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Research Article

Comparison of Polymerase Chain Reaction and Conventional Methods for the Diagnosis of *Listeria monocytogenes* in Stuffed Mussels

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Abstract: The aim of this study was to evaluate the role of stuffed mussel as a source of *Listeria monocytogenes*. Polymerase chain reaction is a rapid procedure with both sensitivity and specificity for quick detection and identification of *L. monocytogenes*. A total of 50 mussel samples were investigated for *L. monocytogenes*. *L. monocytogenes* was not identified by the conventional method. However, PCR amplification products demonstrated that 5 out of 50 samples showed positive reactions with *L. monocytogenes*. The PCR positive samples showed specific amplification at approximately 343 bp for *L. monocytogenes*.

Key Words: *Listeria monocytogenes*, stuffed mussel, conventional methods, PCR

Midye Dolmalarda *Listeria monocytogenes* Tanısı için Polimeraz Zincir Reaksiyonu ve Konvensiyonel Metotlarının Karşılaştırılması

Özet: Bu araştırmanın amacı, *Listeria monocytogenes* kaynağı olarak midye dolmaların rolünün varlığını ortaya çıkarmaktır. Polimeraz Zincir Reaksiyonu (PZR), *L. monocytogenes*'in identifikasyonu ve hızlı tanısında spesifite ve sensitivitesi yüksek bir metottur. Çalışmada, toplam 50 adet midye dolma örneği *L. monocytogenes* yönünden incelendi. Konvensiyonel metotlar ile *L. monocytogenes* identifiye edilemedi. Ancak, PZR'nda, *L. monocytogenes* yönünden 50 örneğin 5'inde pozitif amplifikasyon ürünleri görüldü. PZR pozitif ürünlerde, *L. monocytogenes* için spesifik, yaklaşık olarak 343 bp aralığındaki bant görüntüsü elde edildi.

Anahtar Sözcükler: *Listeria monocytogenes*, midye dolma, konvensiyonel metotlar, PZR

Introduction

Listeria monocytogenes is a foodborne pathogen capable of causing sporadic and epidemic out-breaks of listeriosis (1). In addition to the very young and elderly, immuno-suppressed individuals such as those with cancer, AIDS and diabetes are also at high risk (2). Disease symptoms vary from flu-like symptoms such as fever, fatigue, vomiting, malaise, nausea and diarrhoea, to more serious illness resulting in meningitis or encephalitis (1). Listeria infections can result in stillbirth, meningitis, meningoencephalitis, septicaemia, and death (3).

Listeria organisms are widely disseminated in the rural environment and, consequently, contaminate the raw materials used in the preparation of industrially processed foods and the production plants as well (4). They have been isolated from soil, decaying vegetable matter, silage, sewage, water, animal feed, fresh and processed meats, raw milk, cheese, slaughterhouse waste, seafood and asymptomatic human and animal carriers.

L. monocytogenes has been isolated from both domestic and imported, fresh, frozen, and processed seafood products, including crustaceans, molluscan shellfish, and finfish (5). Contaminated seafoods, such as

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smoked cod roe, mussels, and undercooked fish, have been assumed to be the sources for sporadic cases of listeriosis (6-8). *L. monocytogenes* is sporadically isolated from a range of seafoods, including ready-to-eat products like cold and hot smoked salmon, gravad salmon, shrimp, fermented fish, and fish salads (9). Colburn et al. (10) reported that *Listeria innocua* was identified in oyster samples. Because of their widespread occurrence, Listeria species have many opportunities to enter food production and processing environments (11).

For many years, clinical Listeria isolates were a mere laboratory rarity, and the epidemiology of the disease was an unresolved mystery (12,13). 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 outbreaks in humans in North America and Europe clearly established listeriosis as an important food-borne infection (14,15).

Since all Listeria species are potential food contaminants, the presence on foodstuffs of any of these species can be considered an indicator of their contamination and of the potential presence of *L. monocytogenes*. However, because of the threats to public health posed by contamination of foods by *L. monocytogenes,* it is very important that it be identified be rapidly and reliably detected (16).

The polymerase chain reaction (PCR) has been shown to have a great potential to speed-up the detection of *L. monocytogenes* in food (17). It is a powerful tool for designing nucleic acid-based assays that are highly specific and sensitive, as well as quantitative (18,19). Initially, the detection of PCR products (and, by inference, estimated target numbers) was accomplished by gel electrophoresis, typically by using ethidium bromide to visualise the amplification products.

