Comparison of three real-time PCR-based methods for the detection of *Listeria monocytogenes* in food

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Summary

Three PCR-based methods for the detection of *Listeria monocytogenes* in food were compared, using EN ISO 11290-1 as the reference method. All methods tested involved culture enrichment followed by the real-time PCR identification and produced final results on the next day after sample collection. Forty selected food samples, artificially contaminated with *L. monocytogenes,* and seven naturally contaminated samples were analysed using TaqMan *Listeria monocytogenes* Detection Kit (Applied Biosystems), iQ-Check *Listeria monocytogenes* Kit (Bio-Rad) and an in-house method, consisting of a two-step selective enrichment followed by duplex real-time PCR employing a TaqMan probe. Using the TaqMan detection kit, the detection limits of the methods for artificially contaminated samples were 10^0 CFU per sample with the exception of chicken and pork liver, and for naturally contaminated raw meat products. With both kits utilizing a single-step enrichment, PCR inhibition was observed with artificially contaminated food matrices containing chocolate. Our in-house real-time PCR-based method produced positive results with all samples. Detection limits for dead *L. monocytogenes* cells were 10^6 CFU per sample for the in-house method and 10^3–10^4 CFU per sample for commercial kits. Real-time PCR-based methods proved to be powerful tools for fast, sensitive and accurate *L. monocytogenes* detection in food. If the price of analysis is a decisive factor, our in-house method is the method of choice.

Keywords

Listeria monocytogenes; detection; real-time PCR; food

Listeria monocytogenes is commonly present in the environment, and as a food-borne pathogenic bacterium is able to cause outbreaks or sporadic cases of listeriosis in susceptible individuals. The infection dose remains uncertain yet. The bacterium tolerates a broad range of pH values, salt concentrations and is capable to grow and multiply at the refrigeration temperatures during the shelf life of particular food, and thus may exceed the highest concentration allowed by the Commission Regulation No 2073/2005 [1] which is 100 CFU per gram. However, there is a zero tolerance for the presence of *L. monocytogenes* in certain foodstuffs according to this regulation. *L. monocytogenes* contamination is important in all areas of food processing, catering and retailing, and poses particular hazards and challenges in relation to ready-to-eat foods. Even a low *L. monocytogenes* incidence could have a significant economical impact. The human and economic consequences of a *L. monocytogenes* outbreak can be minimized by implementation of appropriate standards and practices in the food industry, and moreover by implementation of rapid methods for the detection of the pathogen in food. Detection methods employing the real-time PCR fulfill these demands.

Several studies of real-time PCR-based detection of *L. monocytogenes* in food have been published [2-4] and several validated real-time PCR-based kits are commercially available (iQ-Check *Listeria*, Bio-Rad, TaqMan *Listeria*, Applied Biosystems, BAX system PCR Assay *Listeria monocytogenes*, DuPont Qualicon). In these complete methods, real-time PCR detection is preceded by a single-step or a two-step enrichment using media of different selectivity. These methods use different enrichment and DNA preparation approaches to reach the goal of increasing the target live cell

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numbers to a detectable level and dilution of dead *L. monocytogenes* cells as well as food-borne PCR inhibitors.

In this study, we tested two commercial real-time PCR based methods and compared them with our previously described, open-formula real-time PCR-based method for the detection of *L. monocytogenes* in food [5, 6].

MATERIALS AND METHODS

Bacterial strain and culture conditions

L. monocytogenes NCTC 11994 used for artificial food contamination was obtained from the National Collection of Type Cultures, London, United Kingdom. It was grown in Brain Heart Infusion broth (Merck, Darmstadt, Germany) at 37 °C with shaking overnight. Decimal dilutions of the culture were prepared in 0.85% NaCl and the cell concentrations were determined by the plate-count technique on BHI agar incubated for 24 h at 37 °C.

Food samples and artificial contamination

Food samples selected based on association with food-borne listeriosis were obtained from retail markets in Slovakia. For artificial contamination of food samples previously tested as negative for the presence of *L. monocytogenes*, counts of 10^0 CFU, 10^1 CFU and 10^2 CFU, respectively were used. Fraser base and Fraser selective supplements were purchased from Merck. Individual enrichment procedures were performed according to manufacturers' instructions.

