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Research Article

Detection of *Listeria monocytogenes* by using PCR in *Helix pomatia*

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Abstract: The detection of *Listeria monocytogenes* in *Helix pomatia*, both raw and cooked is presented here. Polymerase chain reaction (PCR) is a rapid procedure with both sensitivity and specificity for quick detection and identification of *Listeria monocytogenes* from raw and cooked *Helix pomatia*. A total of 30 bags (10 g each) of *H. pomatia* samples were investigated for the *Listeria monocytogenes* inlB gene with the PCR method. PCR amplification products demonstrated that 18 of the 30 samples showed positive reactions to *Listeria monocytogenes* in the PCR. All PCR positive samples showed specific amplification of the 343 bp fragment for *Listeria monocytogenes*.

Key Words: *Listeria monocytogenes*, *Helix pomatia*, PCR

Kara Salyangoz (*Helix pomatia*)'unda *Listeria monocytogenes*'in PCR (Polimeraz Zincir Reaksiyonu) Metodu Kullanılarak Tanımlanması

Özet: Bu çalışmada, çiğ ve pişirilmiş Kara Salyangoz (*Helix pomatia*)'larında *Listeria monocytogenes*'in varlığı Polimeraz Zincir Reaksiyonu (PCR) metodu ile incelenmiştir. PCR, çiğ ve pişirilmiş Kara Salyangoz (*Helix pomatia*)'larından *Listeria monocytogenes*'in identifikasyonu ve hızlı tanısında spesifite ve duyarlılığı yüksek bir metottur. PCR metodu kullanılarak *Listeria monocytogenes* inlB geninin varlığının ortaya çıkarılması amacıyla toplam 30 grup Kara salyangozu örneği kullanılmıştır. PCR'da *Listeria monocytogenes* yönünden 30 örneğin 18'inde pozitif amplifikasyon ürünleri saptanmıştır. PCR pozitif ürünlerin hepsinde, *Listeria monocytogenes* için spesifik olan 343 bp aralığındaki bant görüntüsü elde edilmiştir.

Anahtar Sözcükler: *Listeria monocytogenes*, *Helix pomatia*, PCR

Introduction

The genus *Listeria* currently includes 6 species: *L. monocytogenes, L. ivanovii, L. seeligeri, L. innocua, L. welshimeri*, and *L. grayi*. Two of these species, *L. monocytogenes* and *L. ivanovii*, are potentially pathogenic (1). *Listeria monocytogenes* are one of the most important foodborne pathogens responsible from serious infections in immunocompromised individuals, newborns, pregnant women, and the elderly. *Listeria* infections can cause abortion, meningitis, meningoencephalitis, septicemia, and death (2).

Listeria organisms are widely disseminated in the rural environment and, consequently, contaminate the raw materials used in the preparation of industrially processed foods as well as the production plants (3). They have been isolated from soil, decaying vegetable matter, silage, sewage, water, animal feed, fresh and processed meats, raw milk, cheese, slaughterhouse waste, and asymptomatic human and animal carriers. Because of their widespread occurrence, *Listeria* species have many opportunities to enter food production and processing environments (2,4,5). These bacteria are well equipped to survive food processing technologies. For example, they tolerate high concentrations of salt and relatively low pHs, and worst of all, they are able to multiply at refrigeration temperatures (6-8). This makes *Listeria* micro-organisms a serious threat to food safety and ranks them among the micro-organisms that most concern the food industry. The foods most frequently implicated are soft cheeses and dairy products, smoked fish, salads, and in general, industrially produced and

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refrigerated ready-to-eat products that are eaten without cooking or reheating (9-11). There are thousands of snail species that exist on land and in water. The typical snails of the genus *Helix* have 2 species in Europe. The common garden snail *Helix aspersa* is very destructive to plants. The Roman snail *Helix pomatia* (*H. pomatia*), which is included in the Gastropoda class of the Mollusca phylum, is 'corralled' for the gourmet food market and is consumed widely, particularly in countries such as France, Belgium, Germany, and Italy. Demand for land snails for consumption is increasing because they have high quality protein, are low in fat, and have high calcium, magnesium, and iron content. Turkey is meeting part of this demand. Yet, very few studies have been carried out related to land snail meat in Turkey. To date, we have not encountered one study indicating the microbiological quality of snail meat in the Turkish literature (12).

