Detection of *Listeria monocytogenes* in food in two days using enrichment and 5'-nuclease polymerase chain reaction with end-point fluorimetry

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Summary

A method for the detection of *Listeria monocytogenes* in food in two days is presented. The method consists of a two-step enrichment, bacterial cells lysis and a closed-tube duplex 5'-nuclease PCR with an internal amplification control, which is read in end-point mode in a 96-well fluorimeter. The 5'-nuclease PCR has an intrinsic detection limit of 10^4 CFU.ml^{-1} and facilitates monitoring of false negative results caused by the failure of amplification. The complete method for the detection of *L. monocytogenes* in food has a detection limit of 10^0 CFU per 10 g and does not produce false positive results due to the presence of dead cells.

Keywords

Listeria monocytogenes; polymerase chain reaction; rapid method; end-point fluorimetry

Listeria monocytogenes is a pathogenic bacterium which relatively frequently contaminates food products, in particular cheese and ready-to-eat meat-containing food products [1, 2]. For most food products, zero tolerance for the presence of *L. monocytogenes* is set by the legislature [3]. The standard analytical method for the detection of *L. monocytogenes* in food and animal feeding stuffs EN ISO 11290-1 suffers from a considerable time requirement of 4 to 6 days [4, 5]. As faster alternatives, several methods based upon enrichment and subsequent polymerase chain reaction (PCR) have been developed and some are commercially available as kits [6-8]. PCR of these methods contains an internal control amplified in one reaction along with the sample in duplex format, which facilitates elimination of potential false negative results. However, the methods employing conventional PCR have been found impractical because of the use of agarose gel electrophoresis. To deal with this problem, 5'-nuclease PCR with continuous fluorimetry (real-time PCR) has been proposed to be used [9]. Because real-time PCR requires expensive equipment, for qualitative analyses, a simplified version using end-point closed-tube detection in a 96-well fluorimeter may be beneficious [10].

In this article, a method for the detection of *L. monocytogenes* in food in two days by 5'-nuclease polymerase chain reaction (PCR) with end-point fluorimetry is presented. The method involves the previously developed two-step enrichment [11] and 5'-nuclease PCR specific for *L. monocytogenes* [12].

MATERIALS AND METHODS

Microbiological methods

Listeria monocytogenes NCTC 11994 (serotype 4b) was cultured in Brain Heart Infusion (BHI) Broth (Merck, Darmstadt, Germany) at 37 °C with shaking (2 Hz) overnight (16 h - 20 h). Decimal dilutions of the cultures were prepared in 0.85% NaCl. Culture density was determined by the plate-count technique on plates of BHI Agar (Merck) incubated at 37 °C for 24 h. Dead cells were prepared from decimal dilutions of the overnight culture by boiling for 25 min and the devitalization efficiency was confirmed by plating on BHI Agar.

Food samples

Food samples were obtained from shops in Bratislava, Slovakia. For artificial contamination,

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the food sample was put in a Stomacher bag with a filter insert, 90 ml of half-Fraser broth (Merck) was poured in and, subsequently, 10^0 CFU of *L. monocytogenes* was added. The sample was homogenized using Stomacher 400 homogenizer (Seward, Basingstoke, England).

Enrichment

A two-step enrichment procedure was used. The homogenized sample was incubated statically at 30 °C for 18–20 h. A volume of 0.1 ml of the primary-enriched sample was transferred into tubes with 10 ml of Fraser broth (Merck) and incubated statically at 37 °C for 24 h.

Preparation of DNA

DNA was released from the cultures using the cell lysis by boiling. A volume of 1 ml of the bacterial suspension was centrifuged at 10 000 *g*, the sediment was resuspended in 0.85% NaCl and centrifuged again at 10 000 *g*. The washed sediment was resuspended in 100 μl of 1x buffer supplied with HotStarTaq DNA polymerase (Qiagen, Hilden, Germany) and incubated at 95 °C for 20 min [13]. The supernatant was used as the DNA template for PCR.

