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Molecular Characterization of *Vibrio Harveyi* in Diseased Shrimp

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Abstract

The objective of this study was to characterize *Vibrio harveyi* phenotypically and by molecular methods. A total number of 420 shrimp post larvae samples were collected, 280 samples of diseased cultured shrimp and 140 samples of apparently healthy marine shrimp. The samples were subjected to bacteriological examination. Thirty three (11.7%) of cultured shrimp samples and 4 (2.8%) of marine shrimp samples were phenotypically positive for *V. harveyi*. Species - specific gene (toxRgene) and two virulence associated genes (vhh - *Vibrio harveyi* haemolysin, and partial hly- haemolysin gene) were investigated using conventional PCR.

This study proved that using of partial haemolysin gene in molecular characterization of *V. harveyi* is the fastest and most accurate method to identify all isolates of *V. harveyi* (highly pathogenic, moderately pathogenic and non-pathogenic strains) due to the presence of a single copy of haemolysin gene encoded in all isolates, although it is not in the same locus in the genome.

1. INTRODUCTION

Bacteria of the genus *Vibrio* have been specifically implicated as shrimp pathogens because they are regularly found in high numbers during periods of high larval mortality and disease outbreak in grow out ponds (Saulnier *et al.*, 2000).

Vibrio harveyi, which now includes *Vibrio carchariae* as a junior synonym, is a serious pathogen of marine fish and invertebrates, particularly penaeid shrimp.

The presence of several *Vibrio* isolates associated with the shrimp from the egg stadia to post larva, as well as their rearing environment. Based on their physiological and genetical characters the isolates were distinguishable from *V. harveyi* that has been proved to be pathogenic on shrimp larva (Widanarni and Suwanto 2000) .Johnson and Shunk (1936) described *V. harveyi* as Achromobacter. Hendrie *et al.*, (1970) studied the systematic study of bioluminescence; the species was later assigned to the genera Lucibacterium. Reichelt and Baumann (1973) described the bacteria as *Lucibacterium harveyi* and *Beneckea harveyi*. Baumann *et al.*, (1981) included the bacterium to the genera Vibrio within the family Vibrionaceae. Grimes *et al.*, (1984a) isolated new organism from a sandbar shark (*Carcharhinus plumbeus*), which died at the National Aquarium in Baltimore, USA and classified as a new species, as *Vibrio carchariae*. Iwamoto *et al.*, (1996) named species *V. carchariae* and *V. trachuri*. Gauger and Gomez-Chiarri (2002) recognized as a result of phenotypic and genotypic studies, including 16S rDNA sequencing that *V.*

harveyi and *V. carchariae* were synonymous.Thompson *et al.,* (2002) reported that the synonyms of *V. harveyi* based on molecular studies.Farmer *et al.,* (2005) described current taxonomic position as *V. harveyi.* Pathogenicity of *V. harveyi* has been related to a number of factors including secretion of extracellular products (ECP) containing substances such as proteases, haemolysins, and lipases (Harris and Owens, 1999; Liu and Lee, 1999; Zhang and Austin, 2000; Teo *et al.,* 2003), a lipopolysaccharide (Montero and Austin, 1999), and a bacteriocin-like substance which were isolated from a pathogenic strain of *V. harveyi* VIB 571, which aided the survival and dominance of the bacteria within the host (Prasad *et al.,* 2005).

Bacteriophages have also been identified as agents responsible for the virulence of these bacteria (Ruangpan *et al.* 1999; Austin *et al.* 2003; Pasharawipas *et al.* 2005; Khemayan *et al.* 2006).

In addition, luminescence (Manefield *et al.,* 2000), quorum sensing (Henke and Bassler, 2004), ability to form biofilms conferring resistance to disinfectants and antibiotics (Karunasagar *et al.,* 1994), sucrose fermentation (Alavandi *et al.,* 2006), and capacity to bind iron (Owens *et al.,* 1996) have all been associated with virulence.

Moreover, it has been shown that environmental factors, such as temperature and varying salinity, also play a role in *V. harveyi*-mediated vibriosis (Alavandi *et al.,* 2006).

