Occurrence of *Vibrio* spp., *Aeromonas hydrophila, Escherichia coli* and *Campylobacter* spp. in crayfish (*Astacus leptodactylus*) from Iran

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Received: September 2013 Accepted: June 2014

Abstract

The aim of this research was to study the occurrence of *Vibrio* spp., *Aeromonas hydrophila*, *Escherichia coli* and *Campylobacter* spp. in crayfish from Azerbaijan Province using culture method and PCR assay. A total of 55 isolates were collected from 97 studied samples. *Vibrio* spp., *A. hydrophila*, *E. coli* and *Campylobacter* spp. were detected in 26 samples (26.8%), 12 samples (12.3%), 15 samples (15.46%) and 2 samples (2.06%), respectively. Among *Vibrio* isolates, *Vibrio vulnificus* (11.3%) was the species most frequently detected followed by *V. harveyi* (7.2%), *V. alginolyticus* (2.06%) and *V. mimicus* (1.03%). The results of this study indicated that crayfish from the studied area contain pathogens relevant to public health.

Keywords: Crayfish, *Vibrio*, *Aeromonas*, *E. coli*, *Campylobacter*.

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Introduction

Raw fish and shellfish can, actively or passively, contain pathogenic bacteria which may be transmitted to human. *Vibrio*, *Aeromonas*, *E. coli* and *Campylobacter* are the pathogenic bacteria which may be found in aquatic animals (Dao and Yen, 2006; Khamesipour *et al*., 2013).

Vibrio and *Aeromonas*, as members of vibrionaceae family, both are native to aquatic environments and have been described as emerging foodborne pathogen for human. The vibrios are gram-negative rod-shaped bacteria that are fermentative, catalase and oxidase positive, halophilic, motile by polar flagella, are usually sensitive to the vibriostatic agent, O/129, and mostly have a requirement for sodium chloride (Farmer *et al*., 2005). These species are opportunistic pathogens with wide distribution in aquatic environments, causing infections to commercially important species of cultured and wild fish, shellfish and even human mostly by the way of sea food poisoning. From the public health point of view, *Vibrio* infections in fish and crayfish can lead to gastroenteritis in humans through ingestion of raw or undercooked crayfish (Eaves and Ketterer, 1994; Bean *et al*., 1998; Rahimi*et al*., 2012; Raissy *et al*., 2012a; Khamesipour 2014b).

A. hydrophila is oxidase positive, facultative anaerobic, gram-negative bacteria and is reported from aquatic environments as well as sea food (Hanninen *et al*, 1997). *A. hydrophila* is described as foodborne pathogen causing gastroenteritis. The bacteria have been isolated from freshwater fish, shrimp, oyster, freshwater prawn and crayfish (Haruo *et al*., 1994; Sung *et al*., 2000; Evangelista-Barreto*et al*., 2006; Khamesipour *et al*., 2014a). It is also isolated from apparently healthy crayfish, but is considered to have the potential to cause problems under culture conditions (Quaglio *et al*., 2006). *A. hydrophila* is found to be highly pathogenic to freshwater crayfish, *Pacifastacus leniusculus*, with 100% mortality following experimental exposure (Jiravanichpaisal *et al*., 2009). This species is also reported as a part of micro flora in wild freshwater crayfish (Khalil *et al*., 2010).

Crayfish appears to be a passive carrier of *E. coli* and *Campylobacter* spp. with no clinical sign. The contamination of these organisms derives from terrestrial sources and crayfish may serve as a vector for these species. Consumption of anchovy has been reported as cause of some secondary pathogens such as *Salmonella* (Minette, 1986). This species is also isolated from fish and water in Egypt (Lotfy *et al*., 2011).

A. leptodactylus naturally inhabits in some inland water bodied of Iran such as Aras reservoir (Abassi, 1969). In recent years, this species has been restocked in some freshwater systems in Iran to establish new populations. *A. leptodactylus* was introduced from Aras reservoir to 34 new water bodies of 13

Provinces between 2000-2005 and currently inhabit in different areas of Iran.

