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Impact of enrichment medium on PCR-based detection of *Listeria monocytogenes* in food

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ABSTRACT

The detection of *Listeria monocytogenes* in food samples using enrichment and PCR is described. The objectives were to determine whether the type of enrichment medium has the influence on the results obtained with PCR and to evaluate proposed method by analysing naturally contaminated food samples. Comparative evaluation of different enrichment media for bacteria of the genus *Listeria* (half Fraser - HF, buffered peptone water - BPW, triptic soy yeast extract broth - TSBYE, universal preenrichment broth - UPB) showed that only the UPB enabled detection of *L. monocytogenes* in artificially contaminated food samples after 24h of incubation. The PCR-based method gave equal results as standard cultural method by analysis of naturally contaminated food samples. Described PCR-based procedure is comparable to some other PCR-based methods of *L. monocytogenes* detection in foods and shows promise as a rapid, routine method which requires 30-72 h, whereas cultural methods require 96-120 h.

Keywords: *Listeria monocytogenes*, universal preenrichment broth UPB, PCR, detection, food hygiene

VPLIV OBOGATITVENEGA GOJIŠČA NA DOLOČANJE BAKTERIJ VRSTE *Listeria monocytogenes* V ŽIVILIH S PCR

IZVLEČEK

V prispevku je predstavljen postopek določanja bakterij vrste *L. monocytogenes* v živilih s PCR po obogatitvi vzorca živila. Namen raziskave je bil določiti, ali vrsta obogatitvenega gojišča vpliva na rezultate PCR, in vrednotenje predlagane metode s preiskavami naravno kontaminiranih živil. Primerjava različnih obogatitvenih gojišč za bakterije rodu *Listeria* (half Fraser - HF, buffered peptone water - BPW, triptic soy yeast extract broth - TSBYE, universal preenrichment broth - UPB) je pokazala, da edino gojišče UPB omogoča določitev bakterij vrste *L. monocytogenes* v umetno kontaminiranih vzorcih živil po 24-urni inkubaciji. Rezultati določanja bakterij vrste *L. monocytogenes* v naravno kontaminiranih vzorcih živil dobljeni po obogatitvi s PCR so bili enaki rezultatom dobljenim s standardno gojitveno metodo. Opisana

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metoda PCR je primerljiva s podobnimi metodami, ki temeljijo na isti encimski reakciji in omogoča hitro, rutinsko določanje bakterij vrste *L. monocytogenes* v živilih v 30-72 urah, medtem ko je za rezultate gojitvenih metod potrebno 96-120 ur.

Ključne besede: *Listeria monocytogenes*, univerzalno predobogatitveno gojišče UPB, PCR, določanje, higiena živil

1 INTRODUCTION

Bacteria of the genus *Listeria* are ubiquitous in the environment and *Listeria monocytogenes* has been recognised as an important foodborne pathogen (Ryser, 1999). Listeriosis can occur as a sporadic disease or as an outbreak and is often related to the consumption of contaminated food. *L. monocytogenes* has been isolated from different foods, such as milk, cheese, ice cream, vegetable, meat, ready-to-eat food (Farber and Peterkin, 1991).

Cultural methods for detection *L. monocytogenes* include selective enrichment of food, isolation of colonies on selective agar, biochemical characterization of suspect isolates and serological confirmation step. These procedures are time-consuming, laborious and may take 5 to 7 days to confirm the isolate (Donnelley, 1999; Scotter *et al.*, 2001). To overcome these limitations, molecular, biological, biochemical and immunological techniques have been applied for the rapid and specific detection of *L. monocytogenes* in food (Manzano *et al.* 1998; Almeida and Almeida 2000; Solve *et al.* 2000). Various methods involving DNA probes and polymerase chain reaction (PCR) have been suggested (Scheu *et al.*, 1998; de Boer and Beumer, 1999; Malorny *et al.*, 2003). Direct detection of *L. monocytogenes* in food samples is a major goal for these methods, but it is difficult to obtain. Direct detection of *L. monocytogenes* in food by PCR has only been performed in very few cases (Makino *et al*, 1995; Herman *et al.*, 1995; Hudson *et al.*, 2001). There are many problems by direct detection of *L. monocytogenes* in food, such as inhibition of PCR by food components and amplification of DNA from dead *L. monocytogenes* cells. To solve these problems several reports dealing with enrichment of food sample prior to PCR detection of *L. monocytogenes* have been published (Bansal *et al.*, 1996; Manzano *et al.*, 1997; Agersborg *et al.*, 1997; O′Connor *et al.*, 2000).

