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The objective of this study was to isolation and characterization of *Listeria* species and determines *Listeria monocytogenes* serotypes in fresh fish, shrimp, crab and lobster in Isfahan and Shahrekord, Iran

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ABSTRACT

The objective of this study was to isolation and characterization of *Listeria* species and determines *Listeria monocytogenes* serotypes in fresh fish, shrimp, crab and lobster in Isfahan and Shahrekord, Iran. From September 2010 to April 2011, a total of 300 samples of fresh fish, shrimp, crab and lobster were obtained from randomly selected retail stores in Isfahan and Shahrekord, Iran. The samples were tested for the presence of *Listeria* species using a ISO 11290 protocol and then only *Listeria monocytogenes* isolates identified by bacteriological methods were tested by polymerase chain reaction (PCR) for serotypes detection and presences of virulence associated genes. From the total 300 samples, 23 (10.45%) fresh fish and 1 (2.5%) shrimp samples were positive for *Listeria* spp., but none of the lobster and crab samples were positive for *Listeria* species. *Listeria monocytogenes* only isolated from 17 fish (7.25%) and 1 shrimp (2.5%) samples while *L. innocua*, *L. ivanovii* and *L. seeligeri* only detected in fish samples (2 (0.9%), 3 (1.36%) and 1 (0.45%)), respectively. All of 18 isolates of *L. monocytogenes* showed the characteristic enhancement of hemolytic zone with *S. aureus*. From these positive *L. monocytogenes*, 66.66%, 5.55% and 27.77% had 4b, 1/2a and 1/2b serotypes, respectively. Consumption of these sea foods, either raw or undercooked, may contribute to food-borne illness due to *L. monocytogenes* in Iran. Also, *L. monocytogenes* in raw seafood may pose a health risk in kitchens if contaminating ready-to-eat food.

Key words: *Listeria* spp., *Listeria monocytogenes*, Serotypes, Seafood, Iran

INTRODUCTION

Some food borne diseases are well recognized, but are considered emerging because they have recently become more common. Though there are various food borne pathogens that have been identified for food borne illness, *Campylobacter*, *Salmonella*, *Listeria* species

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(*Listeria* spp.), and *Escherichia coli* O157:H7 have been generally found to be responsible for majority of food-borne outbreaks [1,2]. *Listeria* species (*Listeria* spp.) are ubiquitous, Gram-positive, facultative anaerobic, non-spore-forming, rod-shaped bacteria which classically classified to six characterized species including *Listeria monocytogenes* (*L. monocytogenes*), *L. ivanovii*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, and *L. grayi* [3]. Mainly, this classification is useful for practical and epidemiological purposes, especially when problems caused by food contamination resulted in human listeriosis [4]. Only the hemolytic species of *Listeria* such as *L. monocytogenes*, *L. ivanovii* and *L. seeligeri*, are associated with human pathogenicity. Against *L. ivanovii* and *L. seeligeri* which respectively have been described to be involved rarely in human pathology and once to be the cause of meningitis in a non immune compromised adult [5], *L. monocytogenes* has been involved in known food-borne outbreaks of listeriosis [6,7], however, there are some reports of *L. seeligeri* and *L. ivanovii* causing illness in humans [8,9]. *L. monocytogenes* is responsible for *listeria* infections that can lead to abortion, bacteraemia, sepsis, and meningoencephalitis [10,11]. In addition to food-borne route of *L. monocytogenes*, its widespread distribution, its ability to survive in a wide range of environmental conditions and finally its ability to grow at refrigeration temperatures caused to it has become a threat to humanity. Approximately 2,500 human listeriosis cases occur annually in the United States, resulting in 500 deaths [12] and recent study showed that several major outbreaks of listeriosis have been associated with consumption of contaminated foods [13]. Vegetables, meat, dairy products and seafoods may be contaminated with *Listeria* spp. and are considered as major sources of infection but seafoods contamination due to the high nutritional value is more important. Since, *Listeria* spp. have been isolated from wide variety of seafoods such as shrimp [14], crab [15], cold-smoked rainbow trout [16], fish products [13] and lobster [16]. Isolation of several species of *Listeria* from fresh seafoods, suggests that there are possible risks associated with the consumption of such seafoods by humans. Besides, the high incidence of *Listeria* species in several seafoods warrants the need for more surveillance and monitoring of such seafoods before they are consumed. Although more than 14 serotypes of *L. monocytogenes* have been described, only three serotypes (1/2a, 1/2b, and 4b) cause the vast majority of clinical cases [17]. Isolation and characterization of *Listeria* species is done mainly by cultural, biochemical and molecular methods. There is a need for quick and reliable molecular methods such as polymerase chain reaction (PCR) for detecting *Listeria* species. The epidemiology and prevalence of *Listeria* spp. is essentially unknown in Iran and this present study was carried out for isolation and characterization of *Listeria* spp. and detection of *L. monocytogenes* serotypes in fresh fish, crab, shrimp and lobster using cultural, biochemical and molecular techniques in Iran.

