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Full Length Research Paper

Molecular detection of *Vibrio* spp. in lobster hemolymph

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The occurrence of various *Vibrio* species in lobster hemolymph from the Persian Gulf was studied. A total number of 60 lobsters (*Panulirus homarus*) were caught from south coast of Iran and were studied to identify *Vibrio* spp. in hemolymph. Four *Vibrio* species including *Vibrio alginolyticus, Vibrio vulnificus*, *Vibrio harveyi* and *Vibrio mimicus* were identified using biochemical and molecular methods. Six lobsters (10%) contained one or more *Vibrio* spp. as 4 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.*

Key word: Persian Gulf, vibriosis, *Panulirus homarus*.

INTRODUCTION

Vibrio species are a normal part of the bacterial flora in aquatic environments and formerly considered to be mostly opportunistic pathogens (Gomez-Gil et al., 1996). However, some more recently occurring diseases of aquatic crustaceans have been caused by *Vibrio* species which behave more like true pathogens than opportunistic invaders (Lightner et al., 1992). 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). Bacterial diseases, mainly due to *Vibrio*, have been reported in penaeid shrimp culture systems implicating at least 14 species and they are *Vibrio harveyi*, *Vibrio splendidus*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Vibrio mimicus, Vibrio anguillarum*, *Vibrio vulnificus*, *Vibrio campbelli*, *Vibrio fischeri*, *Vibrio damsella*, *Vibrio pelagicus*, *Vibrio orientalis*, *Vibrio ordalii*, *Vibrio mediterrani* and *Vibrio logei* etc (Eaves and Ketterer, 1994). The major *Vibrio* species reported from diseased shrimp and other crustaceans are *V. parahaemolyticus, V. harveyi, V. alginolyticus* and *V. vulnificus* (Ruangpan and Kitao, 1991; Jiravanichpaisal et al., 1994). Of these, the *V. harveyi* is the causative agent of luminous disease with 80 to 100% mortality in *Penaeus monodon* hatcheries. *V. harveyi* are 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 (Otta et al., 1999). *V. harveyi* was also reported as the causative agent of vibriosis in tiger shrimp (*P. monodon*), kuruma shrimp (*Penaeus japanicus*), pearl oyster (*Pinctada maxima*), (Lavilla-Pitago et al., 1990; De la Pena et al., 1993). *V. anguillarum*, *V. campbelli*, *V. nereis*, *V. cholerae* (non 01) and *V. splendidus* have also been reported in association with disease outbreaks in crustaceans (Chen 1992; Lavilla-Pitago et al., 1990; Sahul-Hameed et al., 1996). Although there have been several studies of the bacteria associated with disease in shrimp in many countries (Lightner, 1993; Sung et al., 2001; Hosseini et al., 2004; Chrisolite et al., 2008), incidence of *Vibrio* in lobster is less studied. The

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Table 1. *Vibrio* species collected from lobster hemolymph.

Bacteria species / Sample no	*V. alginolyticus*	*V. harveyi*	*V. vulnificus*	*V. mimicus*	*V. parahemolyticus*	*V. cholera*
Lob-3	+					
Lob-12			+			
Lob-24		+		+		
Lob-35	+					
Lob-36	+					
Lob-43	+					
Total	4	1	1	1	0	0

objective of this study was to investigate the incidence of Vibriosis in wild population of lobster from the Persian Gulf.

MATERIALS AND METHODS

Overall 60 lobsters (*Panulirus homarus*) were caught from south coast of Iran during June 2011 to September 2011. The samples were transferred into cool boxes with an internal temperature of +2 to +5°C. During the transport to the laboratory the temperature was continually recorded with a logger (Testo 174, Testo GmbH & Co., Lenzkirch, Germany). All samples were processed within a short time after arrival.

Sampling of hemolymph was done by a process involving disinfection of the surface of the lobster's exoskeleton in the vicinity of the junction between the second and third abdominal somites. This was followed by insertion of a 26 gauge needle attached to a 1 ml tuberculin syringe through the somite junction into the dorsal sinus and withdrawal of hemolymph. The biochemical analysis for *Vibrio* spp. took place according to the method described by Bockemuhl (1992) and Austin and Austin (1999). Briefly, the samples of hemolymph were added to alkaline peptone water (APW) and incubated at 37°C. The positive samples were subcultivated on Thiosulfate Citrate Bile Salts Sucrose agar (TCBS). 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). The exact identification of bacteria was done by PCR for that purpose, the 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 for polymerase chain reaction (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), 5 µl of 10×PCR buffer (100 mM Tris–HCl, pH 8.3, 500 mM KCl, 60 mM $MgCl_2$, 0.1% gelatin and 1% Triton X-100), 1 µl each of the primers (50 pmol/µl), 1 µl each of the 10 mM dNTPs, 0.2 µl units Taq DNA polymerase (5 units/µl) and 40 µl 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 pairs used for *V. parahaemolyticus*, *V. alginolyticus*, *V. harveyi*, *V. vulnificus, V. mimicus* and *V. cholera* respectively, were Vp.flaE-79F (5'-GCAGCTGATCAAAACGTTGAGT-3') and Vp.flaE-934R (5'-ATTATCGATCGTGCCACTCAC-3') based on flaE gene producing a 897-bp fragment (Tarr et al., 2007), VA-F (5'-CGAGTACAGTCACTTGAAAGCC-3') and VA-R (5'-CACAACAGAACTCGCGTTACC-3') based on Collagenase gene producing 737-bp fragment (Di Pinto et al., 2005), VH-F (5'-CTTCACGCTTGATGGCTACTG-3') and VH-R (5'-GTCACCCAATGCTACGACCT-3') based on vhh gene targeting 235-bp fragment (Maiti et al., 2009), Vv.hsp-326F (5'-GTCTTAAAGCGGTTGCTGC-3') and Vv.hsp-697R (5'-CGCTTCAAGTGCTGGTAGAAG-3') based on hsp gene producing 410- bp fragment (Tarr et al., 2007), Vm.sodB-F (5'-CATTCGGTTCTTTCGCTGAT-3') and Vm.sodB-R2 (5'-GAAGTGTTAGTGATTGCTAGAGAT-3') based on sodB gene producing 121-bp fragment (Tarr et al., 2007), *Vc.sodB*-F (5'-AAGACCTCAACTGGCGGTA-3') and *Vc.sodB*-R (5'-GAAGTGTTAGTGATCGCCAGAGT-3') based on sodB gene producing 248-bp fragment (Tarr et al., 2007).

