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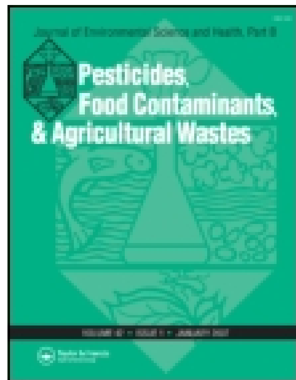

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Detection of *Listeria monocytogenes* in ready-to-eat food by Step One real-time polymerase chain reaction

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The aim of this study was to follow contamination of ready-to-eat food with *Listeria monocytogenes* by using the Step One real time polymerase chain reaction (PCR). We used the PrepSEQ Rapid Spin Sample Preparation Kit for isolation of DNA and MicroSEQ® *Listeria monocytogenes* Detection Kit for the real-time PCR performance. In 30 samples of ready-to-eat milk and meat products without incubation we detected strains of *Listeria monocytogenes* in five samples (swabs). Internal positive control (IPC) was positive in all samples. Our results indicated that the real-time PCR assay developed in this study could sensitively detect *Listeria monocytogenes* in ready-to-eat food without incubation.

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

Introduction

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.[1] *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.[2] This psychrotrophic bacterium causes a disease with mild flu-like symptoms and rarely gastroenteritis in healthy adults, but it may manifest as severe infection in immunocompromised persons and during pregnancy, leading to abortion, stillbirth or perinatal morbidity.[3–7] 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.[8] It is also important to consider that minimally processed vegetables are often used as ingredients to prepare salads, which may have longer refrigerated shelf lives, representing a risk of increasing of *L. monocytogenes* population.[9] Most cases of listeriosis are caused by the ingestion of *L. monocytogenes* - contaminated RTE foods that do not require heating prior to consumption.[7] 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.[10] 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 end-point detection have hampered the transition from research to routine use in food microbiology laboratories.[10–12] 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.[13] Considerable reagent cost is one of the key factors, beside standardization and validation issues,[14] 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. However, savings cannot reach its full potential if the robustness and reliability of real-time PCR

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technology are compromised in any way.[15] In the effort to combat *L. monocytogenes* in foodstuffs, several researchers focused on the development of new methods, based on molecular biology, which could detect this pathogen more rapidly and reliably with respect to traditional microbiological methods. In this context, a new group of methods, based on the polymerase chain reaction (PCR) could detect target pathogens without the need of their cultivation. Nowadays, with the second generation of PCR methods, in which a quantification of the target microorganisms is also possible, new applications have become available. Several quantitative PCR (qPCR) protocols have been recently published, highlighting that this method can be advantageously used to detect and quantify *L. monocytogenes* in food.[16–17]

The aim of this study was to follow the contamination of ready-to-eat milk and meat products with *Listeria monocytogenes* by Step One real-time PCR.

Materials and methods

Food samples

A total of 30 samples of ready-to-eat foods were used in this study. The samples (5 salads, 6 sheep's cheeses, 5 sheep's cheeses from unpasteurized sheep's milk, 7 sausages and 7 smoked salmons) were analyzed for the presence of *Listeria monocytogenes* (Table 1). Samples were obtained by taking swabs from the ready-to-eat food. After sampling, procedure shown in the Scheme 1 was used.

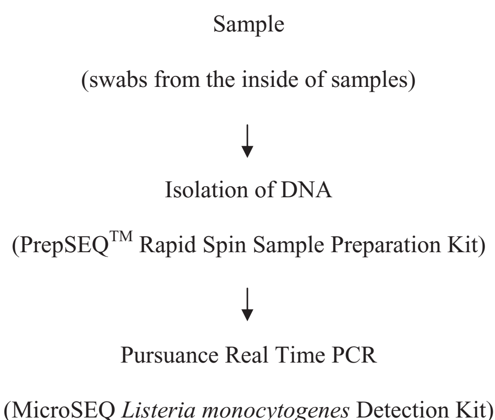
DNA extraction

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). 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.

Table 1. Samples of ready to eat food and their source used in this study.

		Ct	
Sample Number	*Source of Food*	*Listeria monocytogenes*	*IPC*
1	Paris salad	negative	30.98
2		32.25	32.01
3		negative	32.04
4		negative	30.07
5		negative	31.52
6	Sheep's cheese	negative	32.13
7		negative	30.15
8		negative	31.32
9		negative	30.35
10		negative	31.24
11		negative	30.75
12	Sheep's cheese from unpasteurized sheep's milk (cheese UM)	30.97	32.30
13		negative	31.01
14		negative	30.07
15		negative	31.14
16		negative	31.97
17	Sausage	negative	30.57
18		negative	31.43
19		32.94	32.28
20		negative	32.14
21		31.90	30.02
22		negative	30.85
23		negative	32.70
24	Smoked salmon	negative	30.56
25		negative	31.90
26		negative	31.45
27		negative	32.41
28		34.39	32.58
29		negative	31.27
30		negative	30.12

Ct - threshold cycle; **IPC** - internal positive control



Scheme 1. Real-time PCR procedure for the detection of strain of *Listeria monocytogenes*.

MicroSEQ® Listeria monocytogenes detection kit

An 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. Eight-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 performed.