MATERIALS AND METHODS

Bacterial strains

The standard strains of *L. monocytogenes* (PTCC 1298), *Listeria ivanovii subsp. ivanovii* (PTCC 1303), *Staphylococcus aureus* (PTCC 1113) and *Rhodococcus equi* (PTCC 1633) were obtained from the Iranian Research Organization for Science and Technology, Iran and used in culture and PCR methods.

Sample Collection

A total of 300 seafoodsamples were collected from September 2010 to April 2011 from supermarket and retail outlets in Isfahan and Shahrekord townships of Iran. The seafoodsamples analysis comprised samples of fresh fish (n=120), crab (n=20), lobster (n=40)

and shrimp (n=120). The samples were transferred to the Food Microbiology Laboratory at the Islamic Azad University of Shahrekord Branch in portable insulated cold-boxes. Samples were analyzed on the day they were collected.

Isolation and Identification of Listeria

Listeria spp. were isolated from seafood samples according to ISO 11290 protocol [18]. Briefly, 25 g of each seafood sample was aseptically taken, blended for 2 min in 255 mL of *Listeria* enrichment broth (UVM I) (Merck, Germany) and incubated at 37 °C for 24 h. One mL of primary enrichments were transferred to 9 mL of UVM II (Frazer broth) (Merck, Germany) and incubated at 37 °C for 24 h. Secondly enrichments were streaked onto Oxford agar (Merck, Germany) and Palcam agar (Merck, Germany) and incubated at 35 °C for 48 h. The plates were examined for *Listeria* colonies (black colonies with black sunken) and at least 3 suspected colonies were subcultured on Trypton Soy agar supplemented with 0.6% of yeast extract (TSAYE) (Merck, Germany) and incubated at 37 °C for 24 h. All the isolates were subjected to standard biochemical tests including Gram staining, catalase test, motility test at 25 °C and 37 °C, acid production from glucose, manitol, rhamnose, zylose, α-methyl-D-mamoside, and nitrate reduction, hydrolysis of esculin, MR/VP test, ß-hemolytic activity, and CAMP test [19].

Phenotypic characterization

1- Haemolysis on sheep blood agar (SBA)

All the *Listeria* isolates were tested for the type (α or β) and the degree (narrow or wider) of hemolysis on 7% sheep blood agar (SBA). Briefly, the isolates were streaked onto 7% SBA plates and incubated at 37 °C in a humidified chamber for 24 h and examined for hemolytic zones around the colonies. Interpretation of the hemolytic reaction was based on the characteristic β-hemolysis in the form of wider and clear zone of hemolysis representing *L. ivanovii* while a narrow zone of α-hemolysis was the characteristic of *L. monocytogenes* or L. seeligeri.

