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Detection of *Lactococcus garvieae* and *Vibrio cholerae* in some aquatic fishes of Persian Gulf using polymerase chain reaction

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ABSTRACT

Lactococcus garvieae (L. garvieae) and Vibrio cholerae (V. cholerae) causes emerging zoonotic disease in all over the world. In this research, we describe the lactococcosis and Vibrio cholerae in some aquatic fishes of Persian Gulf using Polymerase Chain Reaction (PCR) method during November and December 2013 in Iran. L. garvieae infections were detected in 0.00%, 0.00%, 10%, 10%,10%, 10%, 20% and 20% of the sampled Stromateidae (n=20), Pleuronectiformes (n=20), Scomberomorus commerson (n=20), Oyster (Pinctada Pearl) (n=20), Shrimp (n=30), Sawfishes (Pristis zijsron) (n=20), and Crab (Brachyura) (n=20) respectively. Also, V. cholerae infections were detected in 0.00%, 0.00%, 0.00%, 10%,13%, 0.00% and 30% of the sampled Stromateidae (n=20), Pleuronectiformes (n=20), Scomberomorus commerson (n=20), Oyster (Pinctada Pearl) (n=20), Shrimp (n=30), Sawfishes (Pristis zijsron) (n=20), and Crab (Brachyura) (n=20) respectively. In the present study, we succeeded in detecting and identifying two major species of lactococcosis and vibriosis disease by PCR technique in some aquatic fishes of Persian Gulf of Iran. Further attempts for molecular identification of these strains and introduction of new species are however needed.

Keywords: *Lactococcus garvieae*, *Vibrio cholerae*, aquatic fishes, Persian Gulf, PCR

INTRODUCTION

Lactococcus garvieae, the etiological agent of lactococcosis, causes emerging zoonotic disease and significant economic losses in freshwater culture of salmonid fish and marine culture species all over the world, especially when water temperature increases over 15°C [1, 2]. Also, this pathogen which has been isolated from cattle, various kinds of food products, cow's milk, buffalos with mastitis and from human [3, 4].

It was proven that the *L. garvieae* is one of the major Gram-positive coccus pathogens for fish and is a salt tolerant bacterium (6.5% salt), non-motile, ovoid cocci, occurs in pairs and short chains, produces a-haemolytic colonies on blood agar, and is oxidase and catalase negative, non-acid fast and non-sporulating [3-5].

Lactococcosis in cultured fish have been reported in many countries, such as Australia, Balkans, Bulgaria, England, France, Greece, Iran, Italy, Israel, Japan, Korea, Portugal, Spain, South Africa, Taiwan, Turkey and United States [1,

2, 6]. The streptococcal/lactococcal infections usually cause highmorbidity and mortality and last a long period of time in the farmed fish [2].

The isolation of the disease in human showing its zoonotic character is also important. The studies were showed the ability of *L. garvieae* to cause late prosthetic, heart bypass grafting or joint prothesis, infection in patient [2]. *L. garvieae* causes hemorrhagic septicaemia, enteritis, ascites, bilateral exophthalmus with haemorrhage; darkening of the skin; congestion of the intestine, liver, kidney, spleen, and brain; and hemorrhagic enteritis in farmed trout [2]. There is a few published data on the histopathological lesions associated with lactococcosis. The main histopathological findings in infected fish are epicarditis, peritonitis, enteritis, meningitis and panophthalmitis [2].

Researchers have used many techniques for *L. garvieae* identification [4]. The symptomatologies of fish infected by the various gram-positive cocci pathogenic for fish are similar and do not allow a rapid identification of the agent responsible for the disease [3]. In addition, the isolation and the bacteriological diagnosis of these gram-positive bacteria are not simple and are time-consuming [3]. Thereafter, PCR assays have became the most widely used technique in identifying *L. garvieae* in various sources [4]. It is a quick, inexpensive and simple technique [4]. Taxonomic studies based on DNADNA hybridization studies and currently, PCR reactions are exploiting the diversity of sequences of 16S rRNA genes in *L. garvieae* [3]. Molecular diagnostic techniques, such as PCR assays, are increasingly used to detect and identify many different bacterial pathogens including the most significant fish pathogens [7- 9].