Polymerase chain reaction with end-point fluorimetry

Each reaction sample (volume 65 μl) contained 500 nmol.l^{-1} of the primer LMrt3F (caa agc gag aat gtg gct ata aat ga), 500 nmol.l^{-1} of the primer LMrt3Rbis (taa ttt ccg ctg cgc tat ccg), 200 nmol.l^{-1} of the probe listP labelled with 6-carboxyfluorescein (FAM) and quenched with 6-carboxytetramethylrhodamine (TAMRA;FAM-cct gga tga cga cgc tcc act tg-TAMRA [12]; all oligonucleotides synthesized by Qiagen Operon, Köln, Germany), approx. 10^3 copies of the internal amplification control plasmid pDP2 [10], 500 nmol.l^{-1} of the primer phtAc11F (tta taa agg cta agc gcg agg tat g), 500 nmol.l^{-1} of the primer phtAc131R (gcg gaa gga acc tcc gtc tt), 200 nmol.l^{-1} of the probe phtAc42P labeled with Yakima Yellow and quenched with Black Hole Quencher 1 (YY-cta cgc gaa ctg tgt cgt tgc ggc c-BHQ1; oligonucleotides synthesized by Qiagen Operon), 400 μmol.l^{-1} of each dNTP (Applied Biosystems, Foster City, California, USA), 4.5 mmol.l^{-1} $MgCl_2$, 2 U HotStarTaq DNA polymerase (Qiagen), 6.5 μl of 10x concentrated PCR buffer supplied with the polymerase and 2.5 μl of the DNA template solution. Reactions were performed in TopYield 8-strips (Nunc, Roskilde, Denmark) in a GeneAmp 9700 thermal cycler (Applied Biosystems) using a programme consisting of the initial denaturation at 95 °C for 15 min and 35 cycles (denaturation at 95 °C for 15 s and annealing with polymerization at 60 °C for 60 s). After PCR, amplification was measured by fluorimetry directly in the microtubes in a Genios 96-well reader (Tecan, Grödig bei Salzburg, Austria) equipped with an excitation filter with a pass maximum of 492 nm (bandwidth, 10 nm) and an emission filter with a pass maximum of 520 nm (bandwidth, 10 nm) for channel 1 and an excitation filter with a pass maximum of 515 nm (bandwidth, 10 nm) and an emission filter with a pass maximum of 546 nm (bandwidth, 10 nm) for channel 2. Ten flashes per measurement, integration time 40 μs, optimal gain and measurement from the bottom orientation were used. To define the positivity threshold, three no-template control samples were always analysed and read in the fluorimeter along with the samples, mean value and the standard deviation (SD) were calculated and the positivity threshold for individual channels was set to (mean + 3 SD).

RESULTS AND DISCUSSION

Intrinsic detection limit

The intrinsic detection limit of the duplex 5'-nuclease PCR with end-point fluorimetry was determined using decimal dilutions of *L. monocytogenes* culture. Fluorescence values above the positivity threshold in channel 1 indicated the positive result (Fig. 1). The determined value of 10^4 CFU.ml^{-1} was further supported by the determination of detection probability (Fig. 2). This detection limit is comparable to that of real-time PCR [12] and is

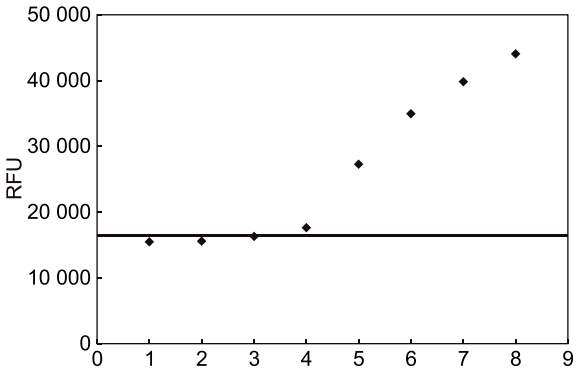

Fig. 1. Results of the duplex 5'-nuclease PCR with decimal dilutions of *L. monocytogenes* culture and with the internal amplification control.
Values of mean fluorescence of three replicates of the *L. monocytogenes*-specific PCR in channel 1 (FAM) are presented; standard deviation values were too low to be depicted at the given scale.