For many years, clinical *Listeria* isolates were a mere laboratory rarity, and the epidemiology of the disease they cause was an unresolved mystery (13). However, at the end of the 1970s and the start of the 1980s, the number of reports on *Listeria* isolations began to increase, and from 1983 onwards, a series of epidemic human outbreaks in North America and Europe clearly established listeriosis as an important food-borne infection (7,14,15).

In recent years, a number of outbreaks of foodborne illness involving a wide range of foods have been linked to *L. monocytogenes* (16).

Since all *Listeria* species are potential food contaminants, the presence on foodstuffs of any of these species can be considered to be an indicator of their contamination and of the potential presence of *L. monocytogenes*. However, because the threat to public health posed by contamination of foods by the *L. monocytogenes,* it is very important that *L. monocytogenes* be identified rapidly and reliably (17).

Inclusion of *L. monocytogenes* in the list of organisms subject to Hazard Analysis and Critical Control Point (HACCP) has recently driven the search for detection methods suitable for on-line monitoring (18).

The standard analytical method for the detection of *L. monocytogenes* in food and animal feed, EN ISO 11290-1, as well as the standard method for the detection of *L. monocytogenes* in milk and milk products, ISO 10560, suffer from an excessive time requirement of 7–10 d (19, 20). Conventional methods for detection and identification of *L. monocytogenes* are laborious, time-consuming, and are not very sensitive (18).

Polymerase chain reaction (PCR) has been shown to have a great potential to speed-up the detection of *L. monocytogenes* in food (21). PCR provides a powerful format for designing nucleic acid-based assays that are highly specific and sensitive, as well as quantitative (22, 23). Initially, detection of PCR products (and by inference, estimated target numbers) was accomplished by gel electrophoresis, typically by using ethidium bromide to visualize the amplification products.

PCR offers a very powerful tool to elaborate specific, sensitive, and rapid detection methods for bacterial pathogens in food products, both clinical and environmental samples (24).

The aim of the study was to detect *L. monocytogenes* in *H. pomatia* in raw and industrially produced, refrigerated cooked products using PCR.

Materials and Methods

Material

A total of 30 bags (10 g each) of *H. pomatia* samples raw and cooked were obtained from one firm in Turkey. Of 30 bags investigated, 2 bags were raw and twenty-eight bags were industrially produced and refrigerated cooked products. Snail meat samples were collected from the following 5 pre-determined processing stages: live snail; after boiling; after meat calibration; after packaging; after freezing.

Reference strains

L. monocytogenes NCTC 11994 was used as the positive control strain.

Enrichment

To determine the detection limit, 10 g of *H. pomatia* samples were homogenized in 90 ml of University of Vermont Modified (UVM) *Listeria* enrichment broth (Difco, BD, 222330, USA) using a Stomacher 400 homogeniser (Seward, Basingstoke, England) and incubated for 24 h at 30 ºC. A volume of 0.1 ml of the primary enriched sample was used to inoculate 10 ml of Fraser broth (Difco, BD, 211767, USA) and incubated for 24 h at 37 ºC (2). A volume of 0.1 ml of the secondary

enriched sample was used to inoculate 10 ml of BHI broth (Merck, 1.10493.0500, Germany) and incubated for 5 h at 37 ºC.

DNA extraction

A volume of 1 ml of the post-enriched sample was centrifuged at 13,000g for 10 min, the sediment was washed with 0.85% NaCl, resuspended in 200 µl of the buffer containing 20 µmol Tris–HCl pH 8.0 and 50 µmol KCl, and incubated at 95 ºC for 25 min. The lysed sample was centrifuged at 13,000g for 3 min and the supernatant was used for further analysis (25).

PCR Primers

Oligonucleotide primers for the PCR assay were selected based on the published nucleotide sequence of the inlB gene (26).