2- Christie, Atkins, Munch and Petersen (CAMP) test

All the *Listeria* isolates were tested by CAMP test. Briefly, the standard strains of *Staphylococcus aureus* and *Rhodococcus equi* were grown overnight on 7% SBA plates at 37oC and their colonies were again streaked onto freshly prepared 7% SBA plates in a manner such that the streaks were wide apart and parallel to each other. In between the parallel streaks of *S. aureus* and *R. equi* the *Listeria* isolates were streaked at 90 °C angle and 3 mm apart before incubating them at 37 °C for 24 h. The plates were examined for enhancement of the hemolytic zone from partial hemolysis to a wider zone of complete hemolysis, if any, between a *Listeria* strain and the *S. aureus* or *R. equi* strain owing to the synergistic effect of their hemolysins in case of a CAMP-positive reaction.
The *Listeria* isolates with CAMP-positivity against *S. aureus* were characterized as *L. monocytogenes* and those with CAMP positivity against *R. equi* were characterized as *L. ivanovii*.

3- Phosphatidylinositol- specific phospholipase C (PI-PLC) assay

All the biochemically characterized *Listeria* isolates were assayed for PI-PLC activity as per the method of Leclercq [20] with certain modifications. In brief, the *Listeria* isolates were

grown overnight onto 7% SBA plates at 37 ℃. All *Listeria* isolates were streaked on L. mono differential agar (Hi Media Ltd, Mumbai, India) in order to assess PI-PLC activity. The inoculated plates were incubated at 37 °C in a humidified chamber for 24 h. On L. mono differential agar, light blue colonies showing a halo formation around the inoculation site were considered positive for PI-PLC assay.

4- Phosphatidylcholine- specific phospholipase C (PC-PLC) assay

The eggyolk opacity test was done to examine the phosphatidylcholine-specific phospholipase C (PCPLC) activity of the isolates. Tryptic soy agar (Hi Media Ltd. Mumbai, India) plates were prepared with 2.5 per cent egg-yolk emulsion (Hi Media Ltd. Mumbai, India) and 2.5 per cent NaCl, pH 6.5-7. *Listeria* isolates were streaked onto the agar surfaces and incubated at 37 ℃ for 36-72 h and observed for formation of opaque zones surrounding the growth [21].

DNA extraction

Chromosomal DNA was prepared as per Zhang et al. [22]. Briefly, 1 mL of overnight culture (from brain–heart infusion) broth was transferred to 1.5-mL microfuge tube and centrifuged at 8,000 rpm for 5 min, and the supernatant was discarded and 500 µL of cetyl trimethylammonium bromide buffer at 60 °C was added to the microfuge tube containing the bacterial pellet; the mixture was held in water bath at 64 °C for 20 min. During incubation, the mixture was briefly mixed several times. After incubation, 500 µL of chloroform/octanol (24:1) was added and mixed vigorously followed by centrifugation at 3,000 rpm for 5 min. The supernatant was transferred to a clean microfuge tube, and an equal volume of ice-cold isopropanol was added and kept on ice bath for 2-h precipitation. The solution was then centrifuged at 8,000 rpm for 8 min. The aqueous phase was discarded and the DNA pellet was rinsed with 80% ethanol, air-dried and resuspended in 50 µL of double distilled water and used for PCR [23].

PCR condition for detection of Listeria spp., L. monocytogenes serotypes L. monocytogenes:

The primers for the detection of *Listeria* spp., *L. monocytogenes* serotypes used in this study were synthesized by CinnaGen, Iran. The details of the primers sequence are shown in Table 1.

Table 1: Primers for amplification of , *Listeria* spp. and serotypes of *L. monocytogenes*

