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Short communication

PCR detection of *Listeria monocytogenes* in 'gravad' rainbow trout

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Abstract

'Gravad' rainbow trout artificially contaminated with *Listeria monocytogenes* was analyzed by use of a 4 h enrichment period followed by extraction of DNA and PCR amplification. This procedure made it possible to detect 10–100 cfu *L. monocytogenes* per gram 'gravad' rainbow trout, within 12 h. After a prolonged enrichment period of 24 h, numbers as low as 1–10 cfu *L. monocytogenes* per gram could be detected. The method described may be a useful tool for screening samples of 'gravad' rainbow trout for the presence of *L. monocytogenes*, since it is sensitive, rapid and simple. © 1997 Elsevier Science B.V.

Keywords: *L. monocytogenes*; Gravad rainbow trout; PCR

1. Introduction

Fish and seafoods have during recent years been focused upon as potential sources of infection for listeriosis (Karim and Embarek, 1994; Loncarevic et al., 1996). 'Gravad' rainbow trout is a popular dish in Sweden. Raw rainbow trout fillets are rubbed with a mixture of sugar, salt and pepper, covered with dill, put into a plastic bag and placed in a refrigerator for 2 days. The plastic bag is opened and the fillets are packaged sliced or whole under vacuum in oxygen-impermeable film and stored up to 21 days at 8°C. Such products are sometimes contaminated with *Listeria monocytogenes* (Jemmi, 1990; Loncarevic et al., 1996). Since *L. monocytogenes* can grow at refrigeration temperature even initial low numbers of the organism may develop into high levels before their 'consume-by date'. As these products

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are consumed without heating, they constitute a potential public health risk for susceptible individuals.

The aim of this study was to develop a rapid and simple method for detection of *L. monocytogenes* in gravad rainbow trout using PCR. A problem when conducting PCR analysis on foodstuffs is that the food often contains factors that have an inhibitory effect on the PCR reaction (Rossen et al., 1992; Simon et al., 1996). Addition of spermidine is one suggestion to facilitate the PCR reaction (Ahokas and Erkkila, 1993; Wan and Wilkins, 1993). Spermidine is a polyamine that is often included in restriction enzyme processes to counteract inhibitory effects of contaminants isolated together with the DNA (Wan and Wilkins, 1993). Furthermore, polyamines are known to stimulate enzymes involved in nucleic acid metabolism like DNA polymerases (Fisher and Korn, 1979). Therefore, we also compared the presence or absence of spermidine in the PCR reaction.

2. Materials and methods

2.1. Sample preparation

Pieces (10.0 g) of gravad rainbow trout were injected with different numbers of *L. monocytogenes* ranging from 10^1 to 10^5 cfu total cells. As a negative control, we used 10.0 g of gravad rainbow trout known to be negative for *L. monocytogenes*. To each sample was added 90 ml prewarmed (30°C) *Listeria* enrichment broth (Difco) supplemented with 4.0 mg nalidixic acid, 1.0 mg acriflavine, 5.0 mg cyclohexamide and homogenized in a stomacher for 1 min. Afterwards, the homogenate was poured into a 100 ml graduated glass and incubated at 30°C for 4 and 24 h. After 4 h the homogenates had separated, leaving a clear middle phase from which 10 ml was taken out and centrifuged for 10 min at $150 \times g$. This was also done after 24 h. After centrifugation, the liquid was discarded and DNA was extracted from the pellet (see below). Portions (0.5 ml) of the homogenate were taken for viable counts from each glass before and after incubation at 4 and 24 h, and were plated onto *Listeria* selective medium (Oxford Formulation agar) plates (Oxoid:agar base CM856 and supplement SR140) and incubated at 37°C for 48 h.