PCR

A reaction mixture of 25 µl contained 200 µM of each dNTP (MBI Fermentas), 250 nmol of each primer (inlB-L: ctggaaagtttgtatttgggaaa; inlB-R: tttcataatcgccatcatcact), 1.5 U Taq DNA polymerase (MBI Fermentas), 2.5 µl of the buffer supplied with the polymerase, and 5 µl of the sample lysate. The primers used have been previously shown to be 100% specific for *L. monocytogenes*. The reaction was performed in a Mastercycler personal thermal cycler (Eppendorf AG, Germany) using the amplification programme consisting of initial denaturation at 94 ºC for 2 min, 35 cycles with a denaturation at 94 ºC for 45 s, annealing at 60 ºC for 45 s and polymerization at 72 ºC for 90 s, followed by the final polymerization at 72 ºC for 8 min (25).

Determination of specificity of the PCR test

L. monocytogenes NCTC 11994 and all suspected DNA samples were tested to determine specificity of the primers used in the PCR procedure. In addition, a broad group of organisms, including *Listeria innocua* (clinical isolates) and *Escherichia coli* ATCC 25922 were used.

Determination of sensitivity of the PCR test

To determine the sensitivity of the PCR, a suspension of *L. monocytogenes* NCTC 11994 containing 4 x 10^4 CFU/ml was serially diluted two-fold to 4 CFU/ml. Twenty µl of each dilution was boiled for 10 min and added directly to the PCR mixture. The bacterial concentration was verified by plating 20 µl of each dilution onto PALCAM *Listeria* selective agar base (Difco, BD, 263620, USA) with the included selective supplement (Difco, BD, 263710, USA) (25).

Detection of the amplification product

Following PCR, a 10 µl portion of the sample was analysed by electrophoresis in agarose gel (1.8%), staining by ethidium bromide, and visualization in UV-light. A DNA molecular weight standard n.100 bp (MBI Fermentas) was analysed along with the samples (25).

Interpretation of PCR results

Presence of a DNA fragment of approx. 343 bp (*L. monocytogenes* NCTC 11994) was used to examine all suspected DNA samples (26). In addition, a broad group of organisms, including *Listeria innocua* (clinical isolates) and *Escherichia coli* ATCC 25922 were used as a control.

Results

In the PCR, 30 bags of *H. pomatia* were analyzed and 18 bags were positive. Of the 18 PCR positive samples, 2 were raw and 16 were cooked products. Six of the PCR positive samples (including 2 raw samples) produced a strong PCR amplification product and 12 samples produced weak amplification products. Amplification revealed a band at approximately 343 bp, which was in agreement with the expected size. Control experiments performed with *L. monocytogenes* NCTC 11994 yielded the same PCR product, but none of the template PCR aliquots yielded any PCR product; however 12 samples showed a negative reaction in the PCR.

Some PCR amplification-products are shown in Figure 1.

Specificity of primers

The PCR test was specific for *L. monocytogenes* strains. The amplification of *L. monocytogenes* yielded the expected 343 bp amplicon. However, none of the other genera (*Listeria innocua, Escherichia coli)* isolates yielded a PCR product when tested with the inlB-L and inlB-R primers (Figure 2).

Sensitivity of the PCR test

The PCR assay had a detection limit of 40-45 cells per ml of PCR mixture (Figure 2), assuming that the lysate procedure was completed, since no viable cells were detected after the boiling treatment. This level equals 4 x 10^1 CFU/ml.