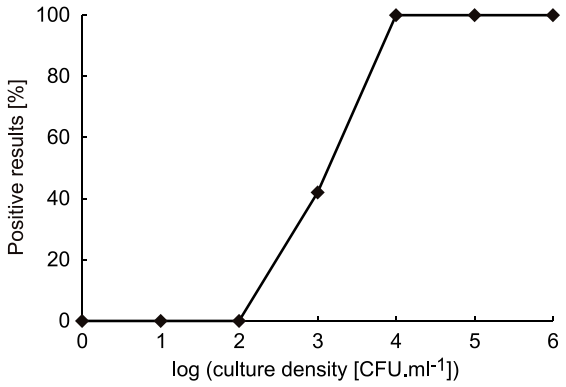

Fig. 2. Detection probability of *L. monocytogenes* in duplex 5'-nuclease PCR with end-point fluorimetry using the internal amplification control.
Individual culture dilutions were analysed in 12 replicates.

compatible with the two-step enrichment [11]. Internal amplification control (IAC) was detectable in all samples, as indicated by fluorescence values above the positivity threshold in channel 2, which means that IAC was functional in monitoring the success of the amplification and thus revealing potential false negative results due to PCR inhibition (data not shown). The IAC signal was not detected in channel 1 and hence the use of IAC in the used amount did not lead to false positive results.

Practical performance of the method with artificially contaminated food samples

The complete method for the detection of *L. monocytogenes* in food consisting of the two-step enrichment, bacterial cell lysis and the duplex 5'-nuclease PCR with end-point fluorimetry was evaluated with food samples artificially contaminated with live *L. monocytogenes* cells at a level of 10^0 CFU per 10 g and with respective dead cells at a level of 10^9 CFU per 10 g . Experiments were carried out with various food matrices to cover cases when different mixed bacterial populations interfere with the enrichment and thus impair the detection limit. The results confirmed the detection limit of 10^0 CFU per 10 g and demonstrated that the method did not detect dead cells in a density of up to 10^9 CFU per 10 g (Tab. 1).

The developed method was found to be equivalent to the standard microbiological method [4, 5], in terms of identical analytical parameters, but it was faster taking only two days. Compared to other conventional PCR-based methods [6-8], this method was safer as regards laboratory contamination because it was entirely carried out in closed microtubes. Compared to real-time PCR-based kits, which also employ the closed-tube format, this method did not require an expensive real-time PCR cycler.

CONCLUSIONS

The presented closed-tube 5'-nuclease PCR with an internal amplification control has a detection limit of 10^4 CFU.ml^{-1} and facilitates monitoring of false negative results caused by the failure of amplification. When coupled to the two-step enrichment and bacterial cell lysis, the complete method for the detection of *Listeria monocytogenes* in food has a detection limit of 10^0 CFU per 10 g , does not produce false positive results due to dead cells and produces results in two days.

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Tab. 1. Results of the complete method for the detection of *L. monocytogenes* in artificially contaminated food samples.

Food sample	Artificial contamination	PCR result	
		L. monocytogenes	IAC
Ice cream	10^0 CFU live cells	+	+
	10^9 CFU dead cells	–	+
Cheese	10^0 CFU live cells	+	+
	10^9 CFU dead cells	–	+
Sausage	10^0 CFU live cells	+	+
	10^9 CFU dead cells	–	+

Results of three replicates are presented. IAC - internal amplification control.

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