Biological Journal of Microorganism
4th Year, Vol. 4, No. 16, Winter 2016
Received: September 8, 2014/ **Accepted:** August 5, 2015. **Page:** 43- 48

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

Samira Kiani
M.Sc. of Microbiology, Department of Microbiology, Falavarjan Branch, Isalmic Azad Unversity, Isfahan, Iran, samira.kiani87@yahoo.com
Nafiseh Sadat Naghavi *
Assistant professor of Microbiology, Department of Microbiology, Falavarjan Branch, Isalmic Azad Unversity, Isfahan, Iran, naghavi@iaufala.ac.ir
Alireza Nazari
Assistant professor of Biotechnology, Department of Biotechnology, Falavarjan Branch, Islamic Azad University, Isfahan, Iran, nazari_a@iaufala.ac.ir

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

*Corresponding Author

Introduction

More than twenty thousand species of freshwater ornamental fish exist in the world. Guppy fish (*Poecillia reticule*) 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 adversed 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 V*ibrio* species especially V*ibrio 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 $MgCl_2$, 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.

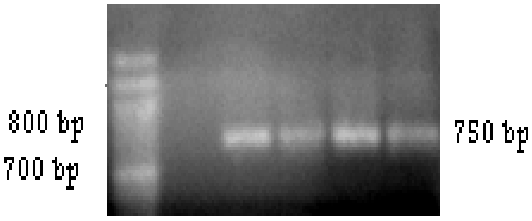

Fig. 1- The amplified fragment for *Vibrio* spp. *16SrDNA*. The amplicon was 750 bp in length.

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

Characteristics	Isolate number 1
Morphology and Gram staining	Gram- negative bacilli
Haemolysis on blood agar	Beta
Indole production	_
Methyl red reaction	+
Voges- Proskauer reaction	_
Citrate utilization	+
Urease	+
SH_2 production	+
Motility	+
Catalase	+
Oxidase	_
Oxidative/Fermentative	Fermentative
Glucose fermentation	+
Xylose fermentation	+
Lactose fermentation	_
Sorbitol fermentation	+
Saccharose fermentation	+
Mannitol fermentation	+
Probable identified species	*Vibrio* Spp.

Table 1: Primer sequences and characteristics of amplification procedures.

Isolated bacterium	Primer sequence	Amplification cycle	Amplified fragment	Reference
Vibrio SPP.	CGGTGAAATGCGTAGAGAT TTACTAGCGATTCCGAGTTC	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.

pH (acidity) of water	water Temperature (°C)	Five- days biochemical oxygen demand (BOD)	Water dissolved oxygen (OD)
9.97	25	89.8	6.98

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

Sequence based on the amplification with forward primer:

ACAGGATGTGAGGCGGCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGT
AGTCCACGCCGTAAACGATGTCTACTTGGAGGTTGTGCCCTAGAGGCGTGGCTTTCGGAGCTAACGCGTTAAGTAGACCGCCTG
GGGAGTACGGTCGCAAGATTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCA
ACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAATCTAGCGGAGACGCTGGAGTGCCTTCGGGAGCTCTGAGACAGGTGC
TGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTGTTTGCCAGCACGT
AATGGTGGGAACTCCAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTTACGAG
TAGGGCTACACACGTGCTACAATGGCGTATACAGAGGGCAGCGATACCGCGAGGTGGAGCGAATCTCACAAAGTACGTCGTAG
TCCGGATTGGAGTCTGCAACTCGACTCCATGAACTCGGACCCCGTAGTAAATCC

Sequence based on the amplification with reverse primer:

TAAAATTTTCTATTTTTGCCCTTGGACAATACTGCCACTCACATGCTACATCGTGGGGAGCAAACACGATTAGATACCCGGGCC
GTTCACCCCGTAAACGATGTCTACTTGTAGGTTGTGTCCTTCAGGCGTGGCTTTCGGAGCTAACGCGTTAAGTAGACCGCCTGG
GGAGTACGGTCGCAAGATTAAAACTCCCATGAATTGTTTGGGGCCCGCACAAGCGGTGGAGCTTGTGGTTTTTTTCGATGCCAC
GCGAACAACCTTACCTACTCTTGACATCCAGAGAATCTAGCGGAGACCCTGGATTGCCTTCGGGATCTCTGAGACTGGTGCTGC
ATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTGTTTGCCAACACGTAA
TGGTGGGAACTCCACGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTTACGAGTAC
GGTTACACCCGGGCTACCATGGCGTATACAGAGGGCTGCGATACCGCGAGGTGGAGCGAATCTCACAAAGTACGTCGTAGTCC
GGATTGTAGTCTGCCACTTGACTCCATGAACTCGCAATCTTTTGTGAAAAAAC