The aim of this research was to study on occurrence of some bacteria including *Vibrio* spp., *A. hydrophila*, *E. coli* and *Campylobacter* spp. in crayfish (*A. leptodactylus*) from Aras reservoir.

Materials and methods

Sample collection and preparation

A total number of 97 crayfish (*A. leptodactylus*) were collected from Aras Dam Lake between November to December 2012. The sampling area is located between 231°20 and 231°25 N latitudes and between 225°25 and 225°50 E longitudes, near Aras town in Qare-Ziaoddin region in west northern border of Iran. The samples were transferred into cool boxes with an internal temperature of +2 to +4 °C and were processed immediately upon arrival to the laboratory using aseptic techniques.

Bacteriological Analysis

Of each meat sample, 25 g was homogenized and transferred to 225 ml of alkaline peptone water (APW). After incubation at 37 °C for 24 h, The samples (0.1 ml) were subcultivated on Thiosulfate Citrate Bile Salts Sucrose agar (TCBS, BD Diagnostics, Heidelberg, Germany) and on Starch Ampicillin Agar (Himedia Laboratories, Mumbai, India) for isolation of *Vibrio* species *A. hydrophila*, respectively (Bockemühl, 1992) and were incubated at 37 °C for 24 h. The isolates were then identified using biochemical tests described by Austin and Austin (1999) and Hosseini *et al*. (2004) including Gram staining, oxidase, catalase tests, acid production from glucose, lactose, mannose, mannitol and arabinose, dehydration of arginine, lysine and ornithine, growth in nutrient broth with 8 and 10% NaCl and nitrate reduction.

For identification of *Campylobacter*, the homogenized flesh samples (25 g) were transferred to Preston Enrichment Broth Base containing *Campylobacter* selective supplement IV (Himedia Laboratories, Mumbai, India) and 5% (v/v) defibrinated sheep blood (225 ml). After inoculation at 42 °C for 24 h in a microaerophilic condition (85% N_2, 10% CO_2, 5% O_2), 0.1 ml of the enrichment was then streaked onto *Campylobacter* Selective Agar Base (Himedia Laboratories, Mumbai, India) with an antibiotic supplement for the selective isolation of *Campylobacter* species(Himedia Laboratories, Mumbai, India) and 5% (v/v) defibrinated sheep blood and was incubated at 42 °C for 48 h under the same condition. One presumptive *Campylobacter* species was performed using standard microbiological and biochemical procedures (Rahimi and Ameri, 2011). For isolation of *E. coli*, twenty-five g of each sample were homogenized in 225 ml tryptone soya broth supplemented with novobiocin (20 mg/L) and incubated at 37 °C for 18-24 h. Then the enrichment samples were streak onto levine eosin methylene blue agar and sorbitol McConkey agar plates supplemented with cefixime (0.5 mg/L)

and potassium tellurite (2.5 mg/L) and incubated as above. Suspected colonies were confirmed by TSI agar and IMViC tests (Stampi *et al*., 2004).

DNA Extraction and PCR

The genomic DNA was prepared using phenol-chloroform DNA extraction method (Ausubel *et al*., 1987). The quality and quantity of genomic DNA in each sample were evaluated by measuring optical densities at 260 and 280 nm. The DNA concentration of each sample was adjusted to 50 ng/µl for PCR. The PCR operation for identification of *V. mimicus*, *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus* was done by using multiplex-PCR and the remaining species were separately identified by PCR.

Two sets of oligonucleotide primers were used for species-specific identification of each species. The primer sequences, targeting genes and amplicon sizes are listed in Table 1. 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 PTC-100 thermal cycler (Eppendorf, Hamburg, Germany) with appropriate thermal cycling. Amplified products were separated by electrophoresis in ethidium bromide stained 1.5% agarose gels at 90 V for 50 min. The gels were visualized and photographed with a UV transilluminator.