References	Size of product (bp)	Target	Primer sequence (5'-3')	Primer name
[24]	870	*L. innocua*	TTATACGCGACCGAAGCCAAC	**Lis1B**
			ACTAGCACTCCAGTTGTTAAAC	**Ino2**
[24]	660	*L. monocytogenes*	TTATACGCGACCGAAGCCAAC	**Lis1B**
			CAAACTGCTAACACAGCTACT	**MonoA**
[24]	1100	*L. ivanovii*	TTATACGCGACCGAAGCCAAC	**Lis1B**
			CTACTCAAGCGCAAGCGGCAC	**Iva1**
[24]	1100	*L. seeligeri*	TTATACGCGACCGAAGCCAAC	**Lis1B**
			TACACAAGCGGCTCCTGCTCAAC	**Sel1**
[24]	1050	*L. welshimeri*	TTATACGCGACCGAAGCCAAC	**Lis1B**
			CCCTACTGCTCCAAAAGCAGCG	**Wel1**
[24]	480	*L. grayi*	TTATACGCGACCGAAGCCAAC	**Lis1B**
			GTGATTTCTGCTTGCCATAG	**MuraI**
[25]	370	All *L. monocytogenes* serovares	GCTGAAGAGATTGCGAAAGAAG	**prsF**
			CAAAGAAACCTTGGATTTGCGG	**prsR**
[25]	691	*L. monocytogenes* serovar1/2a	AGGGCTTCAAGGACTTACCC	**lmo0737F**
			ACGATTTCTGCTTGCCATTC	**lmo0737R**
[25]	471	*L. monocytogenes* serovar1/2b	AGCAAAATGCCAAAACTCGT	**ORF2819F**
			CATCACTAAAGCCTCCCATTG	**ORF2819R**
[25]	597	*L. monocytogenes* serovar 4b	AGTGGACAATTGATTGGTGAA	**ORF2110F**
			CATCCATCCCTTACTTTGGAC	**ORF2110R**

DNA amplification was performed in a DNA thermal cycler (Eppendrof Mastercycler 5330; Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany). The amplification conditionsfor identification of *L. monocytogenes, L. innocua, L. ivanovii, L. seeligeri, L. welshimeri,* and *L. grayi* for the PCR assays were those described by Bubert et al. [24]. The multiplex PCR assay was standardized for the detection of three major serovars of *L. monocytogenes* namely 1/2a, 1/2b and 4b, following the methodology as described by Doumith et al. [25]. PCR products were analyzed by 1.5% agarose gel electrophoresis and the specific DNA bands were visualized using ethidium bromide staining under UV illumination. The types and numbers of the seafood samples analyzed in this study are presented in Table 2. Of the 300 samples screened, 23 (10.45%) of the fresh fish samples and 1 (2.5%) of the shrimp samples were positive for *Listeria* spp., but none of the lobster and crab samples were not positive for *Listeria* spp.. *L. monocytogenes* was isolated from 17 (7.72%) samples of fresh fish. *Listeria* spp. from fresh fish were identified, with the order of predominance as *L. innocua* (2) (0.9%), *L. ivanovii* (3) (1.36%) and *L. seelgeri* (1) (0.45%) (Table 2). In shrimp samples, *L. monocytogenes* was isolated from 1 (2.5%) of the samples.

Table 2: Prevalence of *Listeria* spp. in marine foods in Iran

Type of sample	No. of samples	No. (%) of *Listeria* spp.	No. (%) of *L. monocyt*	No. (%) of *L. innocua*	No. (%) of *L. ivanovii*	No. (%) of *L. seeligeri*
Fish	220	23 (10.45)	17 (7.72)	2 (0.9)	3 (1.36)	1 (0.45)
Shrimp	40	1 (2.5)	1 (2.5)	-	-	-
Lobster	20	-	-	-	-	-
Crab	20	-	-	-	-	-
Total	300	24 (8)	18 (6)	2 (0.66)	3 (1)	1 (0.33)

All the 18 isolates of *L. monocytogenes* showed the characteristic enhancement of hemolytic zone with *S. aureus*. All the 18 isolates of *L. monocytogenes* were found to be pathogenic by PI-PLC and PC-PLC.

The most common serotype found in the *L. monocytogenes* isolates was 4b, which occurred in 12/18 (66.66%). Other common serotypes included 1/2a and 1/2b, with a total of 1/18 (5.55%) and 5/18 (27.77%) occurrences, respectively.

DISCUSSION

Listeriosis is one of the most important zoonotic bacterial diseases with worldwide distribution. Disease has considerable economic and public health significance. *L. monocytogenes* has been described as opportunistic pathogen affecting mainly children, pregnant women, the aged and immune-challenged individuals [26,27]. In addition a wide variety of animals including sheep, cattle, goats, pigs, rabbits, mice, birds, and fish are also infected. An atypical foodborne disease due to *listeria* with a high fatality rate, ranging from 25 to 30% in susceptible populations [28]. Since 1975, food borne listeriosis outbreaks have been reported in industrialized countries in Europe, North America and Oceania with few or no reports from Africa, Asia and Latin America [29,30]. Despite all alarms for this bacterium, studies on occurrence of the important food borne pathogens like *L. monocytogenes* in seafoods have not yet been carried out in detail in Iran except for a few reports [14,31,32] but there is no previous study about detection of *Listeria* spp. in crab and lobster in Iran.