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Real-time PCR

TaqMan® probes labeled with both a fluorophore and a quencher dye were used in real-time PCR assays to detect amplification of specific DNA targets. We used three fluorophore detection chemistries that include FAM™ and VIC® dye-labeled TaqMan® MGB probe-based assays, VIC® and TAMRA™ dye-labeled probe-based assays and ROX™ as passive reference dye. FAM™, which has an emission of 520 nm, has become the most commonly used fluorophore for singleplex qPCR reactions. TAMRA™ will efficiently quench the fluorescence of FAM™, until the probe hybridizes to the target and is cleaved by the 5' exonuclease activity of the polymerase. 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 plotted the normalized reporter signal, ΔRn, (reporter signal minus background) against the number of amplification cycles and also determined the threshold cycle (Ct) value; i.e. the PCR cycle number at which fluorescence increases above a defined threshold level were used.

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Results and discussion

Ready-to-eat (RTE) products can become contaminated with *L. monocytogenes* during production through contact with contaminated surfaces, poor personal hygiene, and cross-contamination between raw materials and finished products.[18] The most sensitive detection of *Listeria monocytogenes* was obtained using PrepSEQ™ Rapid Spin Sample Preparation Kit. The MicroSEQ® *Listeria monocytogenes* Detection Kit compatible with StepOne™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 the DNA extraction procedure used to prepare the template DNA. In our samples without incubation we have detected strain of *Listeria monocytogenes* in five out of thirty samples (swabs) and negative control was negative (Figure 1), as well as in internal positive control (IPC), which was positive in all samples, as it is shown in Figure 2. The threshold value was 0.94 in listeria samples and 0.39 in internal positive control (IPC). The (Ct) value of positive listeria samples was on average 32.49 and IPC (Ct) value was on average 31.61. The lowest value of positive listeria samples was found at 30.97 and the highest was at 34.49. The lowest IPC value was detected at 30.12 and the highest was 32.96. 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.[19] Berrada et al.[5] 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.[20] also used the technique of real-time PCR to quantify *L. monocytogenes* in 66 different samples of

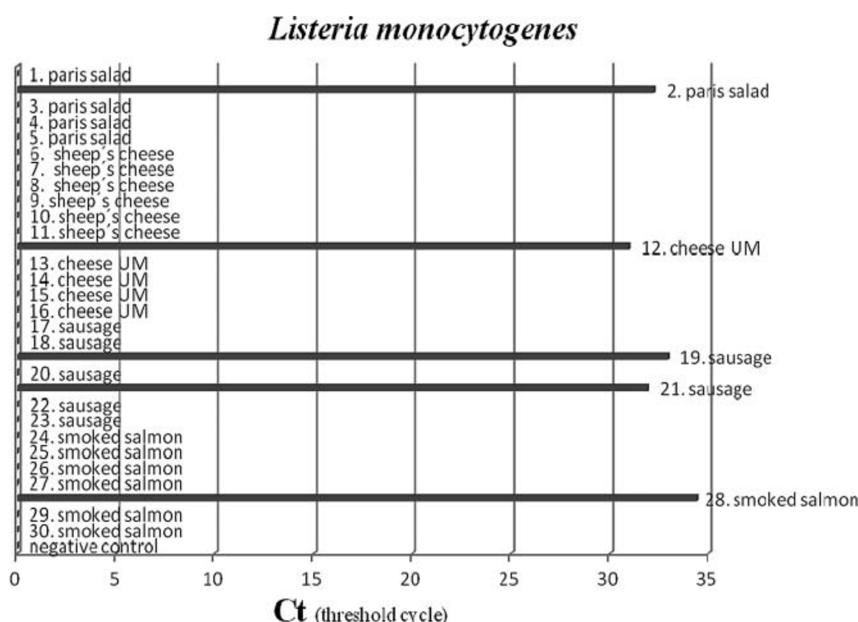

Fig. 1. Real-time PCR detection of *Listeria monocytogenes* without incubation.

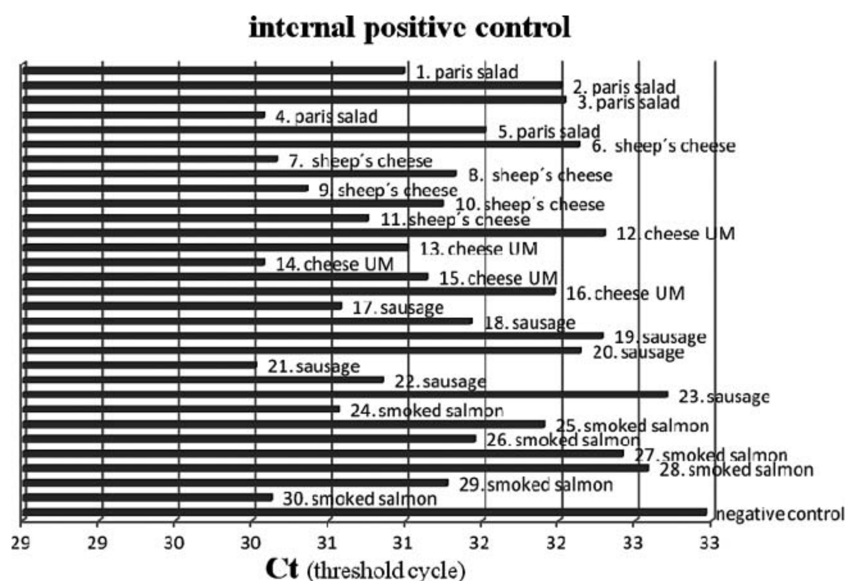

Fig. 2. Real-time PCR detection of internal positive control.

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 μL 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.[21–22] Strict criteria for analytical and diagnostic accuracy, high detection probability, high robustness and low carryover contamination were implemented in order to fulfill standardization requirements.[23] Furthermore, to provide quality assurance and to increase the reliability of the application of real-time PCR assays, a control strategy including internal amplification control (IAC) was applied.[24–25] 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.[26] 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.[27] 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.[28]

Conclusion

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 ready-to-eat food. 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

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