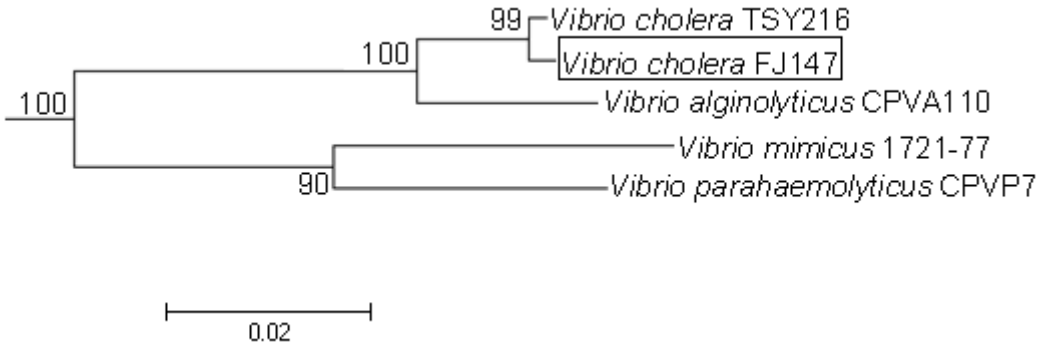

Fig. 2. Phylogenic tree confirmed the isolate *Vibrio cholera* FJ147 after alignment in MEGA version 4.

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 phylogenic tree which resulted from alignment in MEGA version 4 illustrated in Fig 2. As shown in phylogenic 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

(8). 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.

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شناسایی باکتری‌های جنس *Vibrio* جداسازی شده از ماهی آکواریومی گوپی (*Poecillia reticule*) در استخرهای پرورش ماهی کاشان، اصفهان، ایران

سمیرا کیانی: کارشناس ارشد میکروبیولوژی، واحد فلاورجان، دانشگاه آزاد اسلامی، اصفهان، ایران، samira.kiani87@yahoo.com
نفیسه سادات نقوی *: استادیار میکروبیولوژی، واحد فلاورجان، دانشگاه آزاد اسلامی، اصفهان، ایران، naghavi@iaufala.ac.ir
علیرضا نظری: استادیار بیوتکنولوژی، واحد فلاورجان، دانشگاه آزاد اسلامی، اصفهان، ایران، nazari_a@iaufala.ac.ir

چکیده

مقدمه: باکتری‌های گرم منفی از مهم‌ترین باکتری‌های بیماری‌زا برای موجودات دریایی از جمله ماهیان زینتی هستند.

مواد و روش‌ها: در این پژوهش گونه‌های *Vibrio* از ماهی زینتی گوپی در استخرهای پرورش ماهی کاشان، اصفهان، ایران جداسازی و بر اساس روش‌های تشخیص مولکولی و توالی یابی ژنتیکی شناسایی شدند. نمونه‌های کبد، کلیه، پوست، مغز و آبشش از ماهی زینتی گوپی در منطقه کاشان، اصفهان، ایران در مراحل لارو، بالغ و آماده خارج شدن از سالن گرفته شد. نمونه‌ها روی محیط‌های کشت غنی شده کشت داده و مراحل خالص‌سازی انجام شد. شناسایی اولیه با استفاده از تعیین ویژگی‌های بیوشیمیایی باکتری‌های جداسازی شده انجام شد. تشخیص مولکولی بر اساس تکثیر توالی *16SrDNA* ژنوم *Vibrio cholera* حاوی ITS (Internal Transcribed Spacer) و توالی یابی نوکلئوتیدهای تکثیر شده انجام گرفت.

نتایج: باکتری‌های جداسازی شده از انواع گونه‌های *Vibrio* شامل *Vibrio cholera* (۹۹ درصد شباهت در توالی)، *Vibrio alginolyticus*، *Vibrio mimicus* و *Vibrio parahaemolyticus* (کمتر از ۹۰ درصد شباهت در توالی) تشخیص داده شدند. حوضچه‌های پرورش ماهی دارای آب قلیایی و مقدار BOD پنج روزه در محدوده سالم نبودند. این شرایط برای گونه‌های *Vibrio* ایده‌آل است.

بحث و نتیجه‌گیری: موجودات آبزی در ایران می‌توانند ناقل عوامل پاتوژن انسانی مانند گونه‌های *Vibrio* باشند. نتایج این بررسی و پژوهش‌های مشابه، باید در سیستم‌های بهداشت پرورش آبزیان مورد توجه قرار گیرد.

واژه‌های کلیدی: گونه‌های ویبریو، ماهی زینتی گوپی، توالی یابی ژنومی، بهداشت آبزیان

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* نویسنده مسؤول مکاتبات

تاریخ دریافت: ۱۳۹۳/۰۶/۱۷- تاریخ پذیرش: ۱۳۹۴/۰۵/۱۴