From 300 seafood samples collected from retail shop in Iran, 6%, 1%, 0.33% and 0.66% of them were positive for *L. monocytogenes*, *L. ivanovii*, *L. innocua* and *seeligeri*, respectively but previous study from Iran on fresh and frozen fish and shrimp showed that *L. monocytogenes* and *L. innocua* were detected in 1.9% and 5.7% of the samples analyzed, respectively [14]. The lower prevalence of *L. monocytogenes* was reported from Iran in raw/fresh fish and shrimp samples (1.4%) [32]. Another study from Iran indicated that *L. monocytogenes* were greater than 1 × 102 g−1 in 2.6% of fresh not smoked fish [31]. Recent study from Urmia, Iran showed that *Listeria* was isolated from 24 fish (12.37%) and the highest prevalence of *Listeria* was observed in both *Abramis brama* and *Abramis leptodactylus* with 25%, while the lowest prevalence of *Listeria* was seen in *Sander lucioperca* (9.7%) and from the total of 24 *Listeria* isolates, five (21%) were confirmed to be *L. monocytogenes;* seven isolates were *L. ivonoi* (29%) while *L. Seeligeri* was not isolated from any examined fish [33]. To our knowledge, the attack of *Listeria* from intestinal contents to other fish tissues is the main cause of this high presence of *Listeria* spp. in seafoods in our study and other researches. Previous study showed that contact with intestinal contents is the risk factor for prevalence of *Listeria* spp. in seafood samples [34]. In addition, cross contamination, using contaminated equipments, fish manipulation and inappropriate transport, were introduced as risk factors [35,36]. An overall prevalence 3% of *L. monocytogenes* was observed in European fish [37] but Miettinen and Wirtanen [38] reported that the prevalence of *Listeria* spp. and *L. monocytogenes* in pooled unprocessed fresh rainbow trout was on average of 35% and 14.6%, respectively. Study in Greece on fish and environment of fish markets showed that *L. innocua* was more common specie and the level of contamination of the environment of fish markets was higher than fish [39]. In Turkey, the incidence of*Listeria* spp. was 30% in freshwater samples and 10.4% in marine fish samples and *L. monocytogenes* (44.5%) and *L. murrayi* (83.5%) was the most commonly isolated species from freshwater fish and marine fish samples, respectively [40] which was higher than our results. Although, our study showed that *L. monocytogenes* was predominantly among *Listeria* species, some study showed that *L. innocua* is more predominant in seafoods [41,42]. Our results showed that 4b followed by 1/2a and 1/2b with incidence of 66.66%, 5.55% and 27.77% were the most common serotype found in the *L. monocytogenes* isolates. Another study revealed that serotype 1/2a is the most frequently isolated from food and serotype 4b causes the majority of human epidemics [43]. Doumith et al. [25] affirms that at least 95% of strains isolated from contaminated foods and infected patients were of serotypes 1/2a, 1/2b, 1/2c and 4b. The data collected by the National Reference Center in France showed that serovars 1/2a, 1/2b, 1/2c, and 4b, which are separated by our test into four distinct PCR profiles, represent over 98% of the 5,000 isolates collected from food and patients during the last 3 years [25]. Another study on mastitis milk samples showed that all the three isolates of *L. monocytogenes* were serotyped as 4b [44]. Wan et al. [45] compared the use of PCR to detect *L. monocytogenes* in salmon with the ISO culturing method 11290-1, and found the two methods gave comparable results in spiked samples if culture enrichment is used prior to PCR to lower the detection limit for *L. monocytogenes*. The only difference is that the PCR method requires only 58–60 h to perform rather than 5 days, while our results showed that in combined application of culture with PCR method, results are acceptable. In fact, PCR allowed the specific detection of a number of serotypes of *L. monocytogenes* while no bands from other non-pathogenic species of *Listeria* were detected. An effective control measure for this pathogen has to target the farm, processing plants and the environments. At these all these stages, strict adherence to standard operating measures must be practiced. In farming, livestock's should be reared in clean dry environments. Soils in particular should not be moist or damp as that will provide a conductive environment for the growth of this pathogen. Livestock houses should be thoroughly cleaned, and disinfected on regularly basis. Prevent entering of wild animals (which may serve as reservoirs) into the farm especially in areas where feeds are stored [46].

