Kafkas Univ Vet Fak Derg
22 (3): 397-401, 2016
DOI: 10.9775/kvfd.2015.14808

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Investigation of *Salmonella* spp. and *Listeria monocytogenes* in Seafood by Cultural Methods and PCR [1]

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[1] *This work was supported by the Research Fund of the University of Istanbul. Project number: 48022*

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Article Code: KVFD-2015-14808 Received: 04.12.2015 Accepted: 31.01.2016 Published Online: 11.02.2016

Abstract

The present study was conducted to investigate the presence of *Salmonella* spp. and *Listeria monocytogenes* in 700 seafood (400 raw fish, 100 raw shrimps and 200 raw molluscs) collected from retailers. Isolations were performed by conventional culture methods. The isolates were also confirmed by PCR assays. *Salmonella* spp. and *L. monocytogenes* were detected in 9.9% and 3.86% fish and shellfish samples, respectively. The highest rates of *Salmonella* spp. (12.5%) were found in fish samples and *L. monocytogenes* (7.0%) were in shrimp samples. Therefore, it is essential to ensure improving the quality of production technology from fishing to retail outlet and developing the sanitation conditions of food contact surfaces and handling areas.

Keywords: *Seafood, Fish, Shrimp, Mollusc, Pathogen*

Deniz Ürünlerinde *Salmonella* spp. ve *Listeria monocytogenes* Varlığının Kültürel Metotlar ve PCR İle Araştırılması

Özet

Bu çalışma perakendecilerden toplanan 700 deniz ürününde (400 çiğ balık, 100 çiğ karides ve 200 çiğ molüsk (yumuşakça)) *Salmonella* spp. and *Listeria monocytogenes* varlığının araştırılması amacıyla yapıldı. İzolasyonda konvansiyonel kültür metotları kullanıldı ve tüm izolatların PCR ile konfirmasyonları gerçekleştirildi. Balık ve kabuklu deniz hayvanlarından *Salmonella* spp. %9.9 ve *L. monocytogenes* %3.86 oranında belirlendi. *Salmonella* spp. en yüksek oranda balık örneklerinde (%12.5) saptanırken *L. monocytogenes* ise karides örneklerinde (%7.0) saptandı. Bu çalışmanın sonuçları, avlanması/yetiştirilmesinden perakende satış aşamasına kadar üretim teknolojisinin kalitesinin iyileştirilmesinin ve gıda temas yüzeyleri ile ambalajlama alanlarında sanitasyon koşullarının geliştirilmesinin gerekliliğinin önemini göstermektedir.

Anahtar sözcükler: *Deniz ürünleri, Balık, Karides, Molüsk, Patojen*

INTRODUCTION

Seafood refers not only fish but also of shellfish which include crustacea (shrimp) and molluscs (mussel and calamari) [1]. Seafood is a rich source for a great number of nutritive and important components that have high amount of the vitamins such as A, D, E and B_{12}, the well balanced content of essential amino acids, the presence of antioxidants such as tocopherols, the exceptional concentrations of essential elements such as selenium and iodine, and the good digestibility of protein due to low amounts of connective tissue [2].

Seafood are highly perishable products. They may harbour pathogens which cause serious food safety problems for consumers. Some pathogens including *Salmonella* spp. and *L. monocytogenes* have been implicated in seafood-borne diseases. These pathogens are naturally

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present in sea water or can contaminate seafood during processing. The handling problems and poor hygiene conditions are the main reasons [1].

There have been some reports on the presence and prevalence of *Salmonella* spp. in Morocco [3], in Spain [4], in Vietnam [5], in India [6]; *L. monocytogenes* in Spain [7], in USA [8], in India [9], in Denmark [10], in Iran [11]. In Turkey the prevalence of these pathogens has not been extensively investigated.

Turkey has a favourable potential in terms of production and consumption of marine products due to surrounded by sea on three sides by the Black Sea in the north, the Mediterranean Sea in the south and the Aegean Sea in the west. In the north-west is also an important sea, the Sea of Marmara. The total shoreline is 8300 km long and half of Turkey's population lives in coastal cities. Considering the consumption, Turkey (8.2 kg per capita per year) ranks in the 7th place among European countries [12].

The present study was carried out to determine the presence of *Salmonella* spp. and *L. monocytogenes* in seafood obtained from retail markets in Istanbul which has approximately an area of 5.343 km^2 and a population of 14.377.018 million people (18.66% of the country). Furthermore Istanbul gets seafood from all the surrounding seas of Turkey, and has a geographical importance due to its location and that represents a transit corridor between Europe and Asia.

MATERIAL and METHODS

Sample Collection

A total of 700 seafood (400 raw fish, 100 raw shrimps and 200 raw molluscs) were collected from retailers in Istanbul. All samples were kept in sterile jars and immediately transferred to the laboratory in cold boxes.

