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# Application of a chromogenic medium and the PCR method for the rapid confirmation of *L. monocytogenes* in foodstuffs

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**Abstract**

Detection of *Listeria monocytogenes* in foodstuffs by conventional cultivation methods carried out according to EN ISO guidelines is rather time-consuming. Therefore, two alternative methods were applied for rapid confirmation of *L. monocytogenes* in foodstuffs. Inoculum from liquid selective broth was plated on PALCAM and OXFORD agar and on chromogenic agar medium RAPID *L. mono*. Suspect colonies from PALCAM were confirmed according to EN ISO standards and by the multiplex PCR method. In total, 990 samples of foodstuffs were investigated and 63 strains of *L. monocytogenes* were isolated. The chromogenic medium RAPID *L. mono* provided results comparable to PCR, it is easier to handle and provides considerable financial savings. 

*Keywords:* *Listeria*; Confirmation; Foodstuffs; Chromogenic medium; PCR

## 1. Introduction

Listeriosis in humans has been known for over 70 years. However, alimentary transmission of this infection has been confirmed only relatively recently. Detection of *Listeria monocytogenes* in foodstuffs by conventional cultivation methods carried out according to ISO 10560 (ISO, 1996) standards for milk and dairy products or according to the newest horizontal method EN ISO 11290-1 (ISO, 1999) is rather time-consuming and takes 4–6 days. Rapid methods (e.g., PCR) have not yet been validated for routine bacteriological analysis of foodstuffs and so classical cultivation techniques still remain the official methods. In foodstuffs, along with *L. monocytogenes* (LM), there are also other *Listeria* species and their isolation and consequent confirmation may take up an additional few days. Such a method for the detection of *L. monocytogenes* in foodstuffs is not ideal and, therefore, more rapid sufficiently sensitive and specific alternative methods are always being sought. LM secretes several proteins that contribute to virulence, listeriolysin O, PIPLC and broad-range phospholipase C (Wadsworth and Goldfine, 1999). Pathogenic and non-pathogenic *Listeria* species can be distinguished on the basis of phosphatidylinositol specific phospholipase C activity (Nottermans et al., 1991) and the chromogenic medium RAPID *L. mono* is based on this principle. Polymerase chain reaction (PCR) based on a DNA fragment of the hlyA gene

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encoding listeriolysin O is a method used for differentiating LM from other *Listeria* species (Border et al., 1990; Bubert et al., 1999).

The aim of this study was the application of newer confirmation methods to shorten the time needed for identification significantly and, most of all, to simplify and render more precise the confirmation of this pathogenic species.

## 2. Material and methods

### *2.1. Food samples*

During 1998 and 1999, 990 samples of foodstuffs were investigated. The food samples were obtained from a retail market; in part they were taken directly from the producers within the framework of routine public health surveillance.

### *2.2. Methods for detecting L. monocytogenes*

In 1998 the detection of listeria in milk, vegetable, and fruit products was carried out according to ISO 10560 guidelines implementing the enrichment medium BLEB (Buffered Listeria Enrichment Medium, OXOID, UK); in meat and meat products detection was made by the USDA method with media UVM I and UVM II (University Vermont Medium, OXOID, UK). Since 1999 samples have been investigated with the aid of a horizontal method (intended for food and animal feed) for detecting *L. monocytogenes* following the standard EN ISO 11290-1 using half-strength and full Fraser medium (OXOID, UK). All the samples after the selective enrichment step were plated on agar media OXFORD, PALCAM (OXOID, UK) and RAPID *L. mono* (SANOFI Diagnostics Pasteur, France).

### *2.3. Confirmatory methods*

#### *2.3.1. EN ISO 11290-1*

Five suspect *Listeria* species colonies from OXFORD and PALCAM agar were confirmed according to the corresponding standard (test for catalase, CAMP test, utilisation of rhamnose and xylose).

#### *2.3.2. Chromogenic medium*

RAPID *L. mono* medium containing substrate 5-bromo-4-chloro-3-indoxyl-myo-inositol-1-phosphate and xylose was used. Phosphatidylinositol phospholipase C (PIPLC) positive strains form convex turquoise colonies on this medium. Two PIPLC positive *Listeria* species (*L. monocytogenes* and *L. ivanovii*) are distinguished by acid production from xylose.

#### *2.3.3. Serotyping*

All *L. monocytogenes* isolates from foodstuffs were serotyped with the aid of antisera from Denka Seiken (Japan).

#### *2.3.4. PCR method*

An isolated colony taken from a non-selective medium (BHI agar) was resuspended in 100 μl of lysing solution (0.05 mol $l^{-1}$ NaOH, 0.0125% sodium dodecyl sulphate) according to Rijpens et al. (1995). Alkaline lysis lasted 20 min at 100°C. The multiplex PCR method was modified according to Lawrence and Gilmour (1994). A volume of 50 μl of reaction mixture for PCR contained 1 μl of cell lysate, 50 mmol $l^{-1}$ KCl, 10 mmol $l^{-1}$ Tris–HCl (pH 8.3), 1.5 mmol $l^{-1}$ $MgCl_2$, 200 μmol $l^{-1}$ dATP, dGTP, dCTP, 160 μmol $l^{-1}$ dTTP, 40 μmol $l^{-1}$ dUTP (Life Technologies, USA), 1 μmol $l^{-1}$ of primers LI1, LM1, LM2, and 2 μmol $l^{-1}$ of primer U1 (Generi Biotech, CZ).