The present study was conducted (1) to develop a shortened PCR-based method that can detect *L. monocytogenes* in enriched food suspension, (2) to determine whether the type of enrichment medium has an influence on the sensitivity and on time required for the results obtained with PCR-based method and (3) to compare this PCR-based method to standard method according to ISO 11290-1 (Anon., 1996) for detecting *L. monocytogenes* in naturally contaminated food samples.

2 MATERIAL AND METHODS

2.1 *L. monocytogenes* strain and media

Listeria monocytogenes ZIM B520 strain was used for artificial contamination of chicken legs. The strain was maintained on triptic soy yeast extract agar (TSAYE, Fluka 22091) plates with weekly subcultivation. Triptic soy yeast extract broth (TSBYE, Fluka 22092) was inoculated

from these plates and incubated at 37°C for 18-20 h for preparing inocula for artificial contamination of food samples.

Half Fraser (HF, Merck 1.10398.0500), buffered peptone water (BPW, Merck 1.07228.0500), TSBYE and universal preenrichment broth (UPB, Difco 223510) were used as enrichment broths. Palcam agar (Merck 1.11755.0500) plate was used as selective medium for listeria isolation. Manufacturers′ directions were followed for all media preparation.

2.2 Artificial contamination of chicken legs and detection procedures

Chicken legs were obtained from local retail butcher shop. The whole skin from the sample was removed aseptically. Twenty-five g of skin were homogenised in 225 ml of enrichment broth using a stomacher (Stomacher 400 homogenizer, Seward, Basingstoke, England) for 3 min. We have tested enrichment of food samples in four enrichment broths (HF, BPW, TSBYE and UPB). At this stage 1 ml of Butterfield′s phosphate buffered dilution water (pH 7.2 ± 0.1) (BP) containing different amounts of *L. monocytogenes* was added. Decimal dilutions of an 18-20-h culture of *L. monocytogenes* were made in sterile BP. Homogenised skin samples were inoculated with 10 cfu, 10^2 cfu, 10^3 cfu and 10^4 cfu of *L. monocytogenes*. The number of *L. monocytogenes* cells in inoculum was determined by plating decimal dilutions of *L. monocytogenes* culture suspension in BP on TSAYE agar, plates were incubated at 37°C for 48 h. Aliquot of 25g of food sample was always checked for the naturally present *L. monocytogenes*.

All enrichment broths were incubated at 30°C for 24 h. Three detection procedures were applied to each sample (Fig. 1).

Procedure A: Two ml of incubated enrichment broth were used for PCR detection ($PCR_{EB, A}$) of *L. monocytogenes*. Enrichment broth was also streaked on Palcam agar plates. The plates were incubated at 37°C for 24 h. Up to five suspect listeria colonies were picked from Palcam agar plates for identification using PCR ($PCR_{CO, A}$).

Procedure B: The 24-h enrichment broth was reincubated for an additional 24 h at 30°C. Two ml of 48-h enrichment broth was used for PCR detection ($PCR_{EB, B}$) of *L. monocytogenes*. 48-h enrichment broth was also streaked on Palcam agar plates. The plates were incubated at 37°C for 24 h. Up to five suspect listeria colonies were picked from Palcam agar plates for identification using PCR ($PCR_{CO, B}$).