Bacteriological Analysis

Conventional culture-based study of samples was performed as recommended by FDA Bacteriological Analytical Manual for the isolation of *Salmonella* spp. Pre-enrichment was done by suspending 25 g of sample in 225 ml lactose broth (LB - Oxoid, USA) followed by incubation at 37°C for 24 h. One ml mixture was transferred to Rappaport-Vassiliadis (RV - Oxoid, USA) and Muller-Kauffmann Tetrathionate Broth Base (MKTTn - Oxoid, USA). MKTTn and RV broth was incubated for 24 h at 42°C. After incubation samples were streaked on Bismuth Sulfite agar (BS - Oxoid, USA), Brilliant Green agar (BG - Oxoid, USA) and Xylose-Lysine Deoxycholate Agar (XLD - Oxoid, USA), incubated for 24 h at 37°C. The typical colonies were identified by biochemicals tests and confirmed with Salmonella antiserum (O and H-Vi polyvalent antisera) [13].

The isolation of *L. monocytogenes* were performed according to International Standardization Organization (ISO) procedures. 25 gr/ml sample was inoculated into 225 ml Buffered Listeria Enrichment Broth Base (BLEB, Oxoid, USA) and the samples were incubated at 30°C for 4 h. At the end of the 4th h 25 mg/L natamycine was added to each sample and the incubation period was continued up to 48 h at 30°C. At the 24th h of the incubation samples were inoculated onto Oxford Agar and Palcam Agar Plates (Oxoid, USA) and were again inoculated for 48 h at 35°C. After 48 h, all the samples (both from Oxford and Palcam Agars and from BLEB) were inoculated onto Chromogenic Listeria Agar (Oxoid, USA). Then, suspected colonies were passaged onto Triptic Soy Agar with Yeast Extract (TSA, Oxoid, USA) for purification. Suspected isolates which matched to all identification parameters according to reference method (Gram staining, catalase activity, motility test, fermentation of maltose, rhamnose, mannitol, and xylose, hydrolization of esculin, reduction of nitrate) were evaluated as positive. CAMP test with *Staphylococcus aureus* and Henry illumination tests were also applied to all suspected samples [14,15].

PCR

All the culture positive samples were confirmed by PCR assays. The DNAs of all the isolates was extracted by Roche High Pure PCR Template Preparation Kit (Roche, France), according to the manufacturer›s instructions. The extracts were kept at –20°C to be used as target DNA for PCR assays.

Salmonella-specific *invA* primers (5'-GTGAAATTATCGCC ACGTTCGGGCAA-3' and 5'-TCATCGCACCGTCAAAGGAACC-3') were used for the detection of Salmonella in this study [6,16]. The PCR program consisted of an initial denaturation step at 95°C for 2 min, followed by 35 cycles of DNA denaturation at 95°C for 30 s, primer annealing at 64°C for 30 s, and primer extension at 72°C for 30 s. After the last cycle, a final extension step at 72°C for 5 min was added. PCR product were analysed by gel electrophoresis with 2% agarose (Sigma-Aldrich, USA) and visualised. Observed bands at 284 bp were evaluated as positive.

L. monocytogenes specific *actA* gene was reproduced by using specific designed primers (5'-GCTGATTTAAGAGA TAGAGGAACA-3'and 5'-TTTATGTGGTTATTTGCTGTC -3') [17]. The PCR program consisted of an initial denaturation step at 94°C for 2 min, followed by 35 cycles of DNA denaturation at 94°C for 60 s, primer annealing at 50°C for 60s, and primer extension at 72°C for 60 s. PCR products were analysed by gel electrophoresis with 1.5% agarose (Sigma-Aldrich, USA) and visualised. Observed bands at 827 bp were evaluated as positive.

Each of PCR test, positive controls *(Salmonella enterica* serovar Typhimurium, ATCC 23564, field isolate of *L. monocytogenes)*, and negative control (sterile dH_2O) were used separately along with seafood samples.

RESULTS

Salmonella spp. and *L. monocytogenes* were detected in 9.9% and 3.86% fish and shellfish samples, respectively *(Table 1)*. All the *Salmonella* spp. and *L. monocytogenes* were confirmed by PCR assay *(Fig. 1)*.

Table 1. *Prevalence of Salmonella spp. and L. monocytogenes in various seafood*

Tablo 1. *Çeşitli deniz ürünlerinde Salmonella spp. ve L. monocytogenes prevalansı*

Products	Number of Samples	Number of *Salmonella* spp. Positive Samples	Number of *L. monocytogenes* Positive Samples
Fish	400	50 (12.5%)	10 (2.5%)
Shrimp	100	2 (2.0%)	7 (7.0%)
Mollusc	200	17 (8.5%)	10 (5.0%)
Total	700	69 (9.9%)	27 (3.86%)