As for penaeid shrimp, Cysteine protease has been reported as the major exotoxin affecting penaeid shrimp (Liu *et al.,* 1997), but (Montero and Austin 1999) suggested that lipopolysaccharide might be the lethal toxin affecting penaeid shrimp.

Vibrio harveyi is a Gram-negative, bioluminescent, marine bacterium in the genus Vibrio. *V. harveyi* is rod-shaped, motile (via polar flagella), facultatively anaerobic, halophilic, and competent for both fermentative and respiratory metabolism. It does not grow below 4°C or above 35°C (Owens *et al.,* 2006).

Over the past decade, strains of this species have been reported to be significant pathogenic agents and one cause of the high rates of shrimp mortality in the shrimp culture industry worldwide (Karunasagar *et al.,* 1994; Saeed, 1995; Liu *et al.,* 1996a, b).

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 (Cano-Gomez *et al.,* 2009).

The design of molecular techniques for specific detection and quantification of potentially pathogenic *V. harveyi* strains is problematic and challenging for several reasons. The relationship between the presence of virulence genes, their expression, and their virulence to different hosts would have to be demonstrated by a combination of molecular methods and traditional diagnostic methods (Cano-Gomez *et al.,* 2009).

The general aim is to diagnose *V. harveyi* phenotypically and using molecular methods.

2.MATERIAL AND METHODS :

2.1. Collection of Shrimp Samples :

A total of 420 shrimp samples consisting of 280 diseased cultured shrimp and 140 apparently healthy marine shrimp were collected for investigation. Cultured shrimp samples were trimmed and under all the accurate method of transportation were transported in ice boxes as muscles and intestine (abdominal parts) from farms at (Damietta – Port Said – Ismailia) to laboratory. Marine shrimp samples were collected and transported in ice boxes as whole shrimp from fishing ships to laboratory to be trimmed and examined rapidly as soon as possible as they arrived.

2.2. Isolation of *V. harveyi* from Shrimp Samples :

Cultured shrimp samples were trimmed and were transported in ice boxes as muscles and intestine (abdominal parts). Therefore strains were isolated from muscles (Jaysinghe *et al.,* 2007) and intestine. Marine shrimp samples were collected and transported in ice boxes as whole shrimp. There for strains were isolated from hepatopancreas (Najiah Musa *et al.,* 2008), muscles and intestine after surface swapping with 70% ethyl alcohol. Samples were incubated in trypticase soy broth (Difco; supplemented with 3% NaCl) at 28 ºC for 24 h.

2.3. Microbiological Identification of *V. harveyi* :

After samples were incubated in trypticase soy broth (Difco; supplemented with 3% NaCl) at 28 ºC for 24 h, they were streaked onto trypticase soy agar (Oxoid; supplemented with 3% NaCl) at 28ºC for 24 h (Pin point transparent to light creamy colonies as shown in (figure 1-a). Since *Vibrio* strains were mainly aquaculture pathogens originally isolated from the marine environment NaCl was added to the trypticase soy broth and the trypticase soy agar

to give them a salinity like that of seawater (Katarina, 2005). Colonies isolated were purified by re-streaking on thiosulphate/citrate/bile salts/sucrose agar (TCBS; Oxoid) at 28ºC for 24-48 h (typical small green colonies as shown in (figure 1 -b). Single pure isolated colonies were picked and stored in cryovials containing 20% glycerol/ broth at −20 ºC for further analysis and biochemical profiling.

Strains were submitted to Gram staining, swarming motility testing on trypticase soy agar (Oxoid; supplemented with 1% NaCl) at 37ºC for 24 h, oxidase and ONPG test (Liu *et al.*, 2004)followed by testing growth at 0% and 8% NaCl, growth in *Vibrio harveyi* agar at 28ºC for 48 h, and growth on horse blood agar at 37ºC for 24 h (Najiah Musa *et al.*, 2008).Also, isolates were subjected to other biochemical tests as indole production, urease, citrate utilization test using Simmon's citrate medium +0.1% of yeast extract (Ottaviani *et al.*, 2003) Voges-Proskauer and methyle red.

