

## Molecular Studies on *Vibrio* Species Isolated from Shrimps (*Penaeus Japonicas*) in Suez Canal Area

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### Abstract

The present study was planned to investigate the phenotypic and genotypic characterization of bacteria isolated from shrimps in Suez Canal area.

A total number of 210 shell diseased shrimps (*Penaeus japonicas*) were collected. Isolation of 732 isolates from shrimp muscles, cuticles, gills, haemolymph and hepatopancreas and were identified according to the morphological and biochemical tests to vibrio species (*Vibrio alginolyticus* and *Vibrio parahaemolyticus*) and others. The number of isolates for *Vibrio alginolyticus* was 568 isolates by incidence of 77.59%, *Vibrio parahaemolyticus* was 131 isolates by incidence of 17.89%. Clinical signs observed on naturally infected shrimps included dark brown to black patches scattered all over the body surface including the cuticle of the carapace, abdominal segments, uropod, pereopod (walking legs) and pleopod (swimming legs). With necrotic foci most frequently located on the cuticle of the abdominal segments. Necrosis and destruction of the pereopods, pleopods and the antennal flagellum. The eyes of some moribund shrimps were affected and became protruded and edematous (Exophthalmia). Postmortem findings included black colored gills with adhesion of gills lamellae, and there were swelling and congestion of hepatopancreas and heart, also the haemolymph changed their colour to orange (bloody haemolymph). PCR protocol used for amplification and detection of *16S rRNA* gene for all *Vibrio* isolates, and *gyrB* gene, *toxR* and *ompK* virulent genes for *V. alginolyticus*, and *flaE* gene, *tdh* and *tlh* virulent genes for *V. parahaemolyticus* isolates to confirm the diagnosis of these isolates, the positive amplification of *16S rRNA* gene showed at 663bp fragment of the extracted DNA of all vibrio isolates, the positive amplification of *gyrB* gene, *toxR* gene and *ompK* gene showed at 340bp, 173bp and 319bp fragment respectively of the extracted DNA of *V. alginolyticus* and the positive amplification of *flaE* gene,

*tdh* gene and *tlh* gene showed at 897bp, 269bp and 450bp fragment respectively of the extracted DNA of *V. parahaemolyticus*.

## Introduction

A crisis in the shrimp industry over the last few years is due to largely to an increase in virulence of pathogens, especially bacterial diseases, *Lightner (1993)*. It was recognized that various bacteria indigenous to estuarine and marine waters are potential human pathogens and that these pathogens can be concentrated in shellfish like shrimp, presenting a human health risk, *Blake et al., (1980)*.

The cuticle of crustaceans consists of a thin outer layer, the epicuticle, consists of proteolipoidal material, covering three inner chitinous layer, the exocuticle, which is pigmented and calcified, the calcified endocuticle, and the non-calcified endocuticle, *Dennell (1947)*. Chemically chitin is a polysaccharide, the outer cuticular layer of the cuticle, the epicuticle is biochemically inert, and shell erosion can occur when this layer is breached by chemical attack, injury, abrasive action of sediments, or possibly enzymatic digestion, exposing the underlying chitinous layers to an adequate number of chitin-destroying microorganisms, *Stewart (1980)*.

## Material and methods

### 1. Samples:

A total number of 210 shell diseased shrimps (*Penaeus japonicas*) were collected and taken alive from port-said governorate.

Shell diseased shrimps were taken monthly from January to October 2016 then transported alive in marine water by glass containers to the microbiological lab. in the Fish Diseases Department, Central Laboratory for Aquaculture Research in Abbassa, Abu Hamad, Sharkia and subjected to clinical, postmortem, bacteriological examinations. A total of 1050 Samples were collected from naturally infected shrimp organs, 5 organs from each shrimp, as following, muscles, cuticle, gills, haemolymph and hepatopancreas,

### 2. Clinical examination of naturally infected adult shrimps:

These were performed according to method described by *Austin and Austin (1989)* to observe the following. Abnormal coloration and lesions on carapace, Abnormal swimming movements, opacity of abdominal muscles tissue, erosions of appendages, eye abnormalities, gill abnormalities, hemolymph color. The external lesions then were recorded and photographed.

### 3. Postmortem examination of naturally infected adult shrimps:

According to *Austin and Austin (1989)* After cleaning the surface of cuticle by cotton soaked in 70% ethyl alcohol using a pair of sterile scissors with fine points and a pair of fine tipped forceps, the carapace was separated from connective tissue and the hepatopancrease

exposed in the situation the color and consistency were observed, examination of gills, foregut, midgut, hindgut, cardiac sinus, muscle, walking legs (periopods), swimming legs (pleopodes) and tail (uropods) were applied.

#### **4. Bacteriological examination:**

**A. Isolation:** adult shrimp exoskeleton was firstly cleaned with cotton socked in 70% ethyl alcohol. The following samples were separately collected; exocuticle, gills, hepatopancreas, haemolymph and muscles. The Hemolymph extracted from cardiac sinus by inserting insulin needle between cephalothorax and 1st abdominal segment after cleaning the site by ethyl Alcohol 70%, *Brady and Ernesto (1992)*. Each sample were inoculated into Tryptic Soya broth, nutrient broth and peptone water (pH 8.2) all with 2% NaCl and incubated at 18-23°C for 24- 48 hrs. Then the inocula were streaked over Thiosulphate Citrate Bile Salt Sucrose agar (TCBS), nutrient agar with 2% NaCl and Tryptic Soya agar with 2% NaCl at 25-28°C for 48 hrs. The purified suspected colonies were picked up and inoculated into nutrient agar slant and Tryptic Soya agar slant with 2% NaCl for further Identification.

**B- Identification of bacterial isolates:** the morphological and biochemical characters were carried according to *Bergey manual of systematic bacteriology (2004)*.

#### **5. Method for molecular studies:**

##### **1. DNA Extraction using QIA amp kit (Shah et al., 2009):**

After overnight culture on nutrient agar plates, one or two colonies were suspended in 20 ml of sterile distilled water, and the suspension was then heated at 100°C for 20 minutes. Accurately, 50-200 µl of the culture were placed in Eppendorf tube and the following steps were carried out:

**1-**Equal volume from the lysate (50-200 µl) was added, addition of 20-50µl of proteinase K, then incubation at 56 °C for 20-30 min. After incubation, 200 µl of 100% ethanol was added