Detection of *Vibrio* Species Isolated from Ornamental Guppy Fish in Kashan, Isfahan, Iran Fish culturing Pounds

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**Abstract**

**Introduction:** Gram-negative bacteria are the most pathogenic bacteria for marine organisms including ornamental fish.

**Materials and methods:** In the present study *Vibrio* species isolated from ornamental guppy fish in Kashan, Isfahan, Iran fish ponds and were detected according to molecular detection and genetic alignment. Liver, kidney, skin, brain and gill samples were taken from ornamental guppy fish in Kashan, Isfahan, Iran. Samples were cultured on enriched culture media and purification steps were performed based on microbiological methods. Primary identification was done using biochemical characterization of the isolated bacteria. Molecular detection was done based on amplification of 16SrDNA sequence of *Vibrio cholera* genome containing ITS (internal transcribed spacer); and sequence alignment of the amplified nucleotides.

**Results:** The isolated bacteria detected as *Vibrio* spp., including *Vibrio cholera* (99% sequence similarity), *Vibrio alginolyticus*, *Vibrio mimicus* and *Vibrio parahaemolyticus* (up to 90% similarity in the genome sequence). The aquaculture ponds had alkaline water and the amount of five-day BOD was not in a safe range, which are favorable conditions for *Vibrio* species.

**Discussion and conclusion:** Aquatic organisms in Iran can be carriers of human pathogens such as *Vibrio* species. The results obtained in the present study and similar investigations should be mentioned in aquaculture healthcare systems.

**Key words:** *Vibrio* spp., Ornamental guppy fish, Genetic alignment, Water quality
Introduction

More than twenty thousand species of freshwater ornamental fish exist in the world. Guppy fish (*Poecilia reticulata*) is one of the most familiar ornamental fish in many countries, including Iran. The length of the fish is up to 7 cm. The fish swims in mid water. The guppy fish can coexist with all other fish. Examples of guppy fish are snakeskin, red tail, yellow tail, singaporean, blue tail, rabbit tail and glass-belly (1).

All fish are in the exposure of infections caused by different kinds of bacteria, fungi and viruses (2), however bacteria particularly Gram-negative species, are the most common agents of infectious diseases in ornamental fish (3). Environmental factors such as stress, poor quality of water, sewage pollution, unhygienic handling of fish and polluted aquaculture feeding can result in increasing of susceptibility to infections among fish (4 & 5). However, immune system deficiency of fish enhances the morbidity of infection (6).

*Streptococcus*, *Mycobacterium*, *Aeromonas*, *Pseudomonas*, *Edwardsiella*, *Yersinia* and *Vibrio* are the most infectious bacterial genera in fish (2). *Vibrio* spp. can cause septicemia in fish. The disease will be adversely by temperature change or environmental stress. It is estimated that vibrios consist approximately 60% of total heterotrophic bacteria in aquarium and are from opportunistic fish pathogens (7). As *Vibrio* species especially *Vibrio cholera* is from powerful human pathogens and possibly transmitted among aquarists in countries which are outside cholera endemic areas (8), it is necessary to be considered in aquaculture healthcare systems in our country.

During the last decade, biologists have employed different molecular techniques in phylogenetics, evolution, and population diversity. Analysis of 16S (small subunit) rDNA and, more recently, 16S internal transcribed spacer (ITS) has been used greatly in these studies on prokaryotic microorganisms (9).

The aim of the present study was to detect *Vibrio* species isolated from ornamental guppy fish using biochemical and molecular species.

Materials and methods

Samples and isolation of the bacteria: In a period of time from January to September 2012, skin, gill, kidney and brain tissue samples were obtained from symptomatic guppy in five aquaculture ponds in Kashan, Iran. The samples obtained from a total of 40 symptomatic guppy fish. The included symptoms were: ulcer, pop eye, cloudy eye, dropsy, columnaris, mouth rot, tail rot and bloated stomach. The samples inoculated on enriched culture media including brain heart infusion broth, tripticase soy broth and blood agar. The culture media were incubated at 32°C for 24 hours. The isolated bacteria were identified using conventional biochemical analysis (10 & 11).

Due to the effect of water quality on the general health of fish; the amount of dissolved oxygen (DO), acidity (pH) and five-day biochemical oxygen demand (BOD) of the pond water was measured on the sites of sampling.
DNA extraction and sequence amplification: DNA content of media was extracted using Roche Applied Science DNA extraction kit. Pairs of primers were used according to the specific genome sequences of the biochemical detected bacteria. The characteristics of primers and information about amplification by polymerase chain reaction (PCR) are shown in table 1. A mixture consists of 1X PCR buffer, 0.2 mM dNTP mix, 1.5 mM MgCl₂, 0.5 μM of each primer, 1 unit.μl⁻¹ Taq DNA polymerase and 1 μg of the extracted DNA was used for each amplification reaction. The products analyzed by gel electrophoresis using 1% agarose.