Table 1: primer sequences, targeting genes and amplicon size of primers.

Target species	Sequence (5'------ 3')	Amplicon Size (bp)	Targeting Gene	PCR program	Reference
V. parahaemolyticus	GCAGCTGATCAAAACGTT GAGT ATTATCGATCGTGCCACTCAC	897	*flaE*	a	Tarr *et al*., 2007
V. cholerae	AAGACCTCAACTGGCGGTA GAAGTGTTAGTGATCGCCAGAGT	248	*sodB*	a	Tarr *et al*., 2007
V. vulnificus	GTCTTAAAGCGGTTGCTGC CGCTTCAAGTGCTGGTAGAAG	410	*Hsp*	a	Tarr *et al*., 2007
V. mimicus	CATTCGGTTCTTTCGCTGAT GAAGTGTTAGTGATTGCTAGAGAT	121	*sodB*	a	Tarr *et al*., 2007
V. alginolyticus	CGAGTACAGTCACTTGAAAGC C CACAACAGAACTCGCGTTACC	737	*collagenase*	b	Di Pinto *et al*., 2005
V. harveyi	CTTCACGCTTGATGGCTACTG GTCACCCAATGCTACGACCT	235	*Vhh*	c	Maiti *et al*., 2009
A. hydrophila	AGAGTTTGATCATGGCTTACGACTT GGTTACCTTGTTACGACTT	1500	*16S rDNA*	d	Jiravanichpaisal *et al.*, 2009
Campylobacter spp.	ATCTAATGGCTT AACCAT TAA ACGGACGGTAACTAGTTTAGTATT	857	*16SrRNA*	e	Dao and yen, 2006
E. coli	AAAACGGCAAGAAAAAGCAG ACGCGTGGTTAACAGTCTTGCG	147	*uidA*	f	Tsai *et al*., 1993

PCR program: a (Multiplex PCR): 35 times (92°C, 40 s; 57°C, 1 min; 72°C, 1.5 min); b: 35 times (94°C, 30 s; 57°C, 30 s; 72°C, 1 min); c: 30 times (95°C, 1 min; 50°C, 1 min; 72°C, 1 min); d: 35 times (94°C, 1 min; 56°C, 1 min; 72°C, 1 min); e: 30 times (94°C, 1 min; 60°C, 1 min; 74°C, 1 min); f: 40 times (95°C, 1 min; 65°C, 1 min; 74°C, 1 min).

Results

Products of 897, 737, 235, 121, 1500, 857 and 147 bp were obtained from PCR amplification of the bacterial isolates including *V. alginolyticus*, *V. harveyi*, *V. vulnificus*, *V.mimicus*, *A. hydrophila*, *Campylobacter* spp. and *E. coli*, respectively (Figs. 1-6).