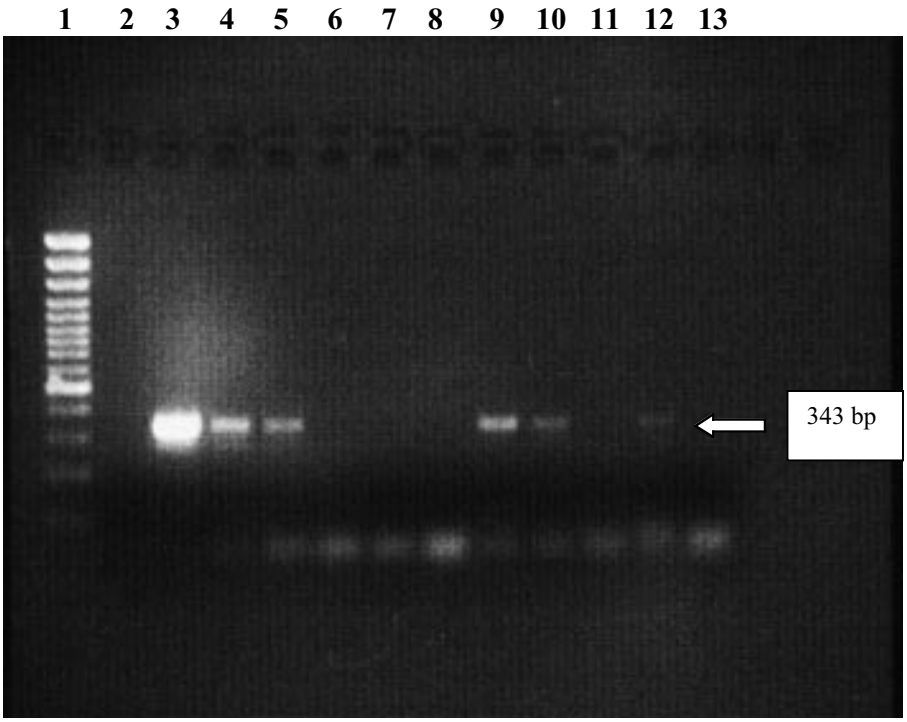

Figure 1. PCR assay for *Listeria monocytogenes* (specific amplification of the 343 bp fragment from the total DNA of *Listeria monocytogenes*). Lane 1: 1-kb plus DNA ladder (MBI Fermentas); Lane 2: no template (negative control); Lane 3: *L. monocytogenes* NCTC 11994 (serotype 4b) (positive control); Lanes 4 to 6 and 9 to 13: DNA from *H. pomatia*; Lane 7: *Listeria innocua*; Lane 8: *Escherichia coli* ATCC 25922.

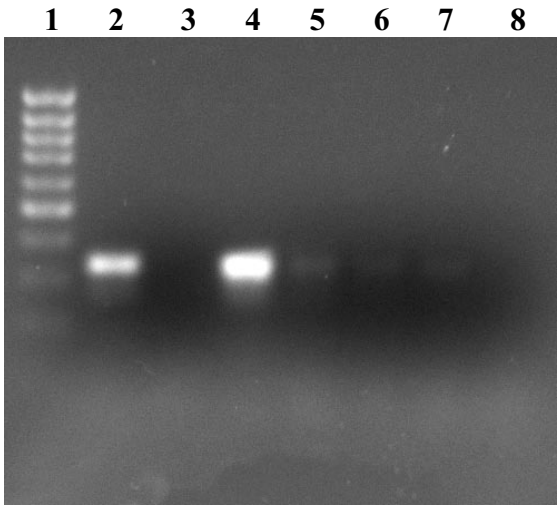

Figure 2. Sensitivity detection of *Listeria monocytogenes* by PCR. Lane 1: 1-kb plus DNA ladder (MBI Fermentas); Lane 2: *L. monocytogenes* NCTC 11994 (positive control); Lane 3: no template (negative control); Lane 4 to 8: 4 x 10^4, 4 x 10^3, 4 x 10^2, 40, and 4 cells *L. monocytogenes* NCTC 11994, respectively.

Discussion

The detection and identification of *L. monocytogenes* have attracted the attention of many researchers. *L. monocytogenes* is one of the most important food-borne pathogens (3,4,11). It is often found in various uncooked foods, such as meat, cheese, and vegetables, and ready-to-eat products. It is widely distributed in the environment and as such, can cause the contamination of food during production and distribution.

L. monocytogenes is commonly found in soil; therefore, live snail samples are often contaminated with this pathogen (3). In this study, our aim was to report the presence of *L. monocytogenes* in live snails by using PCR as a rapid and reliable method, and consequently, 2 raw samples showed strong positive reactions for *L. monocytogenes*. This result showed that *L. monocytogenes* were found to be in the rural environment.