Procedure C: One ml aliquot from the 24-h enrichment broth was subcultured to a 9-ml portion of enrichment broth and reincubated for an additional 24 h at 30°C. After that 2 ml of enrichment broth were used for PCR detection ($PCR_{EB, C}$) of *L. monocytogenes*. Enrichment broth was also streaked on Palcam agar plates. The plates were incubated at 37°C for 24 h. Up to five suspect listeria colonies were picked from Palcam agar plates for identification using PCR ($PCR_{CO, C}$).

2.2.1 PCR template preparation

PCR templates were prepared from 24-h enrichment and 48-h enrichment broths and from up to 5 suspect listeria colonies on Palcam plates with optimized lysis of bacterial cells (Klančnik *et al*., 2003). Lysates were kept on ice until PCR was performed.

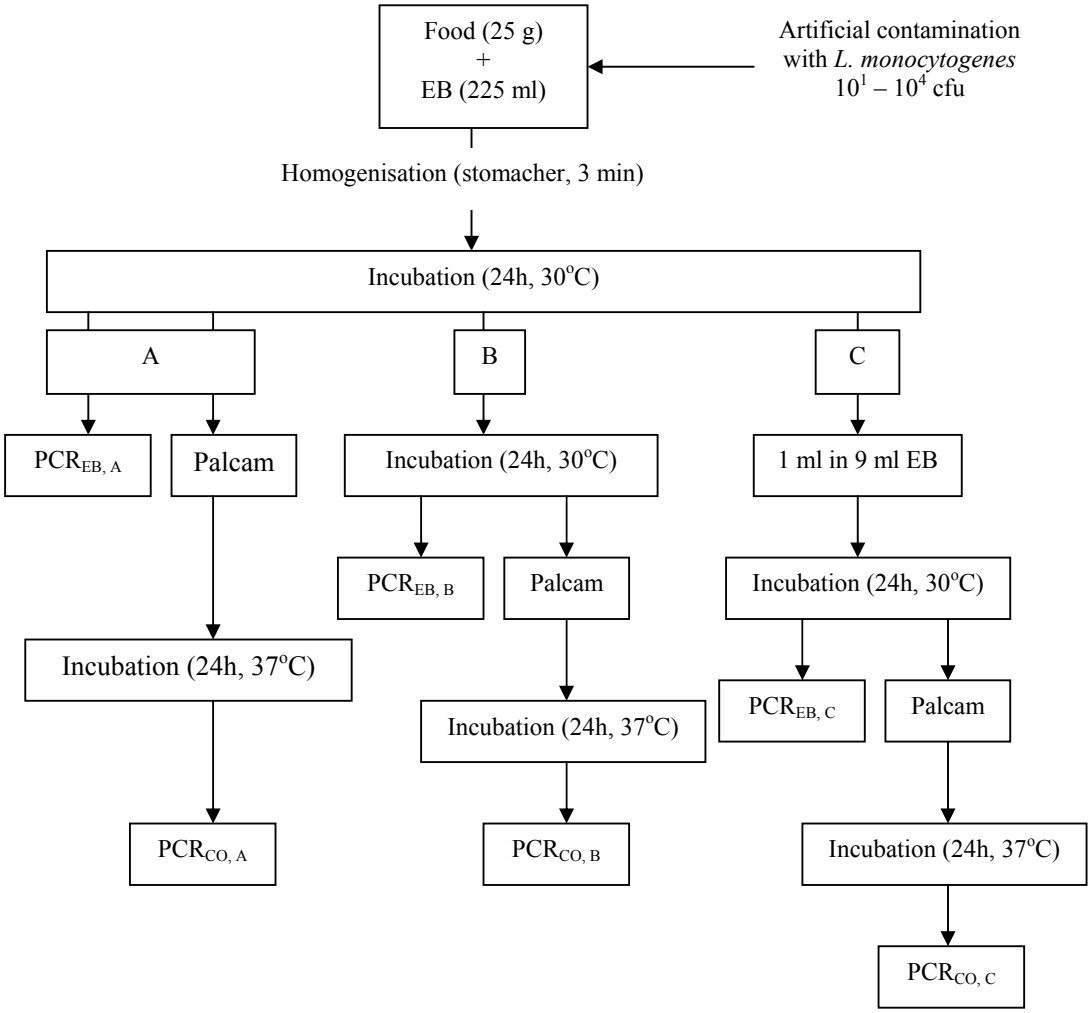

Figure 1: Tested procedures for detection of *L. monocytogenes* in artificially contaminated chicken legs