Vibrio cholerae is important water borne facultative human pathogen of worldwide gastrointestinal disease significance. Cholera is a life threatening diarrhoeal disease, still kills thousands annually and remains one of the few bacterial diseases known for its pandemicity [9, 10].

Cholera has been recognized as one of the emerging and re-emerging infections in developing countries and is associated with plankton and other aquatic organisms [10]. Sea foods including molluscan shellfish, crustaceans and finfish are most often incriminated in food borne cholera cases in many countries [10]. Therefore ingestion of raw or undercooked seafood such as shrimp and drinking water contaminated with *V.cholerae* are risk factors in humans [9].

The conventional isolation procedures includes growth in enrichment broth (Alkaline Peptone Water) followed by plating on selective media i.e., Thiosulfate Citrate Bile salt Sucrose Agar (TCBS). The process, however, is laborious and time consuming. Further, close relatedness among *V.cholerae* and certain other members of the *Vibrio* spp (e.g. *V.mimicus*) or *Aeromonas* spp. with respect to their biochemical properties has often made unambiguous identification of the organism quite difficult [10]. The PCR represents a rapid procedure with both high sensitivity and specificity for the immediate detection and identification of specific pathogenic bacteria from different food materials [10].

In this article, we describe the lactococcosis and *Vibrio cholerae* in some aquatic fishes of Persian Gulf using PCR method During November and December 2013 in Iran.

MATERIALS AND METHODS

Sampling

During November and December 2013, sampling from 20 *Scomberomorus commerson*, 20 *Stromateidae*, 20 *Pleuronectiformes*, 20 *Sawfishes* (*Pristis zijsron*), 20 oyster (*Pinctada Pearl*), 20 Crab (*Brachyura*) and 30 shrimp in Persian Gulf (south of Iran) was done. Were randomly sampled and then from some organs such as liver, hepatopancreas, intestine and meat. Samples were aseptically obtained from organs such as liver, hepatopancreas, intestine, and meat using sterile swabs and transferred to the laboratory for analysis.

Isolation of Lactococcus garvieae and biochemical analysis

Sterile swabs were streaked on TSA plate (Tryptic-soy-agar, Difco, Mi, USA) and plates were transferred to the Laboratory of Islamic Azad University of Shahrekord beside the ice. Plates transferred to the laboratory were incubated at 25°C for 48 h for growing the colonies. Single colonies from plates with pure culture growth were re-streaked on the blood agar media *(*Merck, Germany*)* to obtain pure isolates. In each of the grown colonies catalase, oxidase and gram staining tests were done and Gram-negative, catalase positive and oxidase negative bacilli were

cultured in Waltman –Shatts medium, after 48h incubation at 25°C pure growth colonies used for PCR test. In each step of PCR testing, distilled water as negative control is used. DNA extracting for isolation, pure colonies were put in tubes beside 100 microliter distilled water. DNA was extracted according to kit of extracting DNA (CinaGen Co, Tehran, Iran). The extracted DNA was quantified via spectrophotometric measurement at 260 nm optical density according to the method described by Sambrook and Russell (2001) [11]. Extracted DNA of each sample was kept frozen at -20°C until used, and then delivered to Biotechnology Research Center of Islamic Azad University of Shahrekord**.**

Primers of Lactococcus garvieae and PCR amplification test for Lactococcus garvieae

The oligonucleotide primers with 16S rRNA targeting gene (PLGF, 5'CATAACAATGAGAATCGC3' and PLGR, 5' GCACCCTCGCGGGTTG 3') that specifically amplified 1100 bp fragments were used for PCR amplification [12]. All primers were synthesized by CinnaGen Co. (Tehran, Iran).

