Molecular Identification of *Vibrio harveyi* From Larval Stage of Pacific White *Shrimp (Litopenaeus vannamei)* Boone (Crustacea:Decapoda)By Polymerase Chain Reaction and 16S rDNA Sequencing

Mirbakhsh, M.*[1]; Akhavan sepahy, A.[2]; Afsharnasab, M[3]; Khanafari, A[2]; Razavi, M.R[1]

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Abstract

A Gram-negative, rod-shaped, motile and halophilic bacterium identified as *Vibrio harveyi* was isolated from hatchery-reared larval of Iran Shrimp Research Centerfollowing mass mortality . Seven isolates of *Vibrio* spp.were collected from nauplii of *Litopenaeus vannamei* and then were evaluated for characteristics including morphology, physiology and biochemistry. The results indicated that the isolates belonged to a single species that grew on Thiosulfate citrate- Bile Sucrose agar and in 1.5 to 6% NaCl, positive lysine and ornithine decarboxylation and susceptible to O/129 disk. Identification of *Vibrio harveyi* strainIS01 (1 of 7 isolates) was confirmed by Polymerase Chain Reaction assay for *Vibrio harveyi* (expected amplicon 1493 bp). The 16S ribosomal DNA sequence (National Center for Biotechnology Information, GenBank accession number GU974342.1) gave 99% sequence identity to *V. harveyi* ATCC: 35084 (GenBank accession number EU130475.1).

Keywords: Vibriosis, shrimp diseases, *Litopenaeus vannamei*, 16S rDNA sequencing

1-Biology Department, Science and Research Branch, Islamic Azad University (IAU), Tehran, Iran

2-Microbiology Department, North of Tehran Branch, Islamic Azad University, Tehran, Iran

3-Iranian Fisheries Research Organization, P.O.Box: 14155-6116, Tehran, Iran

*Corresponding author's email: maryam.mirbakhsh@gmail.com

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Introduction

The shrimp culture industry is one of the fastest growing food production in the world (Subasinghe, et al., 1998). Currently, the aquaculture industry in Iran and other parts of the world has been facing serious problems due to microbial (Hosseini, et al., 2004) and viral diseases (Afsharnasab, et al., 2007). Two genera of bacteria cause considerable disease in different phases of shrimp culture including *Leucothrix* spp. and *Vibrio* spp. (Lavilla-Pitogo, 1995). Vibriosis, especially luminous disease, has caused serious loss in shrimp hatcheries. Shrimp larvae are particularly susceptible to *Vibrio harveyi* and infection with this bacterium can lead to luminescent bacterial disease (Lavilla-Pitogo, et al., 1990). Mass mortalities in shrimp hatcheries and culture ponds were caused by outbreaks of vibriosis have been recorded from many regions (Lightner, 1988, Lightner and Lewis, 1975, De la Pena et al., 1993, Jiravanichpaisal et al., 1994, Mohney et al., 1994, Won and Park, 2008, Lavilla-Pitogo C.R. et al., 1992) such as, Korea (Won K.M. and Park S.I., 2008), Philippines (Lavilla-Pitogo et al., 1990, Lavilla-Pitogo C.R. et al., 1992, Lavilla-Pitogo and de la Pena, 1998, Lavilla-Pitogo et al., 1998) and Thailand (Ruangpan L and Kitao T, 1991) hatcheries.