Stuffed mussel, made from mussel and rice, cooked separately then put together in the shell, is commonly consumed, and people eat it generally without any hesitation. However, this special food might be an important source of *L. monocytogenes* as this agent can easily be found in the environment, such as soil, contaminated water and the people who prepare them and the premises where they are cooked and sold. They are generally sold in the streets on tables with no cooling feature, while they are still in their shells.

The aim of this study was to compare the PCR and conventional methods for isolating *L. monocytogenes* in stuffed mussels.

Materials and methods

Processing of mussels food samples

A total of 50 stuffed mussel samples were obtained from street sellers.

Reference strains

L. monocytogenes NCTC 11994 (serotype 4b) was used as the positive control strain.

Enrichment

Twenty five grams of mussel samples were homogenized in 225 ml of UVM enrichment broth (Difco) using a Stomacher 400 homogeniser (Seward, Basingstoke, England) and incubated for 24 h at 30 °C. A volume of 0.1 ml of the UVM culture was used to inoculate 10 ml of Fraser broth (Difco), followed by incubation for 24-40 h at 35 °C. After incubation, a sterile cotton swab was dipped into the Fraser's broth and stroked on a Palcam agar plate. Palcam agars were incubated at 35 °C and examined for typical round Listeria colonies surrounded by a black zone. Listeria isolates were identified according to the USDA FSIS procedures (11). For the PCR, a volume of 0.1 ml of the Fraser culture was used to inoculate 10 ml of BHI broth, followed by incubation for 5 h at 37 °C (20).

DNA extraction

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

PCR Primers

Oligonucleotide primers for the PCR assay were selected based on the published nucleotide sequence of the inlB gene. The product of the inlB gene is essential for the *L. monocytogenes* to enter the hepatocytes, which is a specific virulence factor for this bacterium (21).

PCR

A reaction mixture of 25 µl contained 200 µM of each dNTP (MBI Fermentas), 250 nM 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* (21). The reaction was performed in a Mastercycler personal (Eppendorf AG) thermal cycler using the amplification programme consisting of the initial denaturation at 94 °C for 2 min, 35 cycles with denaturation at 94 °C for 45 s, annealing at 60 °C for 45 s and polymerisation at 72 °C for 90 s, followed by the final polymerization at 72 °C for 8 min.

Detection of the amplification product

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

Interpretation of PCR results

The presence of a DNA fragment of 343 bp with or without the presence of a DNA fragment of approx. *L. monocytogenes* NCTC 11994 and all suspected DNA samples were tested to determine the 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 as a control.

Determination of the 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 2-fold to 4 CFU/ml. Twenty microliters 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 with including selective supplement.

Results

A total of 50 mussel samples were microbiologically examined for *L. monocytogenes* by the conventional method. This method included 2 steps of enrichment, UVM and Fraser broth. Following the enrichment procedures, samples were plated out on Palcam Agar Base. No Listeria spp. or *L. monocytogenes* colonies were observed on the plates, but *Staphylococcus aureus* colonies were found in 2 samples examined.

In the PCR, a total of 50 mussel samples were analysed and 5 were detected as positive. PCR amplification revealed a band at approximately 343 bp, in agreement with the expected size. Control experiments performed with *L. monocytogenes* NCTC 11994 (serotype 4b) yielded the same PCR product, but no template PCR aliquot yielded any PCR product. However, 45 samples showed a negative reaction in the PCR.

Some PCR amplification products are demonstrated in Figure 1.

Specificity of primers

The PCR test was specific for *L. monocytogenes* strains. The amplification of *L. monocytogenes* yielded the expected amplicon at approximately 343 bp. 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 1).

Sensitivity of the PCR test

The PCR assay had a detection limit of 40-45 cells per millilitre 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.

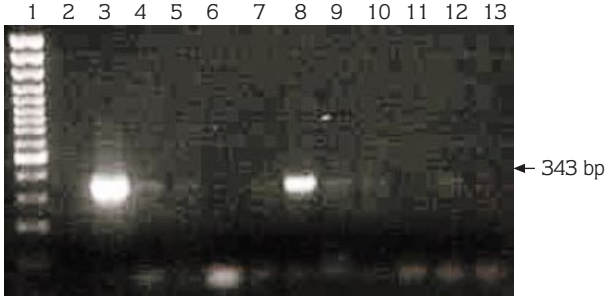

Figure 1. PCR assay for *Listeria monocytogenes* (specific amplification at approximately 343 bp fragment from the total DNA of *Listeria monocytogenes*). Lane 1, 1-kb plus DNA ladder (MBI Fermentas); lane 2, no template (negative control); lane 3, *L. monocytogenes* NCTC 11994 (serotype 4b) (positive control); lanes 4 to 11, DNA from mussels.; lane 12, *Listeria innocua*; lane 13, *Escherichia coli* ATCC 25922.