The amplification reactions were performed in 50 µl reaction mixtures containing 0.1 mM of each dexoynucteotide, 15 pmol of each primer, 50 mM KCl, 10 mM Tris-Hcl (pH = 9), 2 mM MgCl2, 10% dimethyl sulfoxide (DMSO, Sigma), 1.5 U of Taq DNA polymerase (Roche applied science, Germany) and 40 ng of template DNA. The PCR reaction was carried out in a PCR programmed thermocycler (Eppendrof, Mastercycler 5330, Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany Co.) using the thermal profiles: initial cycle 95°C for 4 min, followed by a further 30 cycles: denaturation at 94°C for 1 min; annealing at 57°C for 1 min, extension by polymerase at 72°C for 1 min, and a final 5-min elongation period at 72°C. The PCR products were detected and their size estimated by electrophoresis of 15 µl of each amplification mixture in 1% agarose gels in 1% Tris-borate- EDTA (TBE buffer) with known molecular weight standards (LifeTechnologies) at 80V for 30 minutes. Gels were stained with 0.5 mg ml–1 Ethidium Bromide and examined under Ultra Violet illumination (Uvitec, UK). A negative control (sterile water) in gel electrophoresis run. The PCR products were identified by 100 bp DNA size marker (Fermentas, Germany).

Isolation of Vibrio cholerae and biochemical analysis

Biochemical analysis for *Vibrio* was done following the method described by Bockemuhl (1992) [13] & Austin (1999) [14]. Briefly, the all samples were added (separately) 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) [15]. After incubation at 37°C for 24 h, the isolates were used for biochemical tests including Gram staining, oxidase and catalase tests, culture in Sulfide-Indole-Motility (SIM) and Triple sugar iron agar (TSI) media and other biochemical tests described by Hosseini et al (2004) [16].

DNA extraction and PCR primers of Vibrio cholerae

The exact identification of bacteria was done by polymerase chain reaction (PCR). Genomic DNA was prepared using a standard DNA extraction method [17] 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. Oligonucleotide primers were used for identification of *Vibrio cholerae* (sodB F, 5' AAGACCTCAACTGGCGGTA 3' and sodB R, 5' GAAGTGTTAGTGATCGCCAGAGT 3') that specifically amplified 248 bp fragments were used for PCR amplification [18]. The PCR reaction was performed in a 50 µL reaction system consisting of 2 µL of purified genomic DNA, 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 cholerae* [18].

Analysis of PCR products for Vibrio cholerae

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

Data were analyzed using Statistical Package for Social Sciences version 16 (SPSS 16.0 statistical software).

RESULTS

L. garvieae infections were detected in 0.00%, 0.00%, 10%, 10%,10%, 10%, 20% and 20% of the sampled *Stromateidae* (n=20), *Pleuronectiformes* (n=20), *Scomberomorus commerson* (n=20), Oyster *(Pinctada Pearl)* (n=20), Shrimp (n=30), Sawfishes *(Pristis zijsron)* (n=20), and Crab *(Brachyura)* (n=20) respectively. Also, *V. cholerae* infections were detected in 0.00%, 0.00%, 0.00%, 10%, 13%, 0.00% and 30% of the sampled *Stromateidae* (n=20), *Pleuronectiformes* (n=20), *Scomberomorus commerson* (n=20), Oyster *(Pinctada Pearl)* (n=20), Shrimp (n=30), Sawfishes *(Pristis zijsron)* (n=20), and Crab *(Brachyura)* (n=20) respectively.

The results indicate that showed in Table 1 to Table 3. Standard biochemical tests were also performed for identification of the isolates according to Austin and Austin (1999). Identification of the strains of both bacteria was done by PCR. After PCR, the 1100 and 248 bp bands were blasted with other sequences associated with *L. garvieae* and *V. cholerae* in the gene bank (NCBI, Gen Bank).