2.4. Molecular Identification of *V. harveyi* :

Table 1: The oligonucleotide primer sequence used in this study:

Primer	***Sequence (5' – 3')***	***Amplicon size (bp)***	***Reference***
Vh_toxR-F	TTCTGAAGCAGCACTCAC	390	Conejero and Hedreyda (2003)
Vh_toxR-R	TCGACTGGTGAAGACTCA		
Partial hly-F	GAGTTCGGTTTCTTTCAAG	647	Haldar et al., (2010)
Partial hly-R	TGTAGTTTTTCGCTAATTTC		
Vhh-F	TTCACGCTTGATGGCTACTG	234	Ruwandeepika et al., (2010)
Vhh-R	GTCACCCAATGCTACGACCT		

Molecular identification was done using conventional PCR assay. Species - specific gene (*toxR gene*) and two virulence-associated genes (*vhh -Vibrio harveyi haemolysin, partial hly- haemolysin gene*) were investigated.

2.4.1.DNA Extraction of *V. harveyi* :

The strains were inoculated into Tryptone soya broth were centrifuged at 5000 rpm for 20 min at 10° C. Subsequently, the obtained pellets were diluted in sterile phosphate buffered saline to obtain 3 X 108 cfu/ml. The chromosomal DNAs (cDNAs) from bacterial cells were extracted using DNA extraction kits (QIAmp DNA mini kit) following the manufacturer's protocol. The target genes oligonucleotide primer sets used are listed in Table 1.

2. 4. 2. PCR amplification protocol:

1-The following components were pipetted in sterile clean PCR tube:

2-The sample tubes were mixed gently, spun and placed in BEOCO Germany thermal cycler.

3-Thermal cycler was adjusted to the following conditions for each set of primers.

+ 3% Nacl and incubated at 25° C for 18 hours. After elapsing of incubation time, the inoculated broth

Table 2: component of PCR mix

Component	***Final volume***	***Component concentration***
5X PCR Master Mix	10 µl	5X
Forward primer	1 µl	10 Pmol/ µl
Reverse primer	1 µl	10 Pmol/ µl
Genomic DNA	----------	100 ng
Nuclease free water	Up to 50 µl	--------

Table 3: Adjustment of Thermal Cycles:

Step	*Temperature*	*Time*	*Number of cycles*
Initial denaturation	95°C	5 minutes	1 cycle
Denaturation	95 °C	45 seconds	
Annealing	58 °C	45 seconds	35 cycles
Extension	72 °C	45 seconds	
Final extension	72°C	10 minutes	1 cycle

2. 4. 3. Electrophoresis detection of PCR products:
It is a simple and highly effective method for separating, identifying and purifying DNA fragments using an agarose concentration appropriate for the size of the DNA fragments to be separated (Sambrook and Russell, 2001).

3. RESULTS :

3.1. Incidence of *V. harveyi* in shrimp :

A total of 420 shrimp samples consisting of 280 diseased cultured shrimp and 140 apparently healthy marine shrimp were collected and analyzed by microbiological, molecular methods. Overall, 33 (11.7%) of cultured shrimp samples and 4(2.8%) of marine shrimp samples were positive for *V. harveyi* species.

3.2. Isolation and Culture Results :

Colonies of *V. harveyi* on trypticase soy agar+ 3% NaCl were Pin point transparent to light creamy colonies as shown in (figure 1-a), while they were typical small green colonies when re-streaked on thiosulphate/ citrate/ bile salts/ sucrose agar (figure 1-b). On *Vibrio harveyi* agar medium, colonies were small; light green with dark green centers (figure 1-c).