Legend:

EB	enrichment broth: half Fraser (HF), buffered peptone water (BPW), triptic soy yeast extract broth (TSBYE), universal preenrichment broth (UPB)
$PCR_{EB, A}$	detection of *L. monocytogenes* with PCR from EB after 24-hour incubation
$PCR_{CO, A}$	detection of *L. monocytogenes* with PCR from suspect colonies on Palcam plate after 24-hour incubation of EB
$PCR_{EB, B}$	detection of *L. monocytogenes* with PCR from EB after 48-hour incubation without subculturing
$PCR_{CO, B}$	detection of *L. monocytogenes* with PCR from suspect colonies on Palcam plate after 48-hour incubation of EB without subculturing
$PCR_{EB, C}$	detection of *L. monocytogenes* with PCR from EB after 48-hour incubation with subculturing
$PCR_{CO, C}$	detection of *L. monocytogenes* with PCR from suspect colonies on Palcam plate after 48-hour incubation of EB with subculturing

2.2.2 PCR detection of L. monocytogenes

A reaction mixture of 50 μl contained 5 μl of PCR buffer 10x (Tris-HCl 100 mmol l^{-1}, KCl 500 mmol l^{-1}, Triton X-100 1 %) (Promega M 1661), 3 mmol l^{-1} $MgCl_2$ (Promega A 351B), 150 μmol l^{-1} of each dNTP (dATP, dTTP, dCTP, dGTP) (Applied Biosystems N808-0260), 1 μmol l^{-1} of each primer (LL5: AAC CTA TCC AGG TGC TC , LL4: CGC CAC ACT TGA GAT AT

(Thomas *et al*., 1991)) (MWG – Biotech AG 38-3271-2/34), 0.2 μl Tween 20 (0.05 g ml^{-1}) (Merck 1.09280.0100), 0.5 U *Taq* DNA Polymerase (Promega M188A) and 1 μl of cells lysate.
PCR assays were performed in a DNA thermal cycler (Gene Amp DNA Thermal Cycler 2400, Perkin Elmer). Template DNA was initially denaturated at 95°C for 5 min followed by 30 cycles of 1 min denaturation at 94°C, 1 min primer annealing at 60°C and 2 min extension at 72°C. After a final 8-min extension step at 72°C tubes were held at 4°C. Control samples were included in each PCR. The positive control was in-house standard containing approximately 10^7 *L. monocytogenes* ZIM B520 cells ml^{-1}. In the negative control, 1 μl of sterile distilled water was used instead of bacterial cell lysate.

2.2.3 Detection of amplification products

A portion (8 μl) of PCR product was analysed by electrophoresis in 1.5 % agarose gel (SeaKem ME Agarose, FMC Bioproducts 50014, USA) in Tris-acetate-EDTA buffer 0.5x at 14 V cm^{-1}. The gels were stained with ethidium bromide (0.5 μg ml^{-1}), rinsed, visualised in UV-light and documented by Gel Doc (Bio-Rad Laboratories, USA). A molecular weight marker 100 bp DNA Ladder (Gibco BRL 15628-019, USA) was analysed along with samples.

2.2.4 PCR results interpretation

Presence of a DNA amplification product of 520 bp was taken as a positive result for the presence of *L. monocytogenes* in food sample.

2.3 Detection of *L. monocytogenes* in naturally contaminated food samples

Retail food samples (n = 31) including smoked fish (n = 14) (salmon, trout, cod-fish), fruit and vegetable (n = 15) (melon, sprouts, minced spinach, frozen Brussels sprouts, frozen peas, cooked frozen mangel, mixed frozen vegetables for soup, frozen peas and carrot), delicatessen food (n = 2) (American salad, delicatessen salad with meat of tuna) were examined for the presence of naturally occurring *L. monocytogenes* using cultural method according to ISO 11290-1 standard (Anon., 1996) and PCR-based method.