Sequence analysis of the amplified fragments: The final products have been sequenced by ABI3730XL system (Bioneer Corporation, Korea) and aligned in BLAST data base. Also the content of amplified fragment was analyzed by Gene runner program version 3.05. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (13).

Results

Water quality parameters: The amount of dissolved oxygen (DO), pH and five-day biochemical oxygen demand (BOD) of the pond water are shown in table 2.

The isolated bacteria: All isolates showed similarity to Vibrio spp., according to morphological and biochemical analysis. Detection of bacteria using biochemical tests is shown in table 3. The amount of 5% of samples (2 fish) was infected to Vibrio spp., according to biochemical tests. Both infected fish were obtained from the same pond.

Molecular detection: As the results of molecular amplification, the 750bp fragment amplicon of Vibrio spp. 16SrDNA were obtained (Fig. 1). Both samples were detected as positive by PCR.

![Fig. 1- The amplified fragment for Vibrio spp. 16SrDNA. The amplicon was 750 bp in length.](image)

Table 3- The morphological and biochemical characteristics of the isolated bacteria, which were similar to Vibrio spp.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Isolate number 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology and Gram staining</td>
<td>Gram- negative bacilli</td>
</tr>
<tr>
<td>Haemolysis on blood agar</td>
<td>Beta</td>
</tr>
<tr>
<td>Indole production</td>
<td>_</td>
</tr>
<tr>
<td>Methyl red reaction</td>
<td>+</td>
</tr>
<tr>
<td>Voges- Proskauer reaction</td>
<td>_</td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>+</td>
</tr>
<tr>
<td>Urease</td>
<td>+</td>
</tr>
<tr>
<td>SH₂ production</td>
<td>+</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>_</td>
</tr>
<tr>
<td>Oxidative/Fermentative</td>
<td>Fermentative</td>
</tr>
<tr>
<td>Glucose fermentation</td>
<td>+</td>
</tr>
<tr>
<td>Xylose fermentation</td>
<td>+</td>
</tr>
<tr>
<td>Lactose fermentation</td>
<td>_</td>
</tr>
<tr>
<td>Sorbitol fermentation</td>
<td>+</td>
</tr>
<tr>
<td>Saccharose fermentation</td>
<td>+</td>
</tr>
<tr>
<td>Mannitol fermentation</td>
<td>+</td>
</tr>
<tr>
<td>Probable identified species</td>
<td>Vibrio Spp.</td>
</tr>
</tbody>
</table>
Table 1: Primer sequences and characteristics of amplification procedures.

<table>
<thead>
<tr>
<th>Isolated bacterium</th>
<th>Primer sequence</th>
<th>Amplification cycle</th>
<th>Amplified fragment</th>
<th>Reference</th>
</tr>
</thead>
</table>
| *Vibrio SPP.*      | CGGTGAAATGCAGTAGGATTTACTAGCGATTCCGAGTTC | Step 1: 94°C, 3 min  
Step 2: 94°C, 1 min; 63°C, 1 min; 72°C, 1 min (35 repeats)  
Step 3: 72°C, 5 min | 16SrDNA (750 bp) | 12 |

Table 2- The average of water quality measures in sampling ponds shows low quality of water for fish culturing.

<table>
<thead>
<tr>
<th>pH (acidity) of water</th>
<th>water Temperature (°C)</th>
<th>Five- days biochemical oxygen demand (BOD)</th>
<th>Water dissolved oxygen (OD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.97</td>
<td>25</td>
<td>89.8</td>
<td>6.98</td>
</tr>
</tbody>
</table>

Table 4- The nucleotide sequence of fragments amplified from *Vibrio* spp.

Sequence based on the amplification with forward primer:
ACAGGAGTGGAGGCGCGCCCTAGCAAGATACGACACTCAGATGCGAAAGCGTGGGGAGCAAACGAGATTAAGATACCCCTGGT
AGTCACCGCCGTAACAGTAGTTCTACATTGCAGTGTTGGGAGCATGGTTTAATGCAGTCA

Sequence based on the amplification with reverse primer:
TAAAATTTTCTATTTTTGCCCTTGGAACTGCGGCTACTGCATGCTACATCGTGGGGAGCAAACGAGATTAAGATACCCCTGGT
AGTCACCGCCGTAACAGTAGTTCTACATTGCAGTGTTGGGAGCATGGTTTAATGCAGTCA

Fig. 2. Phylogenetic tree confirmed the isolate *Vibrio cholera* FJ147 after alignment in MEGA version 4.
**Sequenceing analysis:** Alignment of the amplified regions of both species in BLAST data base detected that the isolated *Vibrio* spp. has the most sequence similarity to *Vibrio cholera* (99%). Also this fragment had partial similarity to *Vibrio alginolyticus*, *Vibrio mimicus* and *Vibrio parahaemolyticus* (up to 90%). The nucleotide sequences of the amplified fragments are shown in table 4. Also the phylogenetic tree which resulted from alignment in MEGA version 4 illustrated in Fig 2. As shown in phylogenetic tree, the sequences belong to *Vibrio cholera* isolate FJ147. The internal transcribed spacer (ITS) sequences detected in the amplicon by Gene Runner version 3.05.