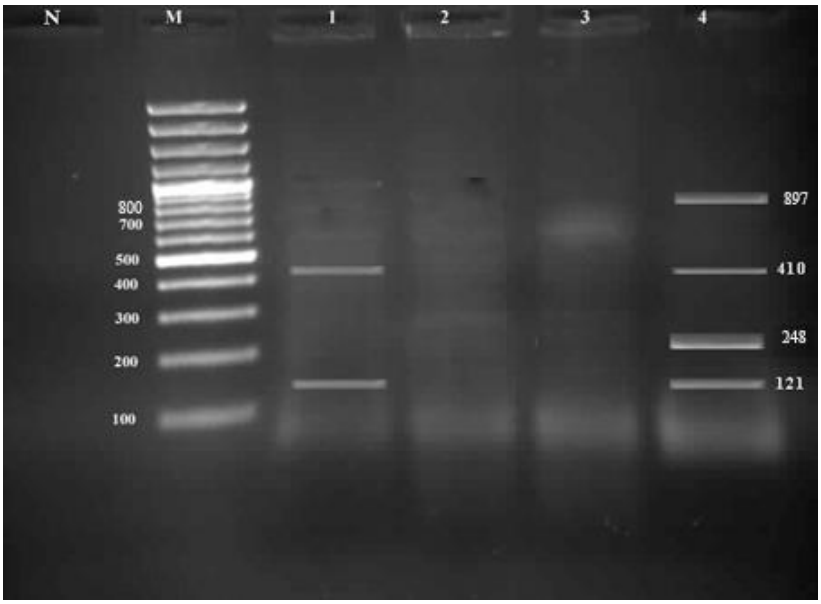

Figure 1:Ethidium bromide-stained agarose gel of Multiplex PCR for detection of *Vibrio* spp. (PCR products of *V. mimicus*: 121 bp, *V. cholerae*: 248 bp, *V. vulnificus*: 410 bp, *V. parahaemolyticus*: 897 bp). Lane N:negative sample; Lane M:100bp DNA ladder (Fermentas, Germany); Lanes 1: positive sample; Lanes 2, 3: negative samples; Lane 4: positive control.

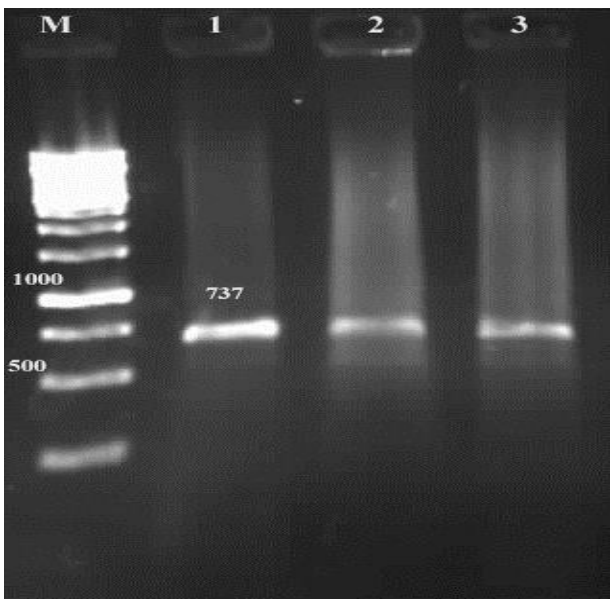

Figure 2: Ethidium bromide-stained agarose gel for the detection of *V. alginolyticus* (737 bp). Lane M: DNA ladder (Fermentas, Germany); Lane 1: Positive ontrol; Lanes 2, 3: Positive samples.

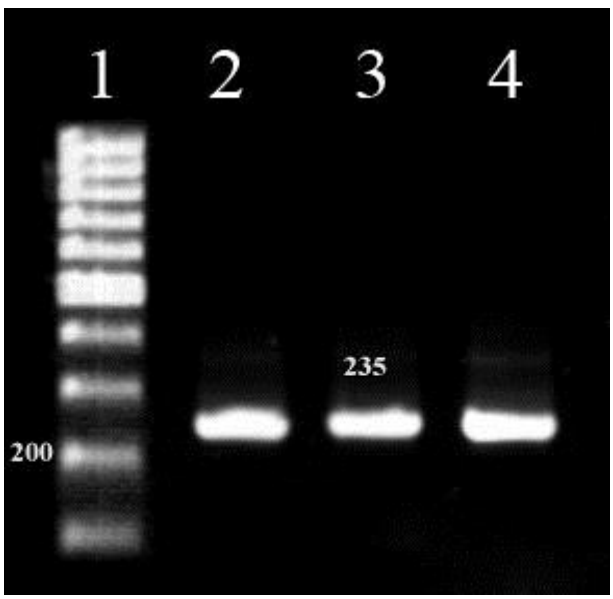

Figure 3: Ethidium bromide-stained agarose gel for the detection of *V. harveyi* (235 bp). Lane 1: DNA ladder (Fermentas, Germany); Lane 2: Positive control; Lanes 3, 4: Positive samples.

