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Prevalence of *Listeria monocytogenes* in the crayfish (*Astacus leptodactylus*) by polymerase chain reaction in Iran

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Key words: Crayfish, (*Astacus leptodactylus*), *Listeria*, occurrence, polymerase chain reaction, Iran.

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Abstract

The Crayfish are the largest freshwater crustacean via high skill opposite wide range of environment variables and The freshwater Crayfish *Astacus leptodactylus* (*A. leptodactylus*) is the one of the important species of Crayfish family that rearing it significant in several nations . Iran has a significant role in export of *A. leptodactylus* to European nations. A number of species have been used aquaria and some use seafood consumer. Listeriosis have become an important topic in biomedical research because of its central role in food microbiology and medical microbiology. In Iran, some reports is available on prevalence of *Listeria* spp. in *A. leptodactylus*. The aim of this study was to find the prevalence of *Listeria monocytogenes* in *A. leptodactylus* meat samples in Iran. From November 2012 to February 2013, a total of 40 meat samples of *A. leptodactylus* samples were obtained from randomly selected localities in "Aras Dam" , Western-Azerbaijan Province , Iran. The samples were tested for the presence of *L. monocytogenes* using Polymerase Chain Reaction (PCR). Three samples (7.5%) were positive for *L. monocytogenes* by PCR method. The results show that crayfish from the studied area regularly contain this pathogen that is important to public health. Consumption of these sea foods, either raw or undercooked, may give to food borne illness in Iran.

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Introduction

The Crayfish are the largest freshwater crustacean via high skill opposite wide range of environment variables and The freshwater Crayfish *Astacus leptodactylus* (*A. leptodactylus*) is the one of the important species of Crayfish family that rearing it significant in several nations (McMahon, 1986; Holdich *et al.,* 1997).Iran has a significant role in export of *A. leptodactylus* to European nations. At the moment, England, Germany, Sweden and France are the main importers of crayfish from Iran (Matinfar, 2007). A number of species have been used for aquaculture aim and more in recent times, there has been an increase in the sale of crayfish for aquaria and some use seafood consumer in the world (Alderman and Polglase, 1988).

Listeriosis is a significant bacterial infection make happen via a gram positive facultative anaerobe, rod-shaped, non-spore-forming and intracellular bacteria (Gawade *et al.,* 2010). The bacteria *Listeria* spp. have become an important topic in biomedical research because of their central role in food and medical microbiology. The genus *Listeria* is composed of six species: *L. monocytogens*, *L. innocua*, *L. grayi, L. ivanovii*, *L. seeligeri*, and *L. welshimeri*. *L. monocytogenes* is the most important human pathogen among *Listeria* spp , even though very rare cases of infection because of *L. seeligeri* and *L. ivanovii* have been described (Mclauchlin, 1997; Guillet *et al.,* 2010). The existence of any *Listeria* species in food is possibly an indicator of poor hygiene. However, since *L. monocytogenes* is the major human pathogen, there is widespread agreement that the goal should be to exclude this organism from the food chain wherever possible, and to keep up conditions that will inhibit its multiplication in foods in which this bacterium can grow (Wyller *et al.,* 1999; Rocurt *et al.,* 2000).

This organism is an acute and regularly fatal illness by clinical manifestations resembling sepsis or meningitis in immunocompromised patients and neonatal babies and flu-like illness or abortion during pregnancy in women, encephalitis, gastroenteritis, arthritis and conjunctivitis (Vázquez-Boland *et al.,* 2001; Delgado, 2008). In ruminants, listeriosis is characterized as encephalitis presentation typical 'circling' symptoms, conjunctivitis, stillbirth, third trimester abortion, etc. (Hoelzer *et al.,* 2012). The case-fatality rate from listeriosis is usually about 20–30% (Farber and Peterkin, 1991). Its public health importance lies in its presence everywhere in nature that shows clearly its wide host range, which includes 40 mammals, 20 birds, crustaceans, fishes and ticks (Gawade *et al.,* 2010). This pathogen is generally distributed in nature and is commonly transmitted to human through contaminated water and food (Kuhn *et al.,* 1988). Biofilm formation on food-contact surfaces via this pathogen is a sign of severe public health hazards (Zameer *et al.,* 2010). *L. monocytogenes* is one of very few pathogenic organisms which can grow at frozen temperatures. Consequently, the storage of food at low temperatures don't growth of this pathogen (Junttila *et al.,* 1988). Most important occurrences of listeriosis have been associated by the consumption of foods of animal origin (Iida *et al.,* 1998; Rocourt *et al.,* 2000), specially sea foods, for example shrimp, mussels and undercooked fish (Brett *et al.,* 1998; Wan Norhana *et al.,* 2010).