**Discussion and conclusion**

Researches on bacterial diseases of ornamental fish (including guppy fish) in Iran are not well established. Many isolated Gram- negative pathogenic bacteria have been reported all over the world which often causes severe symptoms such as septicemia in aquatic organisms (3). These bacteria can be transmitted and be pathogenic for human being (14). Kashan, is one of the largest ornamental fish culture areas in Iran, so this area was selected for fish sampling in the present study. Because guppy fish is one of the most favorable ornamental fish in Iran and is from the most susceptible fish to infection (3), the samples were obtained from the guppy culturing ponds.

Risks of infections and poisoning are one of the challenges facing ornamental aquaculture industry. Microbial diseases of ornamental fish are divided into two categories of infectious and parasitic diseases. Infectious diseases mainly caused by bacteria, viruses and fungi. The bacterial strains that are associated with fish infection are mostly usual saprophytic species which exist extensively in the aquatic environments. A few of these species are of the real obligate pathogens. These microorganisms exist on the external surface of body or tissue and may be present in healthy- appearing fish. These bacteria almost show their pathogenic features, when the environmental conditions are not suitable for fish or fish exposed to different kinds of stresses (1 & 5). As shown in table 4 Kashan aquaculture ponds had alkaline water. Also the amount of five- day BOD was not in a safe range. These conditions are favorable for *Vibrio* species.

The genus *Vibrio* includes symbionts and commensals that are found inside and on the surface of marine animals. Indeed many species are pathogenic to animals (15). In the present study *Vibrio* species were isolated from guppy ornamental fish. 16SrDNA analysis detected the most similarity to *Vibrio cholera* (99%), although the alignment in other specie showed up to 90% similarities to *Vibrio alginolyticus*, *Vibrio mimicus* and *Vibrio parahaemolyticus*. All species, especially *Vibrio cholera*, the etiologic agent of cholera, have been established as human pathogens (12 & 15).

In a previous report, *Vibrio cholerae* 01 has been detected from aquarium water and fish imported in Thailand and Sri Lanka
This bacterium has been detected as autochthonous microorganisms to various aquatic environments (16).

The present study in Iran as well as the previous reports all over the world shows that aquatic organisms can be carriers of human pathogens.

References


(16) Senderovich Y., Izhaki I., Halpern M. Fish as reservoirs and vectors of *Vibrio cholerae*. *Public Library of Science since One* 2010; 5 (6): e8607.
شناسایی بلاکتری های جنس Vibrio جداسازی شده از ماهی آکواریومی گویی (Poecilia reticulate) در استخرهای پرورش ماهی کاشان، اصفهان، ایران

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شیما کیانی

فیبه سادات نقوی

علیرضا نظری

چکیده

مقدمه: باکتری‌های گرم منفی از مهم‌ترین باکتری‌های بیماری‌زا برای موجودات دریایی از جمله ماهیان زینتی هستند. این در اثر اکثریت گونه‌های Vibrio از ماهی‌های ایران بخصوص ماهیان زینتی گویی. در این مطالعه، سه گونه باکتری‌های مولکولی و توالی‌های ITS (Internal Transcribed Spacer) 16SrDNA و توالی‌های حاوی genotyp Vibrio cholerae به اساس تکثیر توالی گرفت. اعضای از این گونه‌ها تاکنون مصنوع به‌کار گرفته شدند.

مواد و روش‌ها: در این پژوهش گونه‌های Vibrio از ماهی‌های گویی در استخرهای پرورش ماهی کاشان، اصفهان، ایران جداسازی و بر اساس روش‌های تشخیص مولکولی و توالی‌های ITS (Internal Transcribed Spacer) شناسایی شدند. نمونه‌های کبد، کلیه، پوست، و آبش از ماهی‌های گویی از ماهی‌های گویی در منطقه کاشان، اصفهان، ایران در مراحل سه‌رواب، به‌کار گرفتند. با استفاده از تکثیر از سلول‌های مولکولی و توالی‌های ITS 16SrDNA نکات‌های تکثیر گرفت. نتایج این پژوهش نشان داد که گونه‌های Vibrio cholerae شامل 99 درصد شاید در استخرهای پرورش ماهی کاشان، اصفهان، ایران می‌توانند ویژگی‌های حساسیت و مقاومت‌های متعددی را داشته باشند. این یک توصیفی از تنوع گونه‌های Vibrio cholerae از این بخش است. 

بحث و نتیجه‌گیری: موارد آزمایش‌های پیشگیری در ایران می‌توانند ناقل عوامل باشند و انسان‌های مبتلا به‌کار گرفته شود. مورد نویج گوینده شدها و ابزار‌های کلیدی: گونه‌های ویبریو، ماهی زینتی گویی، توالی‌های ITS، یک بی‌گم‌کر گردید. 

نویسندگان مسئول مکاتبات

* نویسندگان مسئول مکاتبات

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