The bacteria in *Vibrio* genus were characterized morphological by Gram negative bacteria possessing a curved rod shape, all members of the genus motile and have polar flagella with sheath, facultative anaerobes and oxidase positive that do not form spores and typically found in seawater (Baumann et al., 1984). Luminous vibriosis is caused by *V. harveyi* as both a primary and opportunistic pathogen of marine animals (Austin and Zhang, 2006), such as Gorgonian corals, oysters, prawns, lobsters, barramundi, turbot, milkfish, and seahorses (Owens and Busico-Salcedo, 2006). The disease has been well described in shrimps and named penaeid vibriosis, penaeid bacterial septicemia, bolitas negricans, luminous vibriosis or red-leg disease. This bacteria was first described as *Achromobacter harveyi*(Johnson and Shunk, 1936) after E.N Harvey, an innovator in the systematic study of bioluminescence, the species was later assigned to the genera *Lucibacterium*(Hendrie et al., 1970)and *Beneckea*(Reichelt J.L. and Baumann P, 1973) , and at last specified in the genera *Vibrio* within the family *Vibrionaceae*(Baumann et al., 1981). Type strains of the two previously recognized species, *Vibrio carchariae* (Grimes et al., 1985) and *Vibrio trachuri*(Iwamoto et al., 1996) were decided to be synonyms of *V. harveyi* according to molecular studies (Pedersen et al., 1998, Gauger and Gomez–Chiarri, 2002, Thompson et al., 2002). Traditional approaches for identification and typing of *vibrio spp.* are according to phenotypic methods such as serological methods, metabolic or fatty acid profiling and profiling of antimicrobial susceptibility. Because of their highly similar phenotypes the specificity power of phenotypic methods is limited for the *V. harveyi* group. Nowadays Full and partial 16S rDNA gene sequencing methods have emerged as useful tools for identifying microorganisms (Janda and Abbott, 2007, Petti et al., 2005).

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Materials and methods

Water and nauplii samples were collected after mass mortality in a hatchery belong to Iran Shrimp Research Center (ISRC) in Bushehr Province, Iran. The water temperature, salinity, pH and dissolved oxygen were27.4 ± 1.3 °C, 33.2 ± 2.2 ppt, 7.81 ± 0.90 and 6.7 ± 1.1 mg L^{-1} respectively.

Fifty shrimp nauplii were homogenized in 0.5 ml physiological saline. All the samples were diluted serially and 0.1 ml aliquots were spread onto Thiosulfate Citrate Bile Salt Sucrose agar (TCBS, Merck, Germany), Tryptic Soy Agar (TSA, Merck, Germany) with 2.5% sodium chloride and incubated at 30 °C. The same method was used for tank water samples; 0.1 ml of each sample was inoculated to both mentioned media and incubated in same condition. The morphology and the number of colonies on TCBS were recorded.

Twenty colonies from each sample were selected and subcultured on TSA with 2.5% sodium chloride and identified according to Gram's staining and biochemical reactions which included catalase, oxidase production, growth on MacConkey agar, OF, nitrate reduction, production of lipase, urea hydrolysis, Lysin decarboxylase, Arginine dihydrolase, Ornithine decarboxylase, H_2S production, motility, Indole, carbohydrate fermentation, MR-VP reaction, inhibition zone diameter around O/129 disc (150 µg) and growth on different concentrations of NaCl (Table 1) (Robertson et al., 1998).

Bacteria were incubated at 30°C in Tryptic soy broth (2% NaCl) for 24 h and collected by centrifugation (7000 × g for 20 min at 4°C). The nucleic acid of pelleted bacteria was extracted by using a Genomic DNA purification Kit (Biorad) and stored at –20°C, for polymerase chain reaction (PCR) tests (Abraham et al., 1997).

The 16S rDNA gene of the isolate was amplified using universal eubacterial primers, forward primer 5'–3'(27F GAGTTTGATCCTGGCTCAG) and reverse primer 5'–3' (1392R ACGGGCGGTGTGTRC) (Sigma Co.). The PCR reaction buffer contained 25mM $MgCl_2$, 5.0µl PCR buffer 10X, 1.0 µl dNTPs, and 0.5 µl of each primer, Taq DNA polymerase 0.5 µl, Water (nuclease free) up to 50 µl and 50-100 ng Template DNA. PCR reactions were carried out as follows: initial denaturation 95°C 1 cycle, 30 cycles of denaturation at 95°C for 1 min, annealing at 57°C for 40 sec and extension at 72°C for 90 sec, followed by 1 cycle final extension at 72°C for 10 min and cooling to 4°C. The PCR fragments were transferred to electrophoresis on a 1% agarose gel for length differences, and amplified DNA fragments were cloned into the pTZ57R/T vector following the directions provided (Fermentas). Recombinant bacteria were discovered by blue-white screening and confirmed by PCR. Plasmids containing the insert were purified and utilized as a template for DNA sequencing.

Nucleotide sequence analysis was carried out by using the dideoxynucleotide chain termination method (Musa et al., 2008) on a DNA sequencer (Model 373A, Applied Bios 2008) on. The 16S rDNA sequences were added to available bacterial 16S rRNA sequences and then were integrated into the database with the automatic alignment tool and

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homology search were done by Megablast program of the National Center for Biotechnology Information (NCBI) and CLC sequence viewer 6.6.2 software.