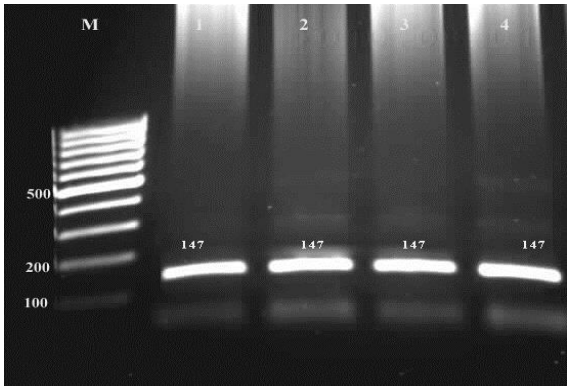

Figure 4: Ethidium bromide-stained agarose gel (147 bp) for the detectionof *E. coli*. Lane M: 100 bp DNA ladder (Fermentas, Germany); lanes 1: Positive control; lane 2-4: positive samples.

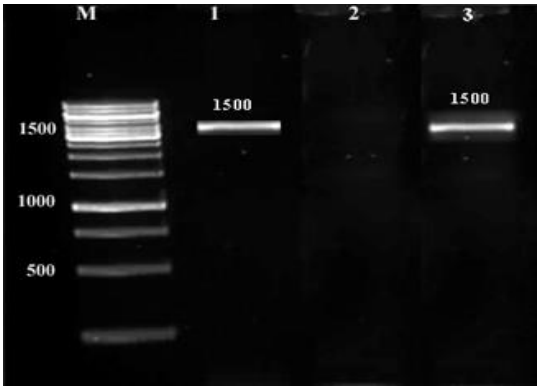

Figure 5: Ethidium bromide-stained agarose gel (1500 bp) for the detection of *A. hydrophila*. Lane M: DNA ladder (Fermentas, Germany); lane 1: positive control; lane 2: negative control. Lane 3: positive sample.

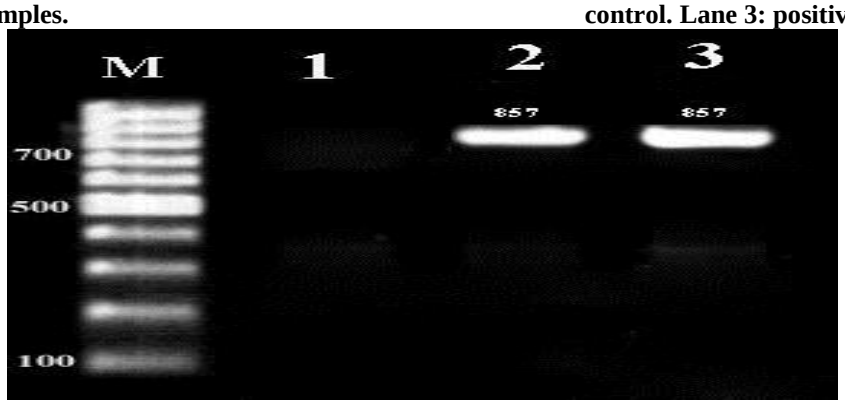

Figure 6: Ethidium bromide-stained agarose gel for the detection of *Campylobacter* spp. (857 bp). Lane M: 100 bp DNA ladder (Fermentas, Germany); lane 1: negative control; lane 2: positive control. Lane 3: positive sample.

The results indicated that 55 samples (56.70%) contained at least one of the studied bacteria including *V. vulnificus* (11 isolates, 11.3% of the studied samples), *V. harveyi* (7 isolates, 7.2%), *V. alginolyticus* (2 isolates, 2.06%), *V. mimicus* (1 isolate, 1.03%), *A.hydrophila* (26 isolates, 26.8%), *E. coli* (15 isolates, 15.46%) and *Campylobacter* spp. (2 isolates, 2.06%). None of the studied samples contained *V. parahaemolyticus* and *V. cholerae*.