Commercially available real-time PCR methods

TaqMan *Listeria monocytogenes* Detection Kit together with PrepMan Ultra sample preparation reagent was obtained from Applied Biosystems Foster City, CA, USA and iQ-Check *Listeria monocytogenes* including the chelex-based lysis reagent was purchased from Bio-Rad, Marnes-la-Coquette, France. Enrichment media and culture conditions were employed according to manufacturers' instructions.

In-house real-time PCR-based method

The method developed and validated in our laboratory [6] consists of a two-step selective enrichment in half Fraser and Fraser broths, DNA preparation by cell lysis using boiling in a buffer [7] and a real-time PCR detection of the *act*A gene sequence unique for *L. monocytogenes* [5].

Tab. 1. Specification of the methods.

Method	TaqMan	iQ-Check	Oravcová et al. (2007)
Enrichment	single-step	single-step	two-step
Medium	Fraser	half Fraser	half Fraser + Fraser
DNA preparation	PrepMan Ultra	lysis reagent	boiling in a buffer
Reaction mixture	partially integrated	partially integrated	non-integrated
Probe type	TaqMan	Molecular Beacon	TaqMan
Reporter dyes used	FAM, VIC	Texas Red, FAM	FAM, JOE
Temperature programme	universal	specific	universal

Tab. 2. Analysis of food samples naturally contaminated with *L. monocytogenes*.

Sample	Results of real-time PCR detection			
	TaqMan	iQ-Check	Oravcová et al. (2007)	EN ISO 11290
Non-smoked parenica 1	+	+	+	+
Non-smoked parenica 2	+	+	+	+
Raw minced meat	–	+	+	+
Beefburger	–	+	+	+
Brawn	+	+	+	+
Salmon filets	+	+	+	–*
Smoked salmon	+	+	+	+

* Only non-*monocytogenes Listeria* colonies were detected using ALOA identification.

Reference method

The method according to EN ISO 11290-1 [8] and EN ISO 11290-1/A1 [9] was used, utilizing a two-step enrichment (up to 72 h) followed by the isolation and differentiation of *L. monocytogenes* from other *Listeria* spp. on the chromogenic agar medium ALOA (Merck).

DNA preparation

DNA from enriched samples was obtained by lysing protocols according to manufacturers' instructions or using cell lysis by boiling in a buffer in the case of the in-house method, respectively.

Real-time PCR

L. monocytogenes-specific DNA sequences were detected in their respective real-time PCRs employing dual-labelled fluorescent probes. An internal amplification control was included in each PCR method. The reactions of TaqMan kit and the in-house method were performed in a PTC-200 thermocycler coupled to the continuous fluorescence detector Chromo 4 (both from MJ Research, Waltham, Massachusetts, USA). Reactions of iQ-Check kit were performed in the iCycler coupled to the iQ5 continuous fluorescence detector (both from Bio-Rad, Hercules, California, USA). Results of all three real-time PCR based methods were analysed using instruments' internal software. Samples with an increasing fluorescence signal were considered positive, regardless of the internal control amplification. Samples with no increasing fluorescence signal but with amplified internal control were considered negative. Samples with no fluorescence signal for both specific target and internal control were considered inhibited. Basic specifications of the methods are summarized in Tab. 1.

Detection of dead cells

For the detection of DNA from dead cells, a decimally diluted overnight culture of *L. monocytogenes* was incubated at 100 °C for 20 min, and further processed as live cells.

RESULTS AND DISCUSSION

Two commercially available real-time PCR-based methods and an in-house real-time PCR based method for the detection of *L. monocytogenes* in food were evaluated in this study along with the reference microbiological method using naturally and artificially contaminated food samples.

Seven food products previously analysed to be contaminated with *L. monocytogenes* and two samples of salmon filets previously analysed to be contaminated with non-*monocytogenes Listeria* using standard method EN ISO 11290-1 with the identification on chromogenic media (ALOA) were assayed. Contamination was positively detected in all food samples by the real-time PCR-based methods but the TaqMan kit afforded false negative results for raw meat samples (Tab. 2). The reference microbiological method was unable to detect *L. monocytogenes* colonies on ALOA in the presence of non-*monocytogenes Listeria* colonies. It is known that other *Listeria* species possibly present in food along with *L. monocytogenes* may overgrow and mask its presence, and thus cause false negative results of analysis [10].

