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Detection of *Vibrio* spp. in shrimp from aquaculture sites in Iran using polymerase chain reaction (PCR)

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Abstract. Shrimp is one of the most important fishery products of the coastal provinces in the Persian Gulf in Iran. Vibriosis has been an important cause of production loss due to bacterial disease in shrimp farms in south Iran in recent years. The objective of this study was to detect the prevalence of *Vibrio* spp. in shrimp samples from farms in the southern provinces of Iran by polymerase chain reaction (PCR). A total number of 36 shrimp were caught from south coast of Iran and were studied to identify *Vibrio* spp. Three *Vibrio* species including *Vibrio parahemolyticus*, *V. alginolyticus* and *V. harveyi* were identified using biochemical and molecular methods. Two shrimp contained one or more *Vibrio* spp. as two samples contained *V. parahemolyticus,* two samples contained *V. alginolyticus* and two contained *V. harveyi*. The results of the present study revealed that the shrimp from the studied area is contaminated with *Vibrio* spp. and molecular detection systems were applied for the investigation of shrimp samples for the presence of the different *Vibrio* spp. The collected data indicate that the PCR systems can be useful for rapid detection and differentiation of *Vibrio* spp. in shrimp samples as basis for preventive protection policy to consumers.
Key Words: prevalence, molecular method, vibriosis, shrimp, Iran.

Introduction. Today the world is witnessing the resurgence in the consumption of shrimp. Shrimp farms have developed during the past twenty years. In 2008, the production of shrimp in the world was reported at 3, 281, 253 metric tons (Pazir et al 2011). Substantial evidence suggests that seafood is high on the list of foods associated with outbreaks of food-borne diseases (Ebrahimzadeh Mousavi et al 2011). It is worth noting that the microbial status of seafood after being caught is closely related to environmental conditions and microbiological quality of the water (Ebrahimzadeh Mousavi et al 2011; Khamesipour et al 2013).

During the last several decades, researchers have reported cases of food-borne infections in humans caused by the consumption of contaminated fresh-raw shellfish, shrimp and other seafoods. Occasionally, *Vibrio* spp. has been identified as the most significant cause of food-borne hospitalizations; even it has been named as the cause of death for some people in some epidemics (Colakoglu et al 2006; Raissy et al 2011; Khamesipour et al 2013).

The members of Vibrionaceae are the natural pathogens of the aquatic environment, which is also inhabited by shellfish, shrimp and other aquatic organisms (Colakoglu et al 2006; Mahbub et al 2011; Ebrahimzadeh Mousavi et al 2011; Raissy et al 2011). Vibrios are among the most important bacterial pathogens of cultured shrimp responsible for a number of diseases, and mortalities up to 100% have been reported due to this disease. *Vibrio* species are a normal part of the bacterial flora in aquatic environments and formerly considered to be mostly opportunistic pathogens (Mahbub et al 2011; Raissy et al 2011). Shrimp pathogenic Vibrios are mainly *V. harveyi, V. fluvialis, V. parahaemolyticus, V. anguillarum, V. damsela* and *V. vulnificus* (Mahbub et al 2011).

Although vibriosis is a common bacterial disease among marine organisms, this disease normally occurs during the warm summer months when the water salinities and organic loads are high (Caipang & Aguana 2011).

Vibrio spp. are Gram-negative, facultative anaerobic, non-spore-forming bacilli which are oxidasepositive and as halophilic bacteria, and they are widely spread in the sea and brackishwater environments worldwide. Most of the *Vibrio* species are pathogenic to humans and are usually responsible for causing alimentary infections in countries with warm coastal waters, where fish and shrimp are consumed raw or lightly cooked (Jaksic et al 2002; Messelhäusser et al 2010). Only a few species, especially *V. parahaemolyticus*, are regularly linked to human foodborne infections caused by consumption of raw, undercooked or recontaminated seafood, but there are also occasional reports of foodborne or waterborne infections caused by environmental *Vibrio* or *Vibrio*-like spp., e. g. *V. mimicus*, *V. algenolyticus*, and *Grimontia* (*Vibrio*) *hollisae*. *Vibrios* are frequently isolated from seafood and in particular, shellfish and shrimp (Jaksic et al 2002; Messelhäusser et al 2010; Mahbub et al 2011).