2.2. DNA extraction

The procedure for extracting DNA was based upon the method described by Pitcher et al. (1989). Briefly, the pellet was dissolved in 1 ml lysozyme (50 mg/ml, Boehringer Mannheim, in 10 mM Tris, 1 mM EDTA, pH 8.0), transferred to an Eppendorf tube and incubated at 37°C for 30 min. The tube was then centrifuged for 2 min at $700 \times g$. The pellet was vortexed with 600 μl 5 M guanidine isothiocyanate (Gibco BRL, Gaithersburg, MD) and left at room temperature for 5 min, followed by the addition of 250 μl cold 7.5 M ammonium acetate and storage on ice for 5 min. A total of a 500 μl chloroform-2-pentanol (24:1; v/v) mixture was added, the tube inverted five times and thereafter centrifuged for 20 min at $1500 \times g$. From the upper phase, 500 μl was transferred to another Eppendorf tube and 270 μl cold ($+4$°C) isopropanol was added for selective precipitation of DNA. The tube was centrifuged for 20 min at $1500 \times g$. The pellet was washed once with ethanol (70%; v/v), dried at 70°C for 10 min and dissolved in 40 μl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). A portion (10 μl) was taken out for PCR analysis.

2.3. PCR analysis

The primers used were 5′-CGGAGGTTCCG-CAAAAGATG-3′ (upstream primer) and 5′-C-CTCCAGAGTGATCGATGTT-3′ (downstream primer) amplifying a 234 bp fragment from the gene encoding listeriolysin O. These primers have previously been described by Furrer et al. (1991), who showed them to be specific for *L. monocytogenes*. The PCR was performed essentially as described by Saiki et al. (1988). The PCR mixture (50 μl) contained 10 mM Tris–HCl pH 8.0, 2.0 mM $MgCl_2$, 50 mM KCl, 0.1% Tween-20, 400 μmol of each deoxynucleoside triphosphate (dATP, dTTP, dCTP, dGTP; Boehringer Mannheim), 0.3 μm of each

primer, and 4.0 U Taq polymerase (Perkin Elmer). PCR was carried out in a Perkin Elmer thermocycler (P13480) and run for 40 cycles (1 min, 94°C; 1 min, 55°C; 1 min, 72°C). An aliquot (6 μl) of the reaction mixture was mixed with 2 μl of gel loading buffer, type IV (Sambrook et al., 1989), fractionated on a 2% agarose gel (SeaKem, LE; FMC) in 0.5 × TBE (45 mM Tris-borate, 1 mM EDTA) at 8 V/cm for 30 min. The PCR product was visualized by ethidium bromide staining (1.5 μg/ml for 15 min) and photographed with a Polaroid MP-3 camera. The result was confirmed by dot blot hybridization.

To evaluate the effect of spermidine we used two tubes for each reaction, one with 0.48 mM spermidine and one without. The concentration used was within the recommended range of 0.4–0.6 (Ahokas and Erkkila, 1993) and 0.2–1.0 mM (Wan and Wilkins, 1993).

2.4. Dot blot hybridization

An aliquot (20 μl) of the PCR product was mixed with 5 μl of 1.6 M NaOH, vortexed, and then left at room temperature for 15 min. Afterwards 5 μl of 6 M ammonium acetate was added and the mixture put on a nylon membrane (Hybond N^+) in a mini dot blot chamber (Ubitech AB, Uppsala, Sweden). The membrane was baked for 4 min on a 312 nm UV table. Afterwards, 100 pmol of the probe (5′-CCATCTGTATAAGCTTTTGAAG-3′) (Furrer et al., 1991), biotinylated in both the 5′ and 3′ end (MedProbe AS, Oslo, Norway), were added. Hybridization was carried out at 45°C for 15 min, followed by 5 min of incubation at 50°C with filter-blocking solution (3% bovine serum albumin, 1% sodium dodecyl sulfate in 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate)). Finally, 2.0 U streptavidin alkaline phosphate conjugate (Boehringer Mannheim) was added, and the hybrid visualized using a stabilized substrate for alkaline phosphatase (Western Blue, Promega).

2.5. Reproducability test

To ensure reproducibility, the whole procedure was repeated five times with different gravad rainbow trouts.