All *V. harveyi* isolates were Beta-haemolytic on horse blood agar and showing swarming motility on trypticase soy agar + 1% NaCl when incubated at 37ºC for 24 h (figure 1-d).Strains were Gram negative, curved rods when submitted to Gram staining, oxidase positive, growing at 0% and 8% NaCl and sensitive to the vibriostatic compound O/129 (10µg and 150µg discs). All isolated strains of V. harveyi (n=14) amplified a 390-bp for primer sets of the V. harveyi toxR gene. Although our PCR assay involves primer sets of V. harveyi haemolysin (vhh-F,vhh-R), no specific amplicons of the predicted size were obtained for 12 of isolated strains of V. harveyi (n=14). However, All isolated strains of V. harveyi (n=14) gave a 647-bp for the primer sets of V. harveyi partial hly- haemolysin gene (Partial hly-F, Partial hly-R).The results were shown in figure 2.

Table 4: Results of biochemical identification of the selected *V. harveyi* :

Biochemical Test	***V. harveyi***
Gram Stain	-ve curved rods
Oxidase	+
V.P.	-
Torky[1]	**Torky**[1]
Indole	+
Citrates	+
Urease	V
ONPG	-
Gelatin	+
O/129	S
H2S (TSI)	-

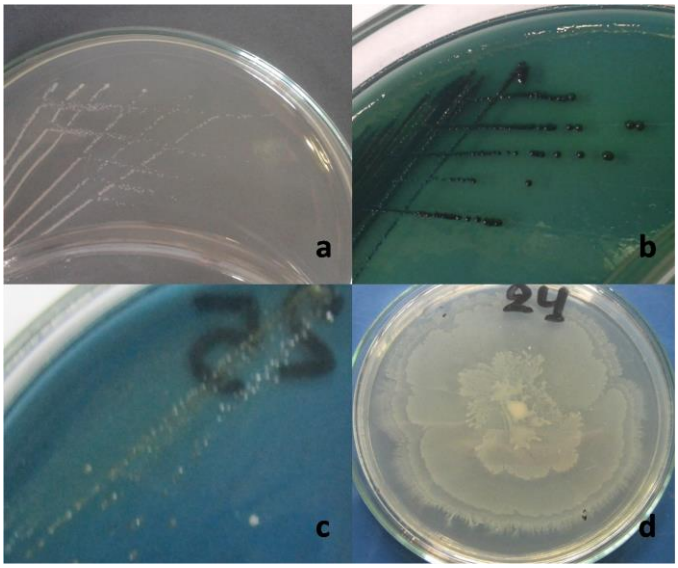

Figure 1: Colonial morphology of *V.harveyi* on different laboratory media:
a: *V. harveyi* on TSA +3% NaCl: Pin point transparent to light creamy colonies, **b:** *V. harveyi* on TCBS after 48h. incubation: Circular, small green colonies, **c :** *V. harveyi* on *Vibrio harveyi* agar after 48h. incubation at 28°C: Small, light green colonies, **d :** *V. harveyi* on TSA +1% NaCl after 24h. incubation at 37°C: Swarming motility pattern.