Bacterial diseases, mainly due to *Vibrio*, have been reported in penaeid shrimp culture systems implicating at least 14 species and they are *V. harveyi*, *V. splendidus*, *V. parahaemolyticus*, *V. alginolyticus*, *V. mimicus, V. anguillarum*, *V. vulnificus*, *V. campbelli*, *V. fischeri*, *V. damsella*, *V. pelagicus*, *V. orientalis*, *V. ordalii*, *V. mediterrani* and *V. logei* etc. (Raissy et al 2011; Raissy et al 2012). Of these, *V. harveyi* is the causative agent of luminous disease, which resulted in 80 to 100% mortality in *Penaeus monodon* hatcheries. *V. harveyi* is found in coastal and marine waters, in association with surface and gut of marine and estuarine organisms and also in shrimp pond water and sediment (Reboucas et al 2011; Raissy et al 2011). *V. harveyi* was also reported as the causative agent of vibriosis in tiger shrimp (*P. monodon*), kuruma shrimp (*Penaeus japanicus*), pearl oyster (*Pinctada maxima*) (Raissy et al 2011; Caipang & Aguana 2011). *V. anguillarum*, *V. campbelli*, *V. nereis*, *V. cholerae* and *V. splendidus* have also been reported in association with disease outbreaks in crustaceans (Ansari & Raissy 2010; Raissy et al 2011). *V. parahaemolyticus*, *V. vulnificus* and *V. alginolyticus* are halophilic organisms that resemble phenotypically, and they exist in warm seawater, river mouths and seafood (Jaksic et al 2002). Vibriosis has been the main cause of production loss due to bacterial disease in shrimp farms in south Iran in recent years (Hosseini et al 2004).

Even though there have been some studies of the bacteria associated with disease in shrimp in several countries (Jaksic et al 2002; Messelhäusser et al 2010; Raissy et al 2011), incidence of *Vibrio* spp. in shrimp is important in Iran. The objective of this study was to detect the prevalence of *Vibrio* spp. in shrimp samples from farms in the southern provinces of Iran by PCR.

Material and Method

Sample preparation. A total of 36 samples of fresh shrimps obtained by random sampling from the south coast of Iran (from shrimp farms) were collected during October and November 2013. All samples were transported to the laboratory, Islamic Azad University of Shahrekord Branch, refrigerated at 4°C or placed on ice and processed within a short period of time after arrival.

Identification. Biochemical analysis for *Vibrio* spp. was done following the method described by Bockemuhl (1992) and Austin & Austin (1999). Briefly, the samples were added to alkaline peptone water (APW) (Merck) and incubated at 37°C. The positive samples were sub-cultivated on Thiosulfate Citrate Bile Salts Sucrose agar (TCBS, Merck). Colony morphology on TCBS agar was determined using API 20E (BioMérieux, Marcy IEtoile, France) (Neogi et al 2010). After incubation at 37°C for 24 h, the isolates were used for biochemical tests including Gram staining, oxidase and catalase tests, culture in SIM and TSI media and other biochemical tests described by Hosseini et al (2004).

DNA extraction and PCR primers. The exact identification of bacteria was done by polymerase chain reaction (PCR). Genomic DNA was prepared using a standard DNA extraction method (Ausubel et al 1987) and stored at -20°C. The purity of genomic DNA in each sample was evaluated by measuring optical densities at 260 and 280 nm wavelengths. The DNA concentration of each sample was adjusted to 50 ng μL^{-1} for PCR. Two sets of oligonucleotide primers were used for species-specific identification of *Vibrio* species. The PCR reaction was performed in a 50 µL reaction system consisting of 2 µL of purified genomic DNA (50 ng μL^{-1}), 5 µL of 10×PCR buffer (100 mM Tris–HCl, pH 8.3, 500 mM KCl, 60 mM MgCl2, 0.1% gelatin and 1% Triton X-100), 1 µL each of the primers (50 pmol μL^{-1}), 1 µL each of the 10 mM dNTPs, 0.2 µL units Taq DNA polymerase (5 units μL^{-1}) and 40 µLl of sterile distilled water. The reactions were performed with a thermal cycler (Eppendorf, Germany) with the program described previously for the detection of *Vibrio* species (Di Pinto et al 2005; Tarr et al 2007; Maiti et al 2009). The primer sequences, targeting genes and amplicon size are listed in Table 1.