Results

Shrimp mortality in ponds affected by disease outbreaks was up to 100% over 5 days. Seven of 20 isolated bacteria were identified as *Vibrio* spp by special biochemical tests and were used to further analyze. All of these isolates were found to be Gram-negative, curve rod shape and produced yellow colonies on TCBS agar and not grow on MacConkey agar. Their colony on Tryptic soy agar had 2mm diameter, cream color, round shape, smooth surface and convex elevation. All strains grew in media containing 1.5 to 6% NaCl and grew weak in 1 and 10% NaCl and were sensitive to O/129 and fermented Manitol, Sucrose and Cellobiose, all of them had Lysin and Ornithine decarboxylase enzyme as shown in characteristics, all strains were similar to *V. harveyi*(Alsina and Blanch, 1994).

Table **1**. Based on their morphological, cultural, physiological, and biochemical

Table 1: Biochemical tests for the identification of *Vibrio harveyi.*

Characteristics	Results
Colony	2mm, cream, round, smooth, convex
Gram staining	Negative curved rode
Catalase	+(w)*
Oxidase	+
Growth on MacConkey Agar	-
OF	Fermentative
Nitrate reduced to nitrite	+
Production of lipase	+
Urea hydrolysis	-
Lysin decarboxylase (2 days)	+
Arginine dihydrolase (7 days)	-
Ornithine decarboxylase (2 days)	-
Ornithine decarboxylase (7 days)	+
SH_2 (SIM medium)	-
Indole	+
Motility	+
MR/VP	-/-
Carbohydrate fermentation:	
Manitol	+
Sorbitol	-
Lactose	-
Sucrose	+
L-Arabinose	-

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Continue table1:	
Raffinose	-
Cellobiose	+
Inhibition zone diameter around O/129 disc(150µg)	20mm
Growth in 0.5% NaCl	-
Growth in 0.8% NaCl	-
Growth in 1% NaCl	+(w)
Growth in 1.5% NaCl	+
Growth in 2% NaCl	++
Growth in 3.5% NaCl	++
Growth in 6% NaCl	+
Growth in 8% NaCl	+(w)
Growth in 10% NaCl	-
Growth in 12% NaCl	-

*Week

Molecular identification of the isolated strains was carried out based on 16S rDNA sequence analysis. The sequence of the 1493 bp 16S rDNA PCR amplicon from isolates were determined and deposited at GenBank under accession number GU974342. Alignment identity of this sequence was compared with some other strains. Sequence showed 99% identity with the sequence of 16S ribosomal RNA gene of *V. harveyi* strain LA08005, LA08008, XC08001 as mentioned in Table 2. A phylogenetic tree was constructed using the neighbor joining method, and is shown in Figure 1. Therefore, the isolated strains were identified as *V. harveyi* strainIS01based on their morphological, cultural, physiological, and biochemical characteristics and 16S rDNA sequence analyses.

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Table 2: Comparison of alignment identity of GU974342 by Megablast program.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
GQ180186.1	*Vibrio harveyi* strain LA08005 16S ribosomal RNA gene, partial sequence	2684	2684	97%	0.0	99%
GQ180185.1	*Vibrio harveyi* strain LA08008 16S ribosomal RNA gene, partial sequence	2684	2684	97%	0.0	99%
FJ605242.1	*Vibrio harveyi* strain XC08001 16S ribosomal RNA gene, partial sequence	2684	2684	97%	0.0	99%
AY750578.1	*Vibrio harveyi* strain S35 16S ribosomal RNA gene, partial sequence	2675	2675	97%	0.0	99%
GQ180184.1	*Vibrio harveyi* strain LA08007 16S ribosomal RNA gene, partial sequence	2673	2673	97%	0.0	99%
EU130475.1	*Vibrio harveyi* ATCC:35084 16S ribosomal RNA gene, partial sequence	2641	2641	96%	0.0	99%