It is known that heat applications at 70 °C for 0.3-2 min are sufficient to destroy *L. monocytogenes* (27); however, recontamination can occur from different sources (28). In this study, snail meat samples were boiled at 100 °C for 4 min (with an internal temperature of 85 °C); yet, cooked samples (16 out of 28) still harboured *L. monocytogenes* after boiling. This indicated that boiling could not entirely eliminate *L. monocytogenes* from the snail meat.

It is essential that the presence or absence of *L. monocytogenes* be rapidly and accurately determined in many food products. The advances in biotechnology over the past decades have resulted in the development of many methods for the detection of pathogenic micro-organisms in food, such as *L. monocytogenes*. Much effort has been expended to facilitate this process and there are now a number of diagnostic methods available, ranging from combinations of selective and differential growth media, coupled with biochemical confirmation, to nucleic acid-based methods such as PCR (29).

Due to the specificity of the primers developed, the protocol was then applied to the direct detection of *Listeria* spp. in food samples. After an overnight enrichment step to increase the number of target cells, and to avoid the amplification of dead cells, DNA was extracted from the enriched broth and subjected to PCR (25).

Pangallo et al. (26) reported that the use of specific primers for *L. monocytogenes* and the inlB gene was 100% specific for *L. monocytogenes*.

The sample preparation should produce a sufficient amount of amplifiable DNA originating in live *L. monocytogenes* cells. For this reason, enrichment by culture seems a good choice.

PCR-based methods are believed to have a great potential to fulfil the requirements for fast, specific, and sensitive detection of *L. monocytogenes* in ready-to-eat products (21). However, this potential may come to practical use only if appropriate sample preparation is used prior to PCR. In the present study, the homogenates were prepared with industrially produced, refrigerated ready-to-eat *H. pomatia* samples and DNA extraction was made on these homogenisates.

In this study, a total of 30 bags of *H. pomatia*, raw and cooked products, were analysed, and 18 samples were positive. Six PCR positive samples produced a strong PCR amplification product and 12 samples produced a weak amplification product in the PCR.

The specificity of our assay was verified by performing the PCR with *L. innocua* and *E. coli*. None of these related bacteria gave the corresponding amplification product. Therefore, the primers described here proved to be specific for *L. monocytogenes* under the conditions assayed.

Our PCR assay could detect as few as 4×10^1 CFU/ml. of *L. monocytogenes* and could detect 40-45 cells/ml of *L. monocytogenes*. These results indicated that this procedure was a highly sensitive and specific method for detecting *L. monocytogenes* in food.

The method can be used for rapid identification of traditionally isolated strains, or it can be applied directly in food samples to detect *L. monocytogenes*, avoiding time-consuming classical isolation and identification. Even if a pre-enrichment step is necessary, in 18 h it is possible to determine the presence of any *Listeria* spp. in the sample (30).

The presented PCR-based method utilizing a selective enrichment plus a short post-enrichment protocol proved to produce definitive results (not requiring further confirmation) on the third day after the sample collection, while being equivalent to the reference methods EN ISO 11290-1 and ISO 10560, respectively (19, 20). The described method was faster than its ISO counterparts by at least 5 d in cases with a negative result and by at least 7 d in cases with a positive one. Because of the multiple dilution of the sample during the enrichment, dead cells were not detected by the method. The method fits well with usual laboratory working hours, making use of the speed of currently available thermal cyclers. The method presented may be used as a faster alternative for the detection of *L. monocytogenes* in food. Because of a similarity of the enrichment protocols utilised, the described PCR-based method may be effectively used along with EN ISO 11290-1 or ISO 10560 during its laboratory introduction and validation (19,20,25).

As a result, using PCR for the detection of *L. monocytogenes* in raw and cooked ready to eat snails (*H. pomatia)* might be a fast, reliable, and sensitive method for detecting the presence of *L. monocytogenes* without reacting to dead *L. monocytogenes* cells.

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