3 RESULTS

3.1 Artificially contaminated chicken legs

Table 1 shows results of *L. monocytogenes* PCR detection after 24-h and 48-h incubation of artificially contaminated chicken legs in four different enrichment broths. Initial concentration of *L. monocytogenes* was in range from 10^1 to 10^4 cfu (25 g)$^{-1}$ food. Different enrichment broths HF, BPW, TSBYE and UPB were tested concurrently for their effect on PCR detection of *L. monocytogenes*.

PCR detections of *L. monocytogenes* from HF, BPW and TSBYE after 24-h incubation were negative irrespective of the above mentioned initial contamination level. When UPB was used as an enrichment broth there were positive results of PCR detection of *L. monocytogenes* after 24-h incubation in samples inoculated with 100 cfu (25 g)$^{-1}$ or more. Detection of *L. monocytogenes* from samples of chicken legs that were contaminated with 10 cfu (25 g)$^{-1}$ was done from suspect listeria colonies on Palcam agar plates ($PCR_{CO, A}$).

Table 1 PCR detection of *L. monocytogenes* in artificially contaminated chicken legs after different enrichment procedures

EB	Contamination level (cfu (25g)$^{-1}$)	Result of PCR detection					
		$PCR_{EB,A}$	$PCR_{CO,A}$	$PCR_{EB,B}$	$PCR_{CO,B}$	$PCR_{EB,C}$	$PCR_{CO,C}$
HF	10	-	+	+	+	+	+
	100	-	+	+	+	+	+
	1000	-	+	+	+	+	+
	10000	-	+	+	+	+	+
BPW	10	-	+	-	+	-	+
	100	-	+	-	+	-	+
	1000	-	+	-	+	-	+
	10000	-	+	-	+	-	+
TSBYE	10	-	+	-	Ng	-	+
	100	-	-	-	+	-	+
	1000	-	+	-	+	-	+
	10000	-	+	-	-	-	+
UPB	10	-	+	+	Nd	+	Nd
	100	+	+	+	Nd	+	Nd
	1000	+	+	+	Nd	+	Nd
	10000	+	+	+	Nd	+	Nd

Legend:

EB	enrichment broth
HF	Half Fraser
BPW	buffered peptone water
TSBYE	triptic soy yeast extract broth
UPB	universal preenrichment broth
Ng	no growth
$PCR_{EB, A}$	detection of *L. monocytogenes* with PCR from EB after 24-hour incubation
$PCR_{CO, A}$	detection of *L. monocytogenes* with PCR from suspect colonies on Palcam plate after 24-hour incubation of EB
$PCR_{EB, B}$	detection of *L. monocytogenes* with PCR from EB after 48-hour incubation without subculturing
$PCR_{CO, B}$	detection of *L. monocytogenes* with PCR from suspect colonies on Palcam plate after 48-hour incubation of EB without subculturing
$PCR_{EB, C}$	detection of *L. monocytogenes* with PCR from EB after 48-hour incubation with subculturing
$PCR_{CO, C}$	detection of *L. monocytogenes* with PCR from suspect colonies on Palcam plate after 48-hour incubation of EB with subculturing
Nd	not done

Longer 48-h incubation of artificially contaminated food samples allowed PCR detection of *L. monocytogenes* in samples that were enriched in HF and in UPB. *L. monocytogenes* in the other two nonselective media (BPW and TSBYE) was not detected when PCR was done directly from enrichment broth, but only from suspect listeria colonies on Palcam plates. There were no differences in PCR sensitivity of procedure B and procedure C where 1-ml aliquot of the pre-enriched sample was transferred to secondary enrichment broth.

3.2 Proposed PCR-based method

Proposed PCR-based method consists of homogenisation of 25 g (ml) of food in 225 ml of UPB and incubation of enrichment broth at 30°C for 48 h. Two ml of each 24-h and 48-h enrichment broths are used for PCR detections of *L. monocytogenes*.