To the reaction mixture, 1 μl (0.1 U) of uracil DNA glycosylase (Life Technologies, USA) and 0.2 μl (1 U) of Platinum® *Taq* DNA polymerase (Life Technologies, USA) were added. The reaction mixture was overlayed with 30 μl of paraffin oil (MJ Research, USA).

Amplification was performed in a thermal cycler PTC-150 (MJ Research, USA) followed by the thermal programme: incubation 10 min at 37°C; initial denaturation 2 min at 96°C; 24 cycles (denaturation 80 s, 94°C; annealing 90 s, 50°C; amplification 120 s, 72°C); and final amplification 10 min at 72°C.

The PCR product was analysed by electrophoresis in 1.5% agarose (Serva, Germany) in 1× TBE buffer (0.089 mol $l^{-1}$ Tris–HCl, 0.089 mol $l^{-1}$ boric acid, 0.002 mol $l^{-1}$ EDTA, pH 8.0). Before solidification, 0.015% ethidium bromide was added to the

gel. Electrophoresis was run for 70 min at 50 V. Visualisation was carried out on a transilluminator (UVB 10, Ultra Lum, USA) and results were registered by a camera (Gel Cam, Polaroid, USA).

In parallel with the samples, a positive control (*L. monocytogenes* CCM 5576 processed in the same way as the other samples), a negative control (instead of the cell lysate, 1 μl $H_2O$ was added to the reaction mixture), and DNA marker pBR 322/Alu I (MBI Fermentas, Latvia) were placed on the gel.

## 3. Results and discussion

In the years 1998 and 1999 a total of 990 food samples were investigated for the presence of *L. monocytogenes*. The spectrum of commodities analysed and the number of positive findings of *L. monocytogenes* and other *Listeria* species are presented in Table 1. A total of 255 listeria isolates were detected from the series of foodstuffs examined, 176 strains of *L. innocua*, 63 strains of *L. monocytogenes*, nine strains of *L. welshimeri*, six strains of *L. seeligeri* and one strain of *L. ivanovii*. Most often LM were demonstrated in poultry, namely in abattoir-processed hens, chickens and turkeys (16.7%) and further in raw beef and pork meat (13.3%). Of heat processed foodstuffs, most LM were isolated from meat products (7.4%), namely white pudding (headcheese), black pudding (blood sausage), liver paté, gammon sausage, and poultry meat frankfurters.

In all cases, *L. monocytogenes* in the foodstuffs was present in the company of other non-pathogenic species, most frequently along with *L. innocua*. Colonies with typical morphology on plates with OXFORD and PALCAM media could mostly be read only after 48 h as recommended in the standard. In all cases, non-pathogenic *Listeria* spp. decidedly outnumbered LM and, therefore, even when five suspect colonies are taken for confirmatory testing, it is probable that LM may not always be detected, although it is present in the sample. A great advantage is to use a chromogenic medium on which LM colonies are easily recognisable from other *Listeria* spp. even after 24 h. The principle of the chromogenic medium lies in the detection of phosphatidylinositol phospholipase C (PIPLC) positive strains and the demonstration of the ability to utilise xylose. A substituted indigo compound is formed after cleavage of chromogenic substrate 4-chloro-5-bromo-indoxylmyoinositol-phosphate. *L. monocytogenes* manifests itself on the chromogenic medium by a varying intensity of turquoise colonies without any yellow zone around them. Likewise, *L. ivanovii* produces phospholipase C, but because it also utilises xylose the colony is surrounded by a zone of yellow (Restaino et al., 1999). Other *Listeria* species grow on this medium as milk-coloured convex colonies, 1–2 mm in diameter, and when they utilise xylose the zone around them is yellow in colour.

Paziak-Domanska et al. (1999) stated that not only LM but also other *Listera* species (*L. innocua* and *L. welshimeri*) can produce PIPLC, but these strains do

Table 1
Commodities investigated and the frequency of *L. monocytogenes* and other *Listeria* spp. positive findings

| Commodity | No. of samples examined | No. of positive findings | |
|---|---|---|---|
| | | LM | Other *Listeria* spp. |
| Meat, raw and semifinished | 240 | 32 | 104 |
| Poultry | 84 | 14 | 24 |
| Fish | 120 | 2 | 18 |
| Dairy products | 209 | 2 | 6 |
| Meat products | 121 | 9 | 8 |
| Delicatessen products | 48 | 0 | 6 |
| Vegetables (frozen) | 156 | 4 | 24 |
| Fruit (dried) | 12 | 0 | 2 |
| Total | 990 | 63 | 192 |

not show lethality for BALB/c mice. On the contrary, there may occur LM mutants that do not produce PIPCL. However, according to Camilly et al. (1991) they are avirulent.