Table 1

Primer sequences, targeting genes and amplicon size of primers

Target species	*Sequence (5'------ 3')*	*Amplicon size (bp)*	*Targeting gene*	*Reference*
V. parahaemolyticus	GCAGCTGATCAAAACGTT GAGT ATTATCGATCGTGCCACTCAC	897 bp	flaE	Tarr et al (2007)
V. cholerae	AAGACCTCAACTGGCGGTA GAAGTGTTAGTGATCGCCAGAGT	248 bp	sodB	Tarr et al (2007)
V. vulnificus	GTCTTAAAGCGGTTGCTGC CGCTTCAAGTGCTGGTAGAAG	410 bp	Hsp	Tarr et al (2007)
V. mimicus	CATTCGGTTCTTTCGCTGAT GAAGTGTTAGTGATTGCTAGAGAT	121 bp	sodB	Tarr et al (2007)
V. alginolyticus	CGAGTACAGTCACTTGAAAGC C CACAACAGAACTCGCGTTACC	737 bp	collagenase	Di Pinto et al (2005)
V. harveyi	CTTCACGCTTGATGGCTACTG GTCACCCAATGCTACGACCT	235 bp	Vhh	Maiti et al (2009)

Analysis of PCR products. Distilled water served as a negative control. PCR product was run using 1.5% agarose gel in 1X TBE buffer at 80V for 30 min, stained with Ethidium Bromide and the images were obtained using UVIdoc gel documentation systems (Uvitec, UK). The sizes of the PCR products were identified by 100 bp DNA size marker (Fermentas, Germany).

Statistical analysis. The data were analyzed using SPSS (Statistical Package for the Social Sciences) software (Version 17. SPSS Inc, USA).

Results and Discussion. A total number of 36 shrimps were studied and 7 samples (19.444%) contained one or two *Vibrio* species. In the present study, biochemical tests confirmed 7 green or blue-green colonies on TCBS agar as *Vibrio* positive samples. The molecular analysis carried out on the isolates gave positive results for all 6 strains using PCR assay. PCR products of 897, 737 and 235 bp were obtained for *V. parahaemolyticus, V. alginolyticus* and *V. harveyi,* respectively, as expected, from PCR amplification of the bacterial isolates. The specificity of the PCR products was confirmed by sequence analysis. According to the results, two samples contained *V. parahaemolyticus*, three samples contained *V. alginolyticus* and two samples contained *V. harveyi* and no sample contained *V. cholerae, V. vulnificus* and *V. mimicus*. The results are presented in Table 2. PCR specimens producing a band of the expected size (897, 737 and 235 bp) were considered positive (Figure 1).

The results showed a high presence of *Vibrio* spp. DNA in shrimp specimens. These results indicated that this infection is an important agent that increases economical costs. These findings suggested that control and eradication programs for *Vibrio spp.*

infection are necessary in Iran. Our findings support the necessity of the PCR test for the detection of *Vibrio* spp. in shrimp samples and could be easily used for routine diagnosis.

Table 2

Vibrio species collected from shrimps samples

Sample no	*Bacteria species*					
	V. alginolyticus	*V.cholera*	*V. harveyi*	*V. mimicus*	*V. parahemolyticus*	*V. vulnificus*
Shrimp-2	+	-	+	-	+	-
Shrimp-3	+	-	-	-	-	-
Shrimp-8	-	-	+	-	+	-
Shrimp-9	+	-	-	-	-	-
Shrimp-10	-	-	-	-	-	-
Shrimp-13	-	-	-	-	-	-
Shrimp-18	-	-	-	-	-	-
Total	3	0	2	0	2	0

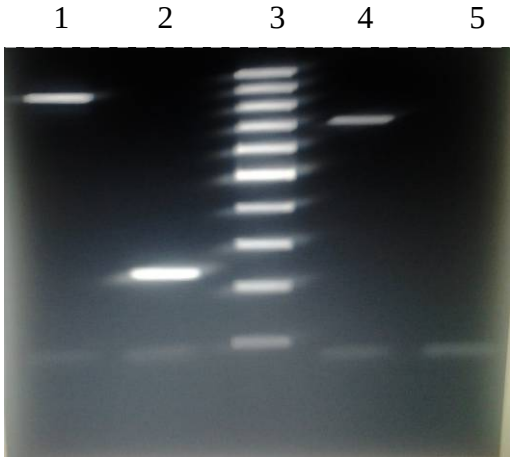

Figure 1. Ethidium bromide-stained agarose gel electrophoresis of PCR products for detection of *Vibrio* spp. in samples after PCR amplification. Agarose gel electrophoresis for identification of *Vibrio spp.* in shrimp samples. Lane 1: positive samples (*V. parahaemolyticus*: 897 bp); Lane 2: positive samples (*V. harveyi*: 235 bp); lane 3: 100 bp DNA ladder (Fermentas, Germany); Lane 4: positive samples (*V. alginolyticus*: 737 bp); Lane 4: Negative control.

Shrimp is one of the most important fishery products of the coastal provinces at the Persian Gulf in Iran. Whilst shrimp farming is a major economic characteristic of these provinces, a large part of the products is exported to other nations especially the European Union countries. Hygienic aspects of fishery industry have improved to the degree, which obtained HACCP certificate for their production. Nevertheless, seafood may be a vehicle for most of known bacterial pathogens (Hosseini et al 2004).

Development of shrimp culture industry has been in parallel with the development of diseases, including Vibriosis. Vibriosis has been an important cause of production loss in shrimp farms in south Iran in recent years, suggesting that there is transfer of the disease from farmed shrimp to wild population in the Persian Gulf (Ansari & Raissy 2010).

Diseases caused by *Vibrio* species have been reported in several aquatic animals such as shrimp, crayfish, fish, oysters and lobster (Chrisolite et al 2008; Raissy et al 2011). While numerous studies have been done on vibriosis in shrimp, the incidence of *Vibrio* in shrimp is of significant importance (Schmidt et al 2000; Sung et al 2001; Ansari & Raissy 2010; Reboucas et al 2011).

In this study the occurrence of *Vibrio* spp. in shrimp was studied. *Vibrio* has been often defined as opportunistic and potential pathogenic of the water bodies especially in warm climate regions (Messelhäusser et al 2010). The increase of bacterial infection especially *Vibrio* infections brought about by the consumption and the manipulation of contaminated fish, shrimp and shellfish have made necessary the precise identification of these bacteria (Rahimi et al 2012; Raissy et al 2012; Khamesipour et al 2013). In the

current study, the results are in agreement with the results of earlier studies in different nations (Jaksic et al 2002; Hosseini et al 2004; Lhafi & Kühne 2007; Ansari & Raissy 2010).

Raissy et al (2011) reported the occurrence of various *Vibrio* species in lobster hemolymph from the Persian Gulf of Iran. A total number of 60 lobsters (*Panulirus homarus*) were studied and were found to harbor *Vibrio* spp. in the hemolymph using biochemical and molecular techniques. Six lobsters (10%) contained one or more *Vibrio* spp. and four samples contained *V. alginolyticus,* one contained *V. vulnificus* and one species contained both *V. harveyi* and *V. mimicus* and none of samples contained *V. parahemolyticus* and *V. cholera* (Raissy et al 2011).

Another study by Amin & Salem (2012) reported the occurrence of *V. parahaemolyticus* in the examined shrimp and crab. The pathogen was found in (4) 20% and (6) 30% via culture technique in shrimp and crab, respectively. On the other hand, the pathogen can be detected in (14) 70% of the shrimps and (10) 50% of the crabs via m-PCR technique. The occurrence of *V. mimicus* in the examined shrimp and crab samples was found to be (5) 25% and (1) 5% by m-PCR method, respectively, while it was not detected in all the examined shellfish samples via culture technique. *V. vulnificus* and *V. cholera*e failed to be detected in all the examined shellfish samples via both the culture and m-PCR techniques (Amin & Salem 2012).

The problems brought about by vibriosis in the shrimp culture industry led to the emergence of alternative strategies to prevent disease outbreaks. These include the so-called ”green-water” technology derived from finfish, use of reservoir, probiotics application and chlorination of ponds during the culture period (Caipang & Aguana 2011). Other than these techniques, routine surveillance of the culture sites has to be done to detect the pathogens during the early phases of infection. Luminous Vibrios as well as presumptive Vibrios are monitored by standard microbiological methods. This involves regular sampling of the rearing water and the cultured stock via plating them on a selective medium, *e.g.* thiosulfate-citrate bile salt agar, TCBS for *Vibrio* spp., that will allow the growth of the target pathogen. However, this technique may not be able to detect the pathogen during the early phases of infection or may have a lower sensitivity of detection. In isolation, other techniques particularly molecular-based detection techniques including PCR have been developed to give a fast and accurate determination of the pathogens at the early phases of infection. In this way, effective management rules could be implemented to prevent severe disease outbreaks and production losses.

Conclusions. The results of the current study revealed that the shrimp from the study region is contaminated with *Vibrio* spp. Even though the source of the bacteria is typically from the aquatic environment, secondary contamination during catching, handling and transportation may also result in their distribution. Since water has also been shown to be contaminated by these species, it is possible that contaminated water or ice may have contributed to the high incidence of the bacteria. It should be considered that occurrence of *Vibrio* in human is highly dependent on the nutritional habits. Iranian cooking processes include a high degree of boiling and roasting which might drop this organism from the seafood, although the toxin may stay in the food stuff.

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