In total, the ability of *L. monocytogenes* to survive in refrigeration and wide environmental conditions increases the plight of achieving zero or minimal tolerant of *L. monocytogens* in foods. Reliable and accurate isolation and detection techniques are important in the surveillance of *L. monocytogenes* and listeriosis. Standard and hygienic operating methods in the farming, processing and marketing of foods are the way forward to reduce the incidence of listeriosis. This study revealed an overall prevalence of *Listeria* spp. in fish, lobster, crab and shrimp obtained from retail shops and super-markets in Isfahan and Shahrekord, Iran. Therefore, for public health matter, it was suggested to eviscerate fish immediately after harvesting to avoid bacteria attacking to other tissues. Furthermore, since cross contamination has been considered as a major cause of seafood contamination with *Listeria* spp., transportation, handling and processing of seafood products should be performed in an extreme hygienic condition. We suggested combined use of cultural, biochemical and molecular techniques for more sensitive detection of *Listeria* spp. in various samples. *Listeria* spp. was 30% in freshwater samples and 10.4% in marine fish samples and *L. monocytogenes* (44.5%) and *L. murrayi* (83.5%) was the most commonly isolated species from freshwater fish and marine fish samples, respectively [44] which was higher than our results. Although, our study showed that *L. monocytogenes* was predominantly among *Listeria* species, some study showed that *L. innocua* is more predominant in seafoods [45,46]. To our knowledge, detection of just one virulence-associated gene by PCR is not always sufficient to identify *L. monocytogenes* and our results showed that all of the 18 *L. monocytogenes* isolates have *plcA, prfA, actA, hlyA* and *iap* virulence genes. Unfortunately, there is no previous published data about detection of *L. monocytogenes* virulence factors in seafoodproducts. To our knowledge, serotype designation is associated with virulence potential. Our results showed that 4b followed by 1/2a and 1/2b with incidence of 66.66%, 5.55% and 27.77% were the most common serotype found in the *L. monocytogenes* isolates. Another study revealed that serotype 1/2a is the most frequently isolated from food and serotype 4b causes the majority of human epidemics [47]. Doumith et al. [25] affirms that at least 95% of strains isolated from contaminated foods and infected patients were of serotypes 1/2a, 1/2b, 1/2c and 4b. The data collected by the National Reference Center in France showed that serovars 1/2a, 1/2b, 1/2c, and 4b, which are separated by our test into four distinct PCR profiles, represent over 98% of the 5,000 isolates collected from food and patients during the last 3 years [25]. Another study on mastitis milk samples showed that all the three isolates of *L. monocytogenes* were serotyped as 4b [48]. Wan et al. [49] compared the use of PCR to detect *L. monocytogenes* in salmon with the ISO culturing method 11290-1, and found the two methods gave comparable results in spiked samples if culture enrichment is used prior to PCR to lower the detection limit for *L. monocytogenes*. The only difference is that the PCR method requires only 58–60 h to perform rather than 5 days, while our results showed that in combined application of culture with PCR method, results are acceptable. In fact, PCR allowed the specific detection of a number of serotypes of *L. monocytogenes* while no bands from other non-pathogenic species of *Listeria* were detected. An effective control measure for this pathogen has to target the farm, processing plants and the environments. At these all these stages, strict adherence to standard operating measures must be practiced. In farming, livestock's should be reared in clean dry environments. Soils in particular should not be moist or damp as that will provide a conductive environment for the growth of this pathogen. Livestock houses should be thoroughly cleaned, and disinfected on regularly basis

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