3. Results and Discussion

Using a 4 h enrichment before the PCR analysis, 10–100 cfu of *L. monocytogenes* per gram in the original sample (gravad rainbow trout) could be detected (Fig. 1). The whole analytical procedure—excluding the hybridization step—could be performed in 1 day. If the enrichment period was extended to 24 h, an initial concentration of as low as 1–10 cfu *L. monocytogenes* per gram could be detected. An enrichment procedure before PCR analysis serves two main purposes. The first is to increase the number of bacteria, i.e. after 4 h of enrichment *L. monocytogenes* approximately doubled in numbers and after a 24 h incubation numbers reached 10^7–10^9 cfu/ml of the homogenate, regardless of the numbers initially added. Secondly, it facilitates the PCR reaction and avoids false-negative results. During the enrichment period the sample separated into three phases, a muddy upper phase mainly consisting of fat, a clear middle phase and a muddy bottom phase of food particles. The clear middle phase proved to be an excellent sampling spot for PCR analysis. Preliminary studies showed that there was approximately the same number of bacteria

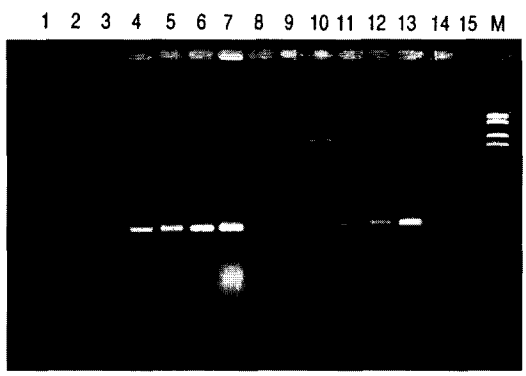

Fig. 1. PCR detection of *L. monocytogenes* in gravad rainbow trout. Lane 1, 0 cfu/g gravad rainbow trout; lane 2, 1 cfu/g; lane 3, 10 cfu/g; lane 4, 100 cfu/g; lane 5, 1000 cfu/g; lane 6, 10 000 cfu/g; lane 7, positive control (purified DNA from *L. monocytogenes*); lanes 8–13, the same number of bacteria as in lanes 1–7 with the addition of 0.48 mM spermidine; lane 14, gravad salmon negative for *L. monocytogenes*; lane 15, negative control (water added); lane M, pBR328 *BglI* plus pBR328 *Hin*fI marker.

in the clear phase as in the bottom sediment. When using the clear phase, as much as a 10 ml sample could be taken out for DNA extraction. This increases the probability of detecting the listeria bacteria with a factor of 10 compared with a sampling volume of 1 ml. Sampling directly after stomaching is difficult since the mixture is very thick and blocks the pipette used for sampling. The food mixture also often contains numerous factors that have an inhibitory effect on the PCR reaction (Rossen et al., 1992; Simon et al., 1996).

The use of 0.48 mM spermidine in the PCR reaction did not increase the sensitivity but, instead, new bands turned up on the gel, indicating a decreased specificity (Fig. 1). We therefore conclude that under the conditions described in this paper, spermidine does not improve the method.

The PCR results in our study were confirmed by dot-blot hybridization. The hybridization takes between 1 and 2 h to perform, but we think this step could be excluded since visualization of the PCR product on the gel will, in most cases, provide sufficient information to give a positive or negative result.

The use of PCR methods for the detection of *L. monocytogenes* in foods has been described by several authors, e.g. Rossen et al. (1991); Niederhauser et al. (1992); Wernars et al. (1991); Simon et al. (1996). However, the methods described are either rapid but not very sensitive, or sensitive but time-consuming and/or laborious. The method described in the present paper is capable of detecting 10–100 cfu *L. monocytogenes* per gram gravad rainbow trout within 1 day.

Since the contamination of rainbow trouts with *L. monocytogenes* most likely takes place in the processing plants (Rörvik et al., 1995), there is a need to screen the products for the presence of *L. monocytogenes* before they leave the manufacturer. The method described in this paper is proposed as being useful for such screenings.

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