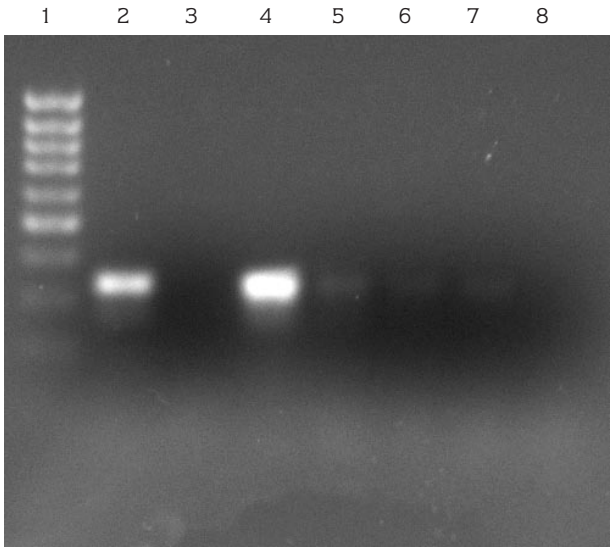

Figure 2. Sensitivity detection of *Listeria monocytogenes* by PCR. Lane 1, 1-kb plus DNA ladder (MBI Fermentas); lane 2, *L. monocytogenes* NCTC 11994 (positive control); lane 3, no template (negative control); Lane 4 to 8, 4 x 10^4, 4 x 10^3, 4 x 10^2, 40 and 4 cells *L. monocytogenes* NCTC 11994,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

Discussion

Listeriosis is an emergent illness with a low incidence, but with a high fatality rate, especially in immunocompromised individuals (22). Different kinds of foods have been reported to be associated with the transmission of *L. monocytogenes* (23), including vegetables, dairy products (pasteurised milk and cheese) and meat products.

L. monocytogenes has been isolated consistently worldwide from a great variety of raw meats, poultry, seafood, dairy products and vegetables (9,24).

The detection and identification of *L.* monocytogenes have attracted the attention of many authors (1,25). It is widely diffused in the environment and this fact can cause the contamination of food during production and distribution. Although the minimal infective dose for humans is unknown, different studies have implied that foods implicated in cases of listeriosis have contained elevated levels of the pathogen (26).

It is essential that the presence or absence of *L. monocytogenes* must be rapidly and accurately determined in many food products. The standard conventional methods for the detection of *L. monocytogenes* in food suffered of high time requirement of 7–10 days (27,28). The advances in biotechnology over the past decades have resulted in the development of many methods for the detection of pathogenic micro-organisms such as *L. monocytogenes* in food (29).

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 (30).

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 (20).

Pangallo et al. (21) reported that DNA extracted directly from food samples was also amplified by the use of specific primers for *L. monocytogenes*, and the inlB gene was 100% specific for *L. monocytogenes*. Colburn et al. (10) identified *Listeria innocua* in oyster samples. They also found *L. monocytogenes* from oysters by using oligonucleotide probe for the haemolysin gene. In our study, the presence of the inlB gene, which is 100% specific for *L. monocytogenes*, was demonstrated in mussel samples.

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.

In this study, a total of 50 stuffed mussel samples were analysed by both PCR and conventional methods. Although no *L. monocytogenes* was isolated by the conventional method, 5 samples were detected as positive in PCR. PCR positive samples produced an amplification product and 45 samples produced no amplification product at all. Although we conducted this experimental work with a limited number of mussel samples, the breaking point was to find 5 positive samples in ready-to-eat stuffed mussels. Because these types of foods are intended to be eaten without reheating, this could cause foodborne disease. We collected mussel samples from

street sellers, who prepare and sell products in public areas. Therefore, many people consume these products as snacks even during their breaks. Thus it is important to analyse food samples as fast as possible, if they are that dangerous.

PCR-based methods are thought to have a great potential to fulfill the requirements for fast, specific and sensitive detection of *L. monocytogenes* in ready-to-eat products (17). However, this potential may be useful only if appropriate sample preparation is done prior to PCR.

In conclusion, ready-to-eat foods, especially those sold in the streets and open areas without any precaution, such as mussels, could be a major cause of food poisoning and foodborne diseases. Therefore, PCR for the rapid detection of *L. monocytogenes* in this sort of foodstuff might be a reliable and sensitive method.

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