In our study, all 63 LM isolates from foodstuffs manifested PIPCL production and we did not detect any other strain of *Listeria* species producing PIPLC on the chromogenic medium. Other bacterial species producing phospholipase C (e.g., *Staphylococcus aureus* and *Bacillus cereus*) were suppressed during the selective enrichment steps and due to the lithium chloride content of the chromogenic medium.

Serotyping of strains isolated from foodstuffs showed that the majority of LM (48) belonged to serotype 1/2, with only nine belonging to serotype 4 and six of the LM isolates grew as the rough form. Results are presented in Table 2.

Another option for rapid and reliable confirmation of results is the application of the multiplex PCR method. By this technique, with two sets of oligonucleotides it is possible to amplify two fragments of the genome of *Listeria*. Selected isolates of 45 *L. monocytogenes* and eight *L. innocua* were analysed by this technique and the results are presented in Table 3. For the identification of *Listeria* species, two sets of specific primers were derived from the gene for listeriolysin O and from the gene for 16S rRNA which is characteristic for the genus *Listeria*. Simultaneously, this PCR product acts in the reaction as an internal control and its presence was registered in all the isolates.

Table 2
Results of serotyping *L. monocytogenes* isolated from foods

| Serotype | 1/2 | 4 | R form |
|---|---|---|---|
| No. of isolates | 48 | 9 | 6 |

Prevention of false-positive results caused by contamination of the polymerase chain reaction by a product from a preceding run was secured by the addition of deoxyuridin triphosphate (dUTP) and uracil N-glycosylase to the reaction mixture (Longo et al., 1990). The total time needed for carrying out the PCR including electrophoresis is no longer than 4 h. Food samples and enrichment media may be inhibitory to PCR and thereby can lower its detection capacity (Wang et al., 1999). Therefore, subculturing on a non-selective medium often precedes the PCR (Border et al., 1990). To shorten the time we used for PCR the colonies were grown on PALCAM and OXFORD agar and our results showed there were no inhibitors present (Fig. 1).

The chromogenic medium RAPID *L. mono* provides results comparable to PCR, namely in 24 h after selective enrichment. In comparison with PCR methods the application of chromogenic media is easier to handle and provides considerable financial savings.

## 4. Conclusion

On the basis of the results obtained we found that, in the detection of LM in foodstuffs, the replacement of a selective medium given in standard EN ISO 11290-1 by the chromogenic medium RAPID *L. mono* is very convenient and useful. The multiplex PCR method is a good replacement for classical confirmatory techniques, namely in laboratories that routinely use molecular biology methods. Both replacements, while retaining the same or providing an even higher specificity, give a non-negligible saving of time.

Table 3
Results of PCR analyses

| No. of isolates tested | Species | Serotype | Result of PCR | |
|---|---|---|---|---|
| | | | LM (702 bp) | *Listeria* spp. (938 bp) |
| 37 | *L. monocytogenes* | 1/2 | + | + |
| 6 | *L. monocytogenes* | 4 | + | + |
| 2 | *L. monocytogenes* | R form | + | + |
| 8 | *L. innocua* | − | − | + |

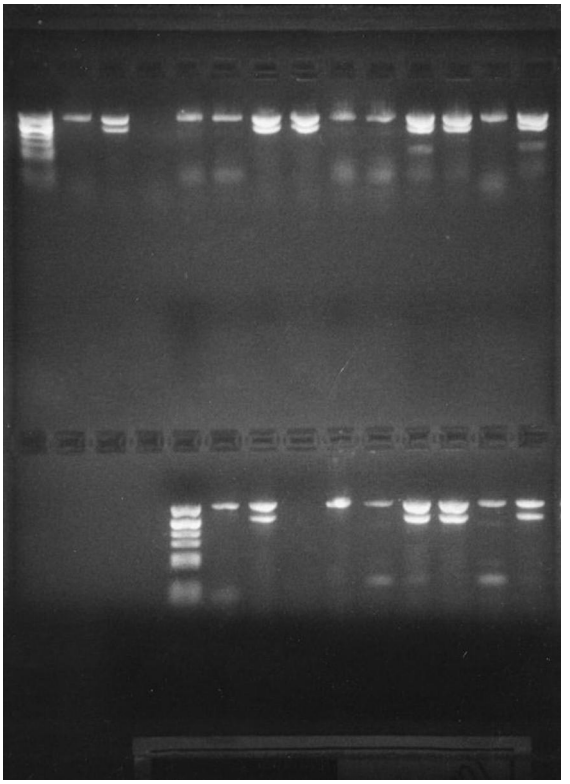

Fig. 1. Amplification products obtained in lanes: M, DNA molecular weight marker pBR32/AluI; 1, positive control *L. innocua* ATCC 33090; 2, positive control *L. monocytogenes* CCM 5576; 3, negative control; 4, *L. innocua* from BHI agar; 5, *L. innocua* from OXFORD agar; 6, *L. monocytogenes* from BHI agar; 7, *L. monocytogenes* from OXFORD agar; 8, *L. ivanovii* from BHI agar; 9, *L. monocytogenes* from PALCAM agar.

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