3.3 Species Specific and Virulence Associated Genes :

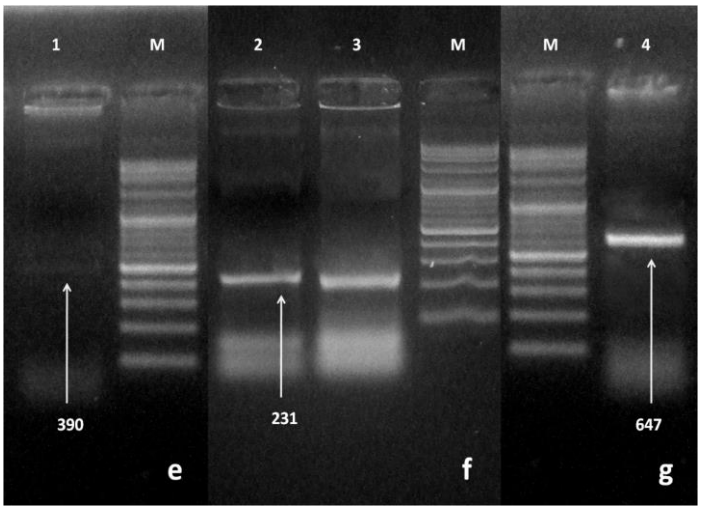

Figure 2 : Electrophoresis analysis of conventional PCR assay for isolated *V. harveyi:*
e:Lane 1, Band of 390 bp of isolated *V. harveyi* strain amplified (Vh_toxR-gene) *Vibrio harveyi* species specific gene, Lane M, 100-bp DNA ladder, **f:** Lane 2 and Lane 3, Bands of 234 bp of two isolated strains of *V. harveyi* that amplified (vhh-gene) *Vibrio harveyi* haemolysin gene, Lane M, 100-bp DNA ladder, **g:** Lane 4, Band of 647 bp of isolated *V. harveyi* strain amplified (Partial hly-gene) *Vibrio harveyi* partial haemolysin gene, Lane M, 100-bp DNA ladder.

4. DISCUSSION :

With increasingly high shrimp consumption, the decline of wild harvests has forced domestication to become the major source of shrimp production. However, the farming industry has been deteriorating due to several factors, in particular the outbreak of disease.

Vibriosis is one of the most important serious infectious bacterial diseases that affect different kinds of marine fishes, molluscs and crustacean over the world.

At this study, diseased cultured shrimp samples were collected from farms at Damietta governorate and apparently healthy marine shrimp samples were collected from fishing ships at Alexandria governorate, Egypt.

Data obtained over 420 diseased cultured shrimp and apparently healthy marine shrimp

(shrimp samples) over 2 years showed recovery of some pathogenic bacterial species.

As a result, a total of 787 bacterial isolates were recovered including 613 (77.85%) Gram negative and 174 (22.14%) Gram positive bacterial isolates. Previous studies revealed that (78 %) fish were found to be infected with Gram negative bacteria and only (22%) with Gram positive ones (Saad, 2013).

Members of the genus Vibrio were quantitatively dominant over other genera, the same as reported by (Oxley *et al,* 2002).

All *V. harveyi* isolated in this study gave the green coloured colonies on TCBS while *V. harveyi* isolated by (Najiah Musa *et al.,* 2008) showed both green and yellow coloured colonies, therefore they explained that isolates that gave yellow coloured colonies on TCBS may be due to the absence of gene that enables isolates to utilize sucrose, on the other hand, isolates that gave green coloured colonies on TCBS may possess the gene csc B in their genomic DNA. Also, (Harris *et al.,* 1996) reported that TCBS agar is not a differential medium in that Vibrio species like *V. harveyi*, which are variable in the utilization of sucrose, cannot be distinguished from other sucrose-positive or sucrose-negative species.

V. harveyi strains when were cultivated on VHA medium were small; light green with dark green centers differentiating them from colonies of *V. alginolyticus* that were large; spreading and light blue in color and these results were the same as described by (Katarina, 2005) when explained that *V. alginolyticus* does not ferment cellobiose and are not able to decarboxylate ornithine so, this property will result in a pH change as its colonies will appear blue or colorless. That were in agreement with (Harris *et al.,* 1996) who used *Vibrio harveyi* agar medium, a selective and differential medium to differentiate *V. harveyi* from 15 other Vibrio species.

V. harveyi isolates were able to hemolyse horse's blood which resulted in breakdown of horse blood agar plate around the colony of bacteria known as B-hemolysis ,the same as described by (Najiah Musa *et al.,* 2008) and this was explained by (Pujalte *et al.,* 2003) as a recognised virulence factor.

Concerning Gram staining that was done from liquid media to show the exact characteristic appearance for Vibrio species, *V. harveyi* isolates were curved rods. This was done according to (UK Standards for Microbiology Investigations, 2015) when mentioned that cells of Vibrio species are Gram negative rods characteristically curved or comma-shaped but can also be straight and that this characteristic appearance is not always observed when the organism is Gram stained from solid media.

Presumptive identification was done on the basis of culture characteristics, Gram staining and oxidase test. Further biochemical testing and results from the API 20E confirmed the identification to the species describtion of *V. harveyi*. All isolates of V. *harveyi* were Gram negative, curved rods, oxidase positive, swarming on TSA (+1%NaCl) and sensitive to the vibriostatic compound O/129 (10µg and 150µg discs). The same results were obtained by (Liu *et al.,* 2004).