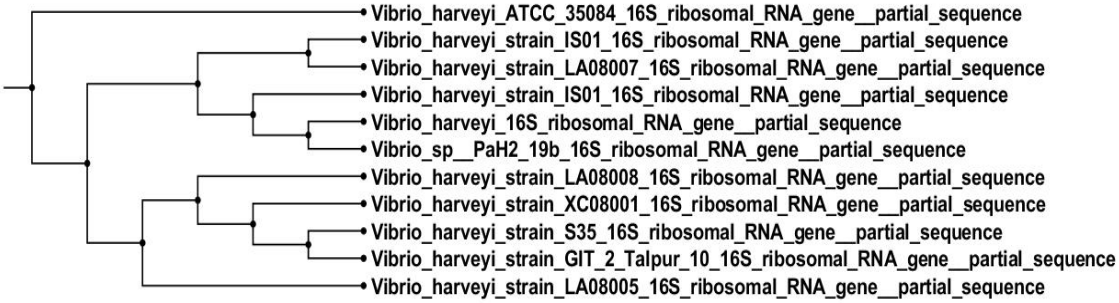


Figure 1: Phylogenetic tree representing the strain under study compared to the whole bacterial database.

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Discussion

Bacterium identified as *V. harveyi* is an economically significant pathogen for the aquaculture industry and methods to identify, type and track *V. harveyi* -related populations are therefore of interest. Due to phenotypic similarities and genome plasticity, traditional phenotypic identification and typing methods are not always able to resolve *V. harveyi* from closely related species(Dworkin et al., 2006). *V. harveyi* has however also been associated with mass mortality events in other aquaculture species, including new potential aquaculture candidates (Liu et al., 2004). The pathogen has proven difficult to eradicate and extensive use of antibiotics in farms has resulted in the development of antibiotic resistant strains. Due to the economic importance of *V. harveyi* infections, there is considerable interest in methods to identify the type and track *V. harveyi* related populations associated with marine reared animals. Identification of *V. harveyi* strains can be a challenging task since species within the Harveyi clade (*V. harveyi*, *Vibrio campbellii, Vibrio alginolyticus, Vibrio rotiferianus, Vibrio parahaemolyticus, Vibrio mytili and Vibrio natriegens*) have a very high degree of both genetic and phenotypic similarity (Sanger et al., 1977). Bacterial typing systems detect differences in the phenotypic or genotypic characteristics of strains, and based on their resolution power can be used to distinguish genera, species or strains. Bacterial typing systems therefore form the basis for the integration of bacterial taxonomy and epidemiology. Pathogen tracking is relevant for epidemiological studies concerned with the ecology and natural history of a disease; or with planning, monitoring and assessment of disease control programs. Methods for pathogen tracking include identification and typing methods as well as methods for direct detection and quantification of the relevant organism in environmental samples.

The 16S and the 23S ribosomal RNAs are essential to the viability of bacterial cells, hence the genes coding for them are highly conserved. However, these genes also contain short variable sequences useful for characterization and discrimination of microbial populations at the level of family and, in many cases, at the level of genus and species. This combination of conserved and variable sites makes these molecules ideal taxonomic markers to identify *vibrio spp.* by PCR amplification and gene sequencing (Cano-Gomez et al., 2009). The 16S rRNA genes are considered the standard marker for *Vibrio* phylogeny though since the gene evolves slowly, the differences between species are limited and therefore often unable to resolve closely related bacterial strains (Sawabe et al., 2007, Nagpal et al., 1998). In the case of *V. harveyi*, it is often difficult to resolve this species from other species of the *Vibrio* core group (*V. alginolyticus, V. campbellii, V parahaemolyticus, and V. rotiferianus*) based solely on 16S rRNA gene heterogeneity. For instance, the species *V. harveyi*, *V. campbellii* and *V. rotiferianus* have

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more than 99% sequence identity of the 16S rRNA gene (Vandenberghe et al., 2003).

Because of the very close phylogenetic relationship of *V. harveyi* to other *Vibrio* species such as *V. parahaemolyticus*, *V. alginolyticus*, *V. campbellii* and *V. carchariae*(Reichelt and Baumann, 1973, Cano-Gomez et al., 2009), identification of this species by conventional biochemical tests is not precise. Hence, a PCR method that could target nucleotide sequences individually to *V. harveyi* may be useful for detection, differentiation and enumeration of closely related *Vibrio* species.

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