Enrichment broths should also be streaked onto Palcam agar plates. If the PCR results from enrichment broth after 24-h and after 48-h of incubation are negative for *L. monocytogenes*, the PCR should be done from suspect listeria colonies on Palcam agar plates. The plates are incubated at 37°C for 24 h and *L. monocytogenes* is detected from up to five suspect listeria colonies with PCR.

3.3 Naturally contaminated food samples

Detection of *L. monocytogenes* in presumably naturally contaminated food samples was done according to cultural method (Anon., 1996) and according to the proposed PCR-based method. Out of 31 samples examined, 8 were found to be positive by both methods and 23 were negative by both methods.

4 DISCUSSION

A large number of rapid methods for detection of *L. monocytogenes* have been developed in the last decade. Most cultural methods are effective in detecting foodborne pathogens but may require a week or more to give the results.

The aim of this study was to develop a PCR-based method for the detection of *L. monocytogenes* in food which would give equivalent results to the standard detection method ISO 11290-1 (Anon., 1996), but in a shorter time period.

We have used previously described *L. monocytogenes*-specific primers LM5 and LM4 (Thomas *et al*., 1991), optimized DNA-template preparation (Klančnik *et al*., 2003) and tested different food sample enrichment procedures. Enrichment of target bacteria by culture is one of the possibilities for obtaining enough DNA for PCR detection. Compared to chemical extraction applied directly to food, enrichment is less laborious, reduces the amount of PCR inhibitors and allows only living bacteria to be detected (Lantz *et al.*, 1994). Selective and nonselective broths in this study included: HF as by cultural detection method according to ISO standard (Anon., 1996), BPW (Duffy *et al.*, 2001), TSBYE (Agersborg *et al.*, 1997) and UPB (Bailey and Cox, 1992; Agersborg *et al.*, 1997; Zhao and Doyle, 2001). The use of nonselective enrichment broths for detection of *Listeria* from food has been recommended as providing some advantages over selective enrichment broths, such as shorter enrichment periods or increased recovery of injured cells (Duffy *et al.*, 2001). Our results showed that PCR-based method could detect *L. monocytogenes* directly in enrichment broth with artificially contaminated chicken legs only when UPB was used as an enrichment broth. The detection limit was 100 cfu $(25\ g)^{-1}$ of food after 24 h incubation and 10 cfu $(25\ g)^{-1}$ after 48 h incubation of enrichment broth. To achieve sensitivity of 1 cfu $(25\ g)^{-1}$ we have still included plating of enrichment broth onto selective medium and detection of *L. monocytogenes* from *Listeria*-like colonies. Achieved sensitivity is comparable to other PCR-based detection methods, such as 1–10 cfu $(25\ g)^{-1}$ (O′Connor *et al.*, 2000), 1-5 cells $(5\ g)^{-1}$ (Agersborg *et al.*, 1997), 10^1 to 10^2 cells ml^{-1} or g^{-1} (Manzano *et al.*, 1997). The described PCR-based detection procedure requires 30–72 h and it is quicker than cultural method according to ISO standard (Anon. 1996) and comparable to some other PCR-based methods, i.e. 55 h (Agersborg *et al.*, 1997), 30 h (Manzano *et al.*, 1997).

To evaluate PCR-based method, food samples potentially naturally contaminated with *L. monocytogenes* were analysed by cultural detection method (Anon., 1996) and by proposed PCR-based method. Out of 31 samples 8 samples were positive and 23 samples were negative with both methods. Application of PCR to procedure which enables *L. monocytogenes* detection within 1.5 to 3 days offers alternative to currently used cultural detection methods. Proposed PCR-based method for detection of *L. monocytogenes* in food is specific, and it is simpler and quicker than the standard detection procedure according to standard ISO (Anon., 1996). However, an additional evaluation of the proposed PCR-based method on naturally contaminated food is still required.

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