The use of the API 20E kit to identify Vibrio species is limited and would require supplementary tests (Katarina, 2005).

At current study *V. harveyi* strains numbers were systematically reduced with wide phenotypic representation and were identified initially by biochemical tests and subsequently byAPI and molecular tools.

The PCR assay provides a simple, rapid, and reliable tool for identification of the major Vibrio pathogens in clinical samples.

The toxR sequence variation could differentiate *V. harveyi* from closely related Vibrio species. A PCR protocol amplifying a 390-bp fragment of the *V. harveyi* toxR was established and could be useful in the specific and rapid detection of the species. Similarly (Conejero and Hedreyda, 2003) identified *V. harveyi* accurately on the basis of species-specific primers designed from toxR gene.

Although our PCR assay involves primer sets of V. *harveyi* haemolysin (vhh-F,vhh-R), no specific amplicons of the predicted size were obtained for 12 of isolated strains of *V. harveyi*(n=14).

The same results were obtained by (Al-Shimaa, 2015) for all isolated strains of *V. harveyi*. These results may be due to genotypic diversity as explained by (Zhang and Austin 2000) who characterized a haemolytic strain of *V. harveyi* (VIB 645) as highly pathogenic to salmonids and found two identical haemolysin genes (vhhA and vhhB), (Zhang *et al.,* 2001) who detected other less pathogenic strains possessed only a single gene or alternatively, no vvh gene was detected and (Conejero and Hedreyda 2004) suggested that the vhh gene is present in all *V. harveyi* strains and it may

be suitable for species specific detection by PCR, with only 85.6% gene identity with the haemolysin gene (tl) from the closely related species *V. parahaemolyticus*.

On contarary, (Ruwandeepika *et al.*, 2010) found that all the virulence genes that are typical for the Harveyi clade vibrios, including (luxR, toxRVh (toxR *V. harveyi*), vhpA (metalloprotease), chiA (chitinase), gene for serine protease, and vhh (*V. harveyi* haemolysin) were present in all the tested forty-eight bacterial isolates belonging to *V. harveyi*. However, the presence of a typical virulence genes in isolates belongs to Harveyi clade vibrios suggesting horizontal gene transfer.

Cano-Gomez *et al.*, (2009) reported that nevertheless, the pathogenicity of strongly haemolytic *V. harveyi* strains suggests that the vhh gene is not suitable as a species identification marker. This is because species-specific markers should be stable in the genome, and at the same time the involvement of vhh in virulence makes this gene susceptible to horizontal gene transfer as reported by (Waldor and Mekalanos, 1996).

Moreover, an amplification product using the set of partial haemolysin gene primers confirmed the presence of amplifiable bacterial DNA of the investigated isolates in the PCR samples with a band of 647-bp as described by (Haldar *et al.* 2010) was used to reconfirm the 16S rRNA gene-based identification of *V. harveyi* using the designed primer partial hly gene. There further analysis revealed probably a single copy of hemolysin gene, a potential virulence factor was encoded in all isolates of *V. harveyi*, although not in the same locus in the genome.

5. CONCLUSION :

In conclusion, through various phenotypic methods used in this study and commercial identification systems, it appeared that VHA is a good choice for perfect identification of *V. harveyi* species specially when concerning separating *V. harveyi* from other *Vibrio* species. Even though it is not the most rapid way to identify this species (Incubation time 48 h), it is easy and gives the perfect identification.

Also, through molecular characterization investigated at this study, it is obvious that the use of primer sets involves (*toxR gene*) is the best as species- specific marker.

All strains of *V. harveyi (n=14)* were positive for hemolysin gene when using primer sets of *partial hly- haemolysin gene* .Further analysis revealed probably a single copy of this gene was encoded in all isolates "Highly pathogenic, moderately pathogenic and non-pathogenic" although not in the same locus in the genome.

However, only two isolated strains of *V. harveyi (n=14)* gave the specific amplicons of the predicted size when using primer sets of *vhh -Vibrio harveyi haemolysin gene.*

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