Table (1): Prevalence of *Lactococcus garvieae* in each samples using PCR

Sample	Number positive/ Total sample	Number negative/ Total sample	Prevalence (%)
Scomberomorus commerson	2/20	18/20	10%
Stromateidae	0/20	20/20	0%
Pleuronectiformes	0/20	20/20	0%
Sawfishes *(Pristis zijsron)*	4/20	16/20	20%
Oyster *(Pinctada Pearl)*	2/20	18/20	10%
Crab *(Brachyura)*	4/20	16/20	20%
Shrimp	3/30	27/30	10%
Total	15/150	135/150	10%

Table (2): Prevalence of *Vibrio cholerae* in each samples using PCR

Sample	Number positive	Number negative	Prevalence (%)
Scomberomorus commerson	0/20	20/20	0%
Stromateidae	0/20	20/20	0%
Pleuronectiformes	0/20	20/20	0%
Sawfishes *(Pristis zijsron)*	0/20	20/20	0%
Oyster *(Pinctada Pearl)*	2/20	18/20	10%
Crab *(Brachyura)*	6/20	14/20	30%
Shrimp	4/30	26/30	13%
Total	12/150	138/150	8%

Table (3): Prevalence of *both bacteria* in each samples using PCR

Sample	Number sampled	Number positive	Prevalence (%)
Scomberomorus commerson	20	0	0%
Stromateidae	20	0	0%
Pleuronectiformes	20	0	0%
Sawfishes *(Pristis zijsron)*	20	0	0%
Oyster *(Pinctada Pearl)*	20	2	10%
Crab *(Brachyura)*	20	3	15%
Shrimp	30	2	7%
Total	150	7	5%

DISCUSSION

Results of this study showed that this PCR procedure has high potential as a rapid screening test for the definitive detection of *L. garvieae* and *V. cholerae* strains in Iran. The PCR method can be employed as a supplementary and complementary test for definitive identification of the bacteria cultured from clinically suspected samples. In future studies, this PCR method can be used as a direct test for the detection of *L. garvieae* and *V. cholerae* in samples of infected fishes.

In past times the epizootic outbreak of lactococcosis caused by *L. garvieae* in farmed rainbow trout in Iran has been reported by Soltani *et al*. (2005), however a sequence comparison between isolates has not been undertaken [19].

Sharifiyazdi et al., (2010), reported a 16% *L. garvieae* from 32 fish of moribund rainbow trout in Iran (1). The results of study by Fadaeifard et al., (2012), showed overcoming *L. garvieae* in isolated samples that indicated epidemiological importance of lactococcosis and the results reveal that 12 samples from 100 total sample were *L. garvieae* (6). Hussein and Hatai (2006) have emphasized on importance of *L. garvieae* as a serious pathogen in aquaculture industry and its impact on the production rate [20].

Molecular diagnostic techniques such as PCR assays are increasingly used to detect and identify important bacterial fish pathogens [7- 9]. Aoki et al. (2000) used a dihydropteroate synthase gene as a target for species-specific PCR of *L. garvieae* [21].

Study by Maheshwari et al., (2011) reported a total of 245 samples (35 each of water, fish, crab, shrimp, meat, milk and clinical stool samples) collected from various sources were subjected to PCR and cultural methods for the presence of *Vibrio cholerae*. Eighty samples (19 water,16 fish, 20 crab, 6 shrimp, 4 meat, 3 milk, and 12 clinical stool samples) were positive by PCR targeting ompW gene, whereas only 59 samples (12 water, 13 fish, 16 crab, 5 shrimp, 3 meat, 2 milk and 8 clinical stool samples) were positive by cultural methods [10].

According to the aim of this study, we isolated the cocci from bacteria earlier approved through classical bacteriology and biochemistry tests and finally confirmed by using the PCR method. These bacteria both in the mediums and some aquatic fishes samples were detected and identified. Simultaneous detection of the two mentioned bacteria in the some aquatic fishes indicated the involvement of important agent that lead to lactococcosis and vibriosis in fishes. In order to identify the two important causative pathogens: *V. cholera* and *L. garvieae*, so that four pairs of specific oligonucleotide primers were used for each of these pathogens.

CONCLUSION

In the present study, we succeeded in detecting and identifying two major species of lactococcosis and vibriosis disease by PCR technique in some aquatic fishes of Persian Gulf of Iran. Further attempts for molecular identification of these strains and introduction of new species are however needed.

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