Since fish and fishery products are perhaps a vehicle for *L. monocytogenes*, it is significant to have data on the prevalence of this pathogen. *L. monocytogenes* has been isolated often from fish and fish products from different parts of the world (Hassan *et al.,* 2001; Mena *et al.,* 2004; Basti *et al.,* 2006; Parihar *et al.,* 2008; Wan Norhana *et al.,* 2010). Therefore, regular screening and constant surveillance of food products including seafood for the presence of this pathogen are required. PCR is a technique which possesses sensitivity, rapidity and specificity and could be employed to help rapid diagnosis of *L. monocytogenes* contamination (Rahimi *et al.,* 2012). Therefore, it is considered that DNA extraction and PCR could become a practical difference to the conventional techniques for detection of *L. monocytogenes* (Rahimi *et al.,* 2012). The current study deals by the isolation and confirmation of *L.*

monocytogenes from sea foods by rapid, reliable and simple PCR method.

In Iran, there are not many reports is available on prevalence of *Listeria* spp. among crayfish (*Astacus leptodactylus*), but is available on prevalence of *Listeria* spp. in several works on other foods, specially sea foods. The aim of the current study was to study prevalence of *L. monocytogenes* in *A. leptodactylus* using PCR, in Iran.

Materials and methods

Sample collection

A total of 40 meat samples of *A. leptodactylus* (Fig. 1.) were collected from November 2012 to February 2013 from the "Aras Dam" Lake in Qare-Ziaoddin region, Western-Azerbaijan Province, Iran. The sampling area is located between 231°20 and 231°25 N latitudes and between 225°25 and 225°50 E longitudes, around Aras town in Qare-Ziaoddin region in west-northern border of Iran. sample were taken from the different crayfish in "Aras Dam". Specimens were captured by baited traps. The samples were transferred to the Food Microbiology Laboratory at the Islamic Azad University of Shahrekord Branch in portable insulated cold-boxes with an internal temperature of +2 to +4 °C in a short time (till 24 hours until being examined).

Total body length was measured to the nearest 0.1 mm with a caliper, from the rostral apex to the posterior median edge of the telson and ranged between 53.4 and 148.1 mm. The carapace length ranged between 35.0 and 62.3 mm. The wet weight was measured to the nearest 0.1 g and ranged between 45.2 and 91.4 g.

Skins of *A. leptodactylus* were cleaned and disinfected using 70% ethanol and Povidone-iodine. Using a sterile scalpel blade the skin was removed from muscle (meat) and the muscle was packaged in sterile plastic sample bags. Alkaline peptone water (APW) was added to each bag and shaken fifty times to wash the muscle evenly.

DNA extraction and PCR conditions

The samples were tested for the presence of *L.monocytogenes* using the selective enrichment and isolation protocol recommended by the United States Department of Agriculture (McClain and Lee 1988). Added to 225 ml of alkaline peptone water (APW) to 25 g of homogenized crayfish flesh and incubated at 37 °C for 24 h and afterward, direct DNA extraction from homogenized pure culture. *L. monocytogenes* isolates identified by bacteriological methods were tested by PCR. The PCR procedures used in this study have been described previously (Zhou and Jiao, 2005). Briefly, 1 ml pure culture was centrifuged at 13,000 g for 5 min at room temperature. Genomic DNA was extracted from specimens using DNA extraction kit (Fermentas, St. Leon-Rot, Germany, K0512) according to the manufacturer's protocol. The total DNA was measured at 260 nm optical density according to the method described by Sambrook and Russell (2001). The extracted DNA of each muscle sample was kept frozen at -20°C until used.

