparation that is time consuming and requires multiple laboratory instruments **(Ibekwe et al., 2003; Jothikumar et al., 2003; Norton et al., 1999; Witham et al. 1996)**.

Figure 1 Process of Real Time PCR



Raw milk contamination could be attributed to the practices on the sanitary management of the animals, the food provided to the cows in which the bacterium can multiply and the water supply. The presence of the pathogen in the environment, where the milk is kept can form biofilms, a source of contamination reported by other authors **(Harvey et al., 2007)**.

The rapid method allows fast and sensitive detection of *L. monocytogenes* in various food matrices and could be used as a screening method. The method is based on the ISO 11290-1 standard, facilitating its integration in routine diagnostics laboratories **(Rossmanith et al., 2006)**.

A critical aspect for the effective implementation of any alternative method in routine laboratories would require a demonstration of its equivalence to the accepted method in terms of relative accuracy **(Rodríguez-Lázaro et al., 2004, 2005a, b)**.

These values were similar to those obtained when bacterial pure cultures were analysed and other molecular methods

have been used for these or other bacterial pathogens **(Rodríguez-Lázaro et al., 2004, 2005a, b; López-Enríquez et al., 2007)**.

Probably because of the presence of PCR inhibitors, the detection of DNA extracted directly from dairy products by PCR was less sensitive than DNA extracted from pure bacterial culture **(Bickley et al., 1996; Romero et al., 1995)**.

CONCLUSION

The rapid real-time PCR based method was 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. Our results indicated that the Step One real-time PCR assay developed in this study could sensitively detect *Listeria monocytogenes* in the investigated samples. This will not only prevent many people from becoming infected with Listeriosis it will also benefit food manufacturing companies by extending their product's shelf-life by several days and saving them the cost of warehousing their food products while awaiting pathogen testing results.

REFERENCES

BICKLEY, J., SHORT, J.K., MCDOWELL, D.G., PARKES, H.C. 1996. Polymerase chain reaction (PCR) detection of *Listeria monocytogenes* in diluted milk and reversal of PCR inhibition caused by calcium ions. In *Letters in Applied Microbiology*, vol. 22, 1996, p. 153–158.

DANIELSSON-THAM, M.L., ERIKSSON, E., HELMERSSON, S., LEFFLER, M., LUDTKE, L., STEEN, M., SORGJERD, S., THAM, W. 2004. Causes behind a human cheese-borne outbreak of gastrointestinal listeriosis. In *Foodborne Pathog. Dis.* vol. 1, 2004, p. 153-159.

DONNELLY, C.W., 2002. Detection and isolation of *Listeria monocytogenes* from food samples: implications of sublethal injury. In *Journal of AOAC International* vol. 85, 2002, p. 495–500.

GRADY, J. O., SEDANO-BALBAS, S., MAHER, M., SMITH, T., BARRY, T. 2008. Rapid real-time PCR detection of *Listeria monocytogenes* in enriched food samples based on the ssrA gene, a novel diagnostic target. In *Food Microbiology*, vol. 25, 2008, n. 1, p. 75–84.

HARVEY, J., KEENAN, K. P., GILMOUR, A. 2007. Assessing biofilm formation by *Listeria monocytogenes* strains. In *Food Microbiology*, vol. 24, 2007, n. 4, p. 380–392.

HEIN, I., KLEIN, D., LEHNER, A., BUBERT, A., BRANDL, E., WAGNER, M., 2001. Detection and quantification of the iap gene of *Listeria monocytogenes* and *Listeria innocua* by a new real-time quantitative PCR assay. In *Research in Microbiology* vol. 152, 2001, p. 37–46.

HOORFAR, J., COOK, N. 2003. Critical aspects in standardization of PCR. In *Methods in Molecular Biology: PCR Detection of Microbial Pathogens*; Humana Press, 2003, p. 51-64.

CHAN, Y. C., BOOR, K. J., WIEDMANN, M. 2007. SigmaB-dependent and sigmaB-independent mechanisms contribute to transcription of *Listeria monocytogenes* cold stress genes during cold shock and cold growth. In *Applied and Environmental Microbiology*, vol. 73, 2007, n. 19, p. 6019–6029.

CHATURONGAKUL, S., BOOR, K. J. 2006. SigmaB activation under environmental and energy stress conditions in *Listeria monocytogenes*. In *Applied and Environmental Microbiology*, vol. 72, 2006, n. 8, p. 5197–5203.

IBEKWE, A.M., GRIEVE, C.M. 2003. Detection and quantification of *E. coli* O157:H7 in environmental samples by real-time PCR. In *J. Appl. Microbiol.* vol. 94, 2003, p. 421–431.

JOTHIKUMAR, N., WANG, X., GRIFFITHS, M.W., 2003. Real-time multiplex SYBR green I-based PCR assay for simultaneous detection of *Salmonella* serovars and *Listeria monocytogenes* In *J. Food Prot.* vol. 66, 2003, p. 2141–2145.