Discussion

The first study on crayfish in Iran was carried out by Abassi (1969), who studied the length frequency of the narrow-clawed crayfish population in Anzali Lagoon (Farmer *et al*., 2005). In 1987, it was stated that there were two species of *Astacus* in Iran. *Astacus leptodactylus* lives in the Anzali Lagoon and *A. leptodactylus eichwaldi* (*A. pachypus*) lives in the Caspian Sea (Matinfar, 2007).

In recent years, some incentive policies have been applied about culturing crayfish as a growing industry. Crayfish has also been introduced to some inland water bodies such as Aras Dam Lake in order to increase the population.

Presence of *Vibrio* in aquatic animals such as fish (Schmidt *et al*, 2000; Messelhausser *et al*., 2010), shrimp (Dalsgaard *et al*., 1996; Reboucas *et al*., 2011), mussel (Lhafi and Kühne, 2007) has been mentioned, although bacterial contamination of crayfish is less studied.

In the present study, four *Vibrio* species including *V. alginolyticus*, *V. vulnificus*, *V. harveyi*, and *V. mimicus* were collected from the examined samples which is in agreement with the results of previous studies in different countries (Jakši *et al*., 2002, Hosseini *et al*., 2004, Lhafi and Kühne, 2007, Ansari and Raissy, 2010; Raissy *et al*., 2012b). Raissy *et al* (2012b) studied 132 lobster and crab samples for *Vibrio* spp. using both biochemical tests and PCR. According to their results, 25% (33 samples) including 29 lobsters (29%) and 4 crabs (12.5%) contained one or more *Vibrio* species (Raissy *et al*., 2012b).

V. vulnificus, the most frequent species in this study, cause gastro-enteritis, and is known to be responsible for primary and secondary infections in human (Feldhusen, 2000). This species was detected in 11.3% of the samples in the present study. *V. alginolyticus* is reported to be the most common species in fish and shell fish in Europe and North America (Di Pinto *et al*., 2005). In the present study, *V. alginolyticus* was found with the frequency of 2/97 (2.06%) among the *Vibrio* isolates identified. *V. mimicus* which was found in 1.03 % of the studied sampleshas been isolated previously from crayfish particularly under culture conditions (Eaves and Ketterer, 1994; Wong *et al*., 1995).

A. hydrophila is considered as a pathogen of emerging importance due to its' special characteristics such as presence in the aquatic environment and multiplicity of virulence factors. Thesebacteriaare also reported from fish and shellfish from different areas (Hanninen *et al*., 1997; Evangelista-Barreto *et al*., 2006). In this study, this species was identified in 12/97 (12.3%) samples.

Although *Campylobacter* spp. and *E. coli* do not originate efficiency from natural aquatic systems, they are reported from aquatic animals in previous studies. *E. coli* is reported from unprocessed fish from Vietnam (Dao and Yen, 2006). *Campylobacter* spp. is reported from the aquatic environment of marine mammals and from shellfish (Wilson and Moore 1996). Low incidence of *Campylobacter* spp. (2.3%) is also reported in fish products in Finland (Lyhs *et al*., 1998). In this study, 15 (15.46%) and 2 (2.06%) of the studied samples were found to contain *E. coli* and *Campylobacter* spp., respectively.

The results of the present study revealed that crayfish from the studied area is contaminated with *Vibrio* spp., *A. hydrophila*, *E. coli* and *Campylobacter* spp. Although the source of the bacteria is mostly from aquatic environment, secondary contamination during catching, handling and transportation may also contribute to their distribution. Since

water can also be contaminated with these species (Burke *et al*., 1984), it is likely that contaminated water or ice may have contributed to the high incidence of the bacteria. The significance for public health is dependent on the health status of the consumer, concentration and pathogenicity of the pathogen as well as on the nutritional habits.

Acknowledgments

The author thanks Mr. Manouchehr Moumeni, Fisheries Research Center, of Islamic Azad University, Shahrekord Branch for the sincere help in performing technical parts of the project and to deputy of Research, Islamic Azad University, Shahrekord Branch for supporting the project.

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