Oligonucleotide primers for the PCR assay were selected based on the published nucleotide sequence of the actA gene (Cai *et al.,* 2002). The pair of primers 01 (5-′GCTGATTTAAGAGATAGAGGAACA-3′) and 02 (5′-TTTATGTGGTTATTTGCTGTC-3′) were used to amplify a 827-bp DNA fragment that corresponds to the region of the 3- end of the actA gene. A 25 µl aliquot of PCR buffer contained 22 µl PCR supermix (0.2 µl of each primer at 12.5 µM, 2.5 µl of 10 × PCR buffer, 1.0 µl of 25 mM MgCl2, 1.0 µl of 1 mm of dNTPs mix, 0.1 µl of 3 U/ml Taq DNA polymerase in 17 µl of ddH2O). A 3 µl aliquot of each supernatant was added to the PCR mix. Thermocycling conditions included an initial hold of 2 min at 94°C, then a denaturation step at 95°C for 10 s, annealing at 60°C for 30 s and a 30 s extension at 72°C for a total of 40 cycles. A final hold at 4°C followed a final extension at 72°C for 10 min. Amplification reactions were carried out in a DNA thermal cycler (Master Cycler Gradient, Eppendorf, Germany). PCR products were examined by electrophoresis in 1.5% agarose gel, stained with 1% solution of ethidium bromide, and viewed under UV illumination.

Results

The results of this study by PCR method are presented in Table 1. In PCR method out of total 40 samples (meat/muscle) tested, 3 samples (7.5%) were positive for *L. monocytogenes*. PCR specimens producing a band of the expected size (827 bp) were considered positive (Fig. 2).The results show that crayfish from the studied area regularly has *Listeria* relevant to public health.

Table 1. Prevalence of *L. monocytogenes* in *A. leptodactylus* in Iran. Results expressed as the number of *Listeria*-positive samples/ number of samples analyzed (%).

Type of food sample	No. of samples	*L. monocytogenes*, n (%)
Meat of Crayfish	40	3 (7.5%)

Discussion

In this study the occurrence of *L. monocytogenes* in crayfish was studied. The present study on crayfish in Iran, revealed the presence of potential human pathogens, as well as bacteria that may cause morbidity and mortality in crayfish or other marine animals.

Fig. 1. Freshwater Crayfish *Astacus leptodactylus* using in study.

There are not many reports of *Listeria* in Crayfish in Iran and other world, but is available on prevalence of *Listeria* spp. in several works on other foods, specially sea foods (Miettinen and Wirtanen, 2005; Soultos *et al.,* 2007 ; Modaresi *et al.,* 2011; Rahimi *et al.,* 2012).

In the study of Modaresi *et al.* (2011) Prevalence of *Listeria* spp, in fish obtained from Urmia fish markets and the highest prevalence of *Listeria* was observed in both *Abramis brama* and *Astacus leptodactylus* with 25%, while the lowest prevalence of *Listeria* was seen in *Sander lucioperca* (9.7%) (Modaresi *et al.,* 2011).

Numerous reports indicate that fish and fishery products can be frequently contaminated by *L. monocytogenes* as this organism has been isolated from fish and fishery products from different parts of the globe (Karunasagar and Karunasagar, 2000; Nakamura *et al.* 2004; Van Coillie *et al.,* 2004).

1 2 3 4

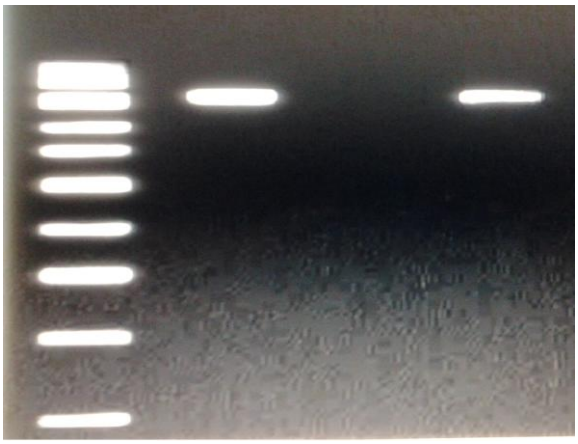

Fig. 2. Ethidium bromide-stained agarose gel electrophoresis of PCR products (827 bp) for detection of *L. monocytogenes* DNA in samples after PCR amplification.

Agarose gel electrophoresis for identification of *L. monocytogenes* DNA in *A. leptodactylus* meat samples. lane 1: 100 bp DNA ladder (Fermentas, Germany); Lanes 2: are positive control; lanes 3: negative samples; lanes 4: positive samples (827 bp).

Additional studies have found that the prevalence of *L. monocytogenes* in raw fish is quite low, ranging from 0–1% (Autio *et al.,* 1999) to 10% (Jemmi and Keusch, 1994). Though, Hartemink and Georgasson (1991) said that 56% of fresh fish on sale in Iceland was contaminated by *L. monocytogenes* and other *Listeria* species (Hartemink and Georgasson, 1991). In another place (food products commercialized in Portugal), Mena *et al.* (2004) stated that 12% of fresh fish samples were contaminated by *L. monocytogenes* (Mena *et al.,* 2004).