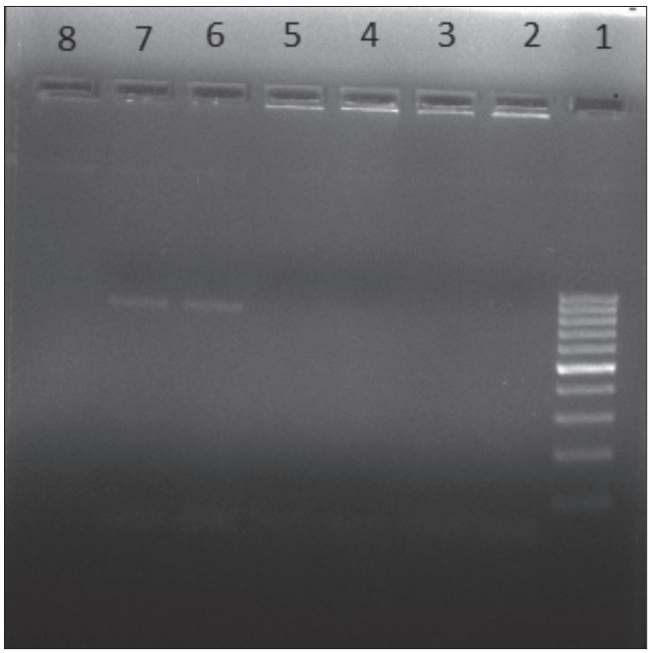

Fig 1. PCR result of a *L. monocytogenes* positive sample

Line 1 DNA marker (100 bp); Line 2-5 Negative samples; Line 6 Positive sample (827 bp); Line 7 Positive control; Line 8 Negative control

Şekil 1. *L. monocytogenes* pozitif örneğe ait PCR sonucu

Hat 1 DNA merdiveni (100 bp); Hat 2-5 Negatif örnekler; Hat 6 Pozitif örnek (827 bp); Hat 7 Pozitif kontrol; Hat 8 Negatif kontrol

DISCUSSION

According to Turkish Food Codex [18], the presence of *Salmonella* spp. and *L. monocytogenes* in 25 g of fish and shellfish is not acceptable.

The present study demonstrated that *Salmonella* spp. was isolated from 12.5% of raw fish samples. Regarding the contamination rate, our results were slightly similar to the study obtained by Kusumaningrum et al.[19] and Hatha & Lakshmanaperumalsamy [20]. In another study, no *Salmonella* spp. was isolated [21]. Contrary to this, the studies which had higher results (90.0%, 43.8% and 30.5%) than ours were reported by Jegadeeshkumar et al.[22], Budiati et al.[23] and Kumar et al.[6] respectively. The reason for high contamination rate should be due to the use of contaminated raw materials, detection methods and the geographical conditions.

The prevalence of *Salmonella* spp. in shrimp samples tested in this study was lower in comparison to those detected by Kumar et al.[6] (29.0%), Kumar et al.[24] (26.7%) and Hatha & Lakshmanaperumalsamy [20] (%15.2). On the other hand, Koonse et al.[25] reported the prevalence rate of *Salmonella* spp. was 1.6% in shrimp samples. Our findings (2.0%) showed similarity with the mentioned results. These differences may be originated from sampling procedures and the sanitation applications. Ahmed [26] stated that the presence of *Salmonella* spp. is an indicator of aldulteration in shrimp industry which is one of the most important commodities seen in global fishery trade.

In this study, *Salmonella* spp. was detected in 8.5% of molluscs samples. This result was in acceptance to the findings reported by Setti et al.[3] and Simental & Martinez-Urtaza [27]. According to the classification of the seafood, the highest risk category includes raw fish and molluscs especially mussels [28]. In Spain, Martinez-Urtaza et al.[4] demonstrated that 3.0% of samples were positive for *Salmonella* spp. The prevalence of *Salmonella* spp. was related with different hygiene applications and poor manufacturing processes.

In our study, *L. monocytogenes* was detected in 2.0% fish samples. Higher results were found by Jeyasekaran et al.[22], Farber [29] and Ellner et al.[30] at rates of 17.2%, 13.3% and 50.0% in fish samples, respectively. Similar results were detected by Gesche & Ferrer [31], Dhanashree et al.[32] and Davies et al.[33]. In contrast, Fuchs & Surendran [34] and Cenet [35] could not detected. Parihar et al.[36] reported that *L. monocytogenes* is not usually found on fish captured from open waters and contamination may take place long before the fish raw material reaches retail trade or processing factories.

According to the results from this study, the prevalence of *L. monocytogenes* was determined as 7.0% in shrimp samples. Likewise, Hofer and Ribeiro [37], Berry et al.[38] and Jeyasekaran et al.[39] demonstrated that *L. monocytogenes* was isolated from 8.8%, 6.7% and 10.7% of shrimp, respectively. On the contrary, lower results were reported by some authors [21,40]. Differences between the findings obtained from several studies can be related to the contaminations after process, preservation conditions and inadequately personal hygiene.

In the present study, of the analysed 200 molluscs

samples, 5% were positive for *L. monocytogenes*. In Spain 7.5% of mussels [41] and in Argentina 4.5% of mussels [42] were investigated *L. monocytogenes* contamination. In another study in Brazil, no *L. monocytogenes* was isolated [43].

In conclusion, the result of this study confirmed that fish and fish products may be contaminated with pathogens which can cause serious public health problems. In Turkey, seafood consumption has been increasing [44]. Therefore, it is essential to ensure improving the quality of production technology from fishing to retail outlet and developing the sanitation conditions of food contact surfaces and handling areas. Also, food safety training should be provided for all staff to increase the level of awareness and the sense of responsibility regarding food hygiene.

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