LIU, D. 2006. Identification, subtyping and virulence determination of *Listeria monocytogenes*, an important foodborne pathogen. In *Journal of Medical Microbiology*, vol. 55 (part 6), 2006, p. 645–659.

LIU, Y., YE, J., LI. Y. 2003. Rapid detection of *Escherichia coli* O157:H7 inoculated in ground beef, chicken carcass, and lettuce samples with an immunomagnetic chemiluminescence fiber-optic biosensor. In *J. Food Protect.* vol. 66, 2003, p.512–517.

LÓPEZ-ENRÍQUEZ, L., RODRÍGUEZ-LÁZARO, D., HERNÁNDEZ, M., 2007. Quantitative detection of *Clostridium tyrobutyricum* in milk by real-time PCR. In *Appl. Environ. Microbiol*. vol. 73, 2007, p. 3747–3751.

MALORNY, B., PACCASSONI, E., FACH, P., BUNGE, C., MARTIN, A., HELMUTH, R., 2004. Diagnostic real-time PCR for detection of *Salmonella* in food. In *Appl. Environ. Microbiol.* vol. 70, 2004, p. 7046–7052.

MALORNY, B., TASSIOS, P.T., RADSTROM, P., COOK, N., WAGNER, M., HOORFAR, J., 2003. Standardization of diagnostic PCR for the detection of foodborne pathogens. In *Int. J. Food. Microbiol.* vol. 83, 2003, p. 39–48.

MAREČEK, J. The fungicides efficacy against *Fusarium graminearum, Fusarium culmorum* and *Microdochium nivale* in *in vitro* conditions. In *Agriculture*. 2005, vol. 51, 2007, n. 12, p. 656-663.

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.B., BATT, C.A. 1999. Detection of viable *Listeria monocytogenes* with a 5′ nuclease PCR assay In *Appl. Environ. Microbiol*. vol. 65, 1999, p. 2122–2127.

RODRÍGUEZ-LÁZARO, D., JOFRÉ, A., AYMERICH, T., HUGAS, M., PLA, M., 2004. Rapid quantitative detection of *Listeria monocytogenes* in meat products by real-time PCR. In *Appl. Environ. Microbiol*. vol. 70, 2004, p. 6299–6301.

RODRIGUEZ-LAZARO, D., JOFRÉ, A., AYMERICH, T., GARRIGA, M., PLA, M. 2005. Rapid quantitative detection of *Listeria monocytogenes* in salmon products: Evaluation of pre-real-time PCR strategies. In *Journal of Food Protection*, vol. 68, 2005, n 7, p. 1467–1471.

RODRÍGUEZ-LÁZARO, D., D'AGOSTINO, M., HERREWEGH, A., PLA, M., COOK, N., IKONOMOPOULOS, J., 2005a. Real-time PCR-based methods for quantitative detection of *Mycobacterium avium* subsp. *paratuberculosis* in water and milk. In *Int. J. Food Microbiol*. vol. 101, 2005, p. 93–104.

RODRÍGUEZ-LÁZARO, D., JOFRÉ, A., AYMERICH, T., GARRIGA, M., PLA, M., 2005b. Rapid quantitative detection of *Listeria monocytogenes* in salmon products: evaluation of pre-real-time PCR strategies. In *J. Food Prot*. vol. 68, 2005, p. 1467–1471.

ROMERO, C., PARDO, M., GRILLÓ, M.J., DÍAZ, R., BLASCO, J.M., LÓPEZ-GOÑI, I., 1995. Evaluation of PCR and indirect-ELISA on milk samples for the diagnosis of brucellosis in dairy cattle. In *Journal of Clinical Microbiology*, vol. 33, 1995, p. 3198–3200.

ROSSMANITH, P., KRASSNIG, M., WAGNER, M., HEIN, I., 2006. Detection of *Listeria monocytogenes* in food using a combined enrichment/real-time PCR method targeting the prfA gene. In *Res. Microbiol.* vol. 157, 2006, p. 763–771.

WITHAM, P.L., LIVAK, K., YAMASHIRO, C., BATT, C.A. 1996. A PCR-based assay for the detection of *Escherichia coli* shiga-like toxin (slt) genes in ground beef In *Appl. Environ. Microbiol.* vol. 62, 1996, p. 1347–1353.

Acknowledgments:
This work was funded by Grant Agency VEGA 1/0372/09 and APVV-VMSP-P-0111-09 and KEGA 430-014SPU-4/2010.

Contact address:
Ing. Jaroslav Pochop, Department of Microbiology, FBFS, SUA in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Tel.: 037 641 5814, e-mail: pochop.jaroslav@gmail.com

doc. Ing. Miroslava Kačániová, PhD., Department of Microbiology, FBFS, SUA in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Tel.: 037 641 4494, e-mail: miroslava.kacaniova@uniag.sk

Ing. Lukáš Hleba, Department of Microbiology, FBFS, SUA in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Tel.: 037 641 5814, e-mail: lukas.hleba@gmail.com

Ing. Simona Kunová, PhD., Department Food Hygiene and Food Safety, FBFS, SUA in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, e-mail: simona.kunova@uniag.sk