The detection limit of the methods tested with 40 artificially contaminated food products was 10^0 CFU per sample which is in accordance with the standard EN ISO 11290-1. There were some exceptions for the TaqMan kit with artificially contaminated ice-cream and two liver samples (Tab. 3), where the detection limit grew to 10^1 CFU per sample. Using naturally contaminated food, similar results for the TaqMan kit were obtained with raw meat products (Tab. 2). This could be caused by the slower growth of *L. monocytogenes* in the selective medium Fraser in comparison to its growth in half Fraser broth used for the single-step enrichment. It is known that single-step enrichment in Fraser broth with fully concentrated selective agents may inhibit the growth of sublethally injured *L. monocytogenes* present in food [11]. Our in house real-time PCR based method produced positive results with all samples.

Using both kits utilizing single-step enrichment, PCR inhibition was observed with artificially contaminated food samples (yoghurt and ice-cream) containing chocolate.

After the sample dilution recommended by the manufacturer in the case of inhibition, the reaction was positive for the internal amplification control but negative for the target template probably due to lower concentration of the latter. The results of analyses were false negative. No inhibitory effect was observed using our in-house method utilizing a two-step enrichment.

The detection limit of the in-house method utilizing the two-step enrichment for dead cells of *L. monocytogenes* was 10^6 CFU per sample. The detection limit for dead *L. monocytogenes* cells was 10^3 to 10^4 CFU per sample using the TaqMan kit and the iQ-Check kit utilizing single-step enrichment (data not shown). The latter detection limits

Tab. 3. Analysis of food samples artificially contaminated with *L. monocytogenes* at a level of 10^0 CFU per sample.

Sample	Results of real-time PCR detection			
	TaqMan	iQ-Check	Oravcová et al. (2007)	EN ISO 11290
Non-smoked parenica 1	+	+	+	+
Non-smoked parenica 2	+	+	+	+
Non-smoked oštiepok 1	+	+	+	+
Non-smoked oštiepok 2	+	+	+	+
Smoked parenica	+	+	+	+
Smoked oštiepok	+	+	+	+
Cottage cheese	+	+	+	+
Mozzarella 1	+	+	+	+
Mozzarella 2	+	+	+	+
Edamer 1	+	+	+	+
Edamer 2	+	+	+	+
Parmiggiano Reggiano	+	+	+	+
Fresh cow's cheese 1	+	+	+	+
Fresh cow's cheese 2	+	+	+	+
Fresh ewe's cheese 1	+	+	+	+
Fresh ewe's cheese 2	+	+	+	+
Feta 1	+	+	+	+
Feta 2	+	+	+	+
Camembert 1	+	+	+	+
Camembert 2	+	+	+	+
Camembert 3	+	+	+	+
Roquefort 1	+	+	+	+
Roquefort 2	+	+	+	+
Red smear cheese 1	+	+	+	+
Red smear cheese 2	+	+	+	+
Limburger cheese 1	+	+	+	–*
Limburger cheese 2	+	+	+	+
Yoghurt white	+	+	+	+
Yogurt with chocolate 1	i	i	+	+
Yogurt with chocolate 2	i	i	+	+
Strawberry ice cream	+	+	+	+
Ice-cream with chocolate	i	i	+	+
Cabbage salad	+	+	+	+
Chicken liver	–	+	+	+
Pork liver	–	+	+	+
Raw minced meat	+	+	+	–*
Sausage	+	+	+	–*
Brawn	+	+	+	+
Salmon filets	+	+	+	–*
Smoked salmon	+	+	+	+

* Only non-*monocytogenes Listeria* colonies were detected using ALOA identification. i - inhibition.

apparently cannot guarantee elimination of false positive results in certain types of food products.

The evaluated real-time PCR-based methods for the identification of *L. monocytogenes* in food were found to be a good rapid alternative to the current standard method EN ISO 11290-1. However, the kits employing single-step enrichment may be of limited use for certain food matrices which contain compounds inhibiting PCR or higher levels of dead *L. monocytogenes* cells. Our in-house method had analytical parameters comparable or better than the commercial kits, required more handling, but was cheaper.

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