In a study in Brazil, Hofer and Ribeiro (1990) detected *Listeria* spp. in 8.8% of frozen shrimp (for export) samples (Hofer and Ribeiro, 1990). In study conducted in the United States, 27 of 74 frozen shrimp samples (20%) analyzed were positive for *Listeria* (Jinneman *et al.,* 1999). In another study in USA, 25% of fresh and frozen shrimp samples were positive for *Listeria* (Buchanan *et al.,* 1989). In France, Ravomanana *et al.* (1993) isolated *Listeria* in 23.5% of fresh shrimp samples (Ravomanana *et al.,* 1993). Manoj *et al.* (1991), Dhanashree *et al.* (2003),

Moharem *et al.* (2007) and Parihar *et al.* (2008) detected *Listeria* spp. in 10.5%, 9.1%, 73.3% and 30.0% of fresh and frozen shrimps in India, respectively (Manoj *et al.,* 1991; Dhanashree *et al.,* 2003; Moharem *et al.,* 2007; Parihar *et al.,* 2008). Higher contamination rates (10–53%) have also been reported (Jeyasekaran *et al.,* 1996; Cordano and Rocourt, 2001; Ellner *et al.,* 1991; Minami *et al.,* 2010; Wan Norhana *et al.,* 2010).

L. monocytogenes causes an estimated 1591 cases of gastroenteritis in normal humans per year in the United States and it contributes to about 0.1 % of total food-borne illnesses, nonetheless is responsible for 2.6 % of hospitalizations and 18.87 % of the deaths caused via food-borne illnesses (Scallan *et al.,* 2011). In the study by Das *et al.* (2012), a total of 324 tropical seafood and fishery environmental samples were screened for *L. monocytogenes*. The occurrence of which was 1.2 %. *Listeria* spp. was detected in 32.3, 27.1, and 5% of fresh, frozen, and dry fish samples, respectively. *Listeria innocua* was found to be the most prevalent *Listeria* spp in the tropical seafood and environmental samples of Kerala (Das *et al.,* 2012).In another study by Rahimi *et al.* (2012), out of the total of 264 samples examined, 20 (7.6%) were found to be positive for *Listeria*. *Listeria* species were isolated in 7.5%, 4.2%, 11.7% and 6.6% of fresh fish, frozen fish, fresh shrimp and frozen shrimp samples, respectively. *L. monocytogenes* and *L. innocua* were detected in 1.9% and 5.7% of the samples analyzed, respectively (Rahimi *et al.,* 2012).

Only a few studies on the prevalence of *Listeria* spp. in seafood in Iran have previously been done. A study by Jalali and Abedi (2008) on the prevalence of *Listeria* species in food products found that, of the 85 fresh and frozen fish and fresh shrimp samples studied, 2 (2.3%) were contaminated by *L. innocua* and 1 (1.6%) by *L. monocytogenes*. Correspondingly, another report from Iran indicated that, in agreement by our findings, 2.6% of fresh fish samples were found to be contaminated with *L. monocytogenes* (Basti *et al.* 2006). The real situation about listeriosis in Iran is unclear, and little info exists on the prevalence of *L. monocytogenes* in foods consumed in the country. It is also important to note that listeriosis is not a notifiable disease in the Iranian health system (Jalali and Abedi 2008).

Isolation of *L. monocytogenes* from seafood suggests that there is a risk of obtaining listeriosis through sea foods in Iran. *L. monocytogenes* will be killed via cooking, and raw or semi-raw seafood are not consumed in Iran. Though, *L. monocytogenes* in raw sea foods may pose a health risk in kitchens as it could contaminate cooked food or other ready-to-eat foods. In view of outbreaks of listeriosis associated by different foods, avoidance of ingestion of not enough cooked sea foods via at-risk people is recommended. Diligent execution of hygienic conditions of food contact surfaces and handling zones, and good personal hygiene practices must decrease the potential contamination of fishery products via *L. monocytogenes* at the marketing level and management aquaculture.

In this study, *L. monocytogenes*. was isolated in 3 out of 40 samples showing the high occurrence rate (7.5 %). Therefore, it can be concluded that constant monitoring and surveillance for the presence of this psychrotrophic pathogen in *A. leptodactylus* are required. Iranian cooking processes involve a high degree of boiling and roasting which might drop this organism from the seafood.

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