RESULTS

A total number of 60 lobsters were studied and 6 samples (10%) contained one or two *Vibrio* species. In the present study, biochemical tests confirmed 6 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 a PCR assay. Products of 897, 737, 235 and 121 bp were obtained for *V. alginolyticus, V. harveyi, V. vulnificus* and V. *mimicus* 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, four samples contained *V. alginolyticus*, one contained *V. vulnificus* and one species contained both *V. harveyi* and *V. mimicus* and no sample contained *V. parahemolyticus* and *V. cholera*. The results are presented in Table 1.

DISCUSSION

Diseases due to *Vibrio* species have been reported in many aquatic animals such as fish, shrimp, crayfish, oyster and lobster (De la Pena et al., 1993; Schmidt et al., 2000; Tall et al., 2003; Reboucas et al., 2011). Although many studies have done on vibriosis in shrimp,

incidence of *Vibrio* in lobster is less studied. Tall et al. (2003) reported *Vibrio fluvialis* like bacteria in American lobster with economic losses exceeding $2.5 million. Lobsters with limb lobster disease display weakness, lethargy, and slow or ineffectual responses to sensory stimuli. Luminous Vibriosis is also reported by Diggles et al. (2000) in rock lobster reared in an experimental culture facility. In this study possibly the first of its kind in the Persian Gulf, four *Vibrio* species identified in 60 studied lobsters. *Vibrio alginolyticus*, *V. vulnificus, V. harveyi, V. mimicus* were collected from the lobsters with no clinical sign. According to the results 10 % of lobster samples contained at least one species of *Vibrio*. Two samples contained two *Vibrio* species. *V. alginolyticus* has been reported to be the most common species in Europe and North America (Di Pinto et al., 2005). In the present study, we also determined *V. alginolyticus* with the frequency of 4/60 among the *Vibrio* isolates identified. According to the results, 4 of 6 infected samples (66.6 %) contained *V. alginolyticus*.

Shrimp is one of the most important fishery products of Persian Gulf coastal provinces of Iran. Whilst shrimp farming is an important economy characteristic of these provinces, a large portion of the products export to other countries especially European Union countries (Hosseini et al., 2004). Development of shrimp culture industry has been accompanied with development of diseases such as Vibriosis. Vibriosis has been an important cause of production loss due to bacterial disease in shrimp farms in south Iran in recent years which supports the hypothesis of transferring the disease from farmed shrimp to wild population of lobster in the Persian Gulf (Ansari and Raissy, 2010).

Gomez-Gil et al. (1996) found the hepatopancreas of apparently healthy *Penaeus vannamei* contained several *Vibrio* species, including *V. alginolyticus*, *V. damsela* and other *Vibrio* spp., But it seems that bacteria are not commonly found in the Hepatopancreas or hemolymph because they are prevented from entering by the gastric sieve which excludes particles larger than 0.1 mm (Hopkin and Nott, 1980). It has been suggested that the sieve may combine with the digestive enzymes to prevent bacteria gaining access to or colonizing the hepatopancreas or hemolymph and therefore the presence of bacteria in internal organs may represent a failure of these mechanisms. However, it is possible for bacteria to enter the hepatopancreas by other routes. There were fewer bacteria and a wider range of distinct isolates recovered from the hepatopancreas compared to the stomach and intestine, however, from these data it is not possible to conclude that the bacterial population in these portions of the digestive tract was significantly different.

The findings presented here suggest that the presence of bacteria in the hepatopancreas is not necessarily indicative of disease and diagnosticians should expect to find a wide range of *Vibrio* spp. isolates in the hepatopancreas or hemolymph of healthy animal. Some authors believe that the presence of bacteria in the haemolymph is indicative of septicemia (Lightner, 1977) and stress (Lightner, 1988). Other authors have recovered bacteria from the haemolymph of apparently healthy shrimp. *Vibrio* spp., *Pseudomonas* spp. and *Aeromonas* spp. have been isolated from the haemolymph of apparently healthy crustaceans such as *H. americanus* (Cornick and Steward, 1966), *C. sapidus* (Haskell et al., 1975), *P. clarkii* (Scott and Thune, 1986) and *Machrobrachium rosenbergii* (Brady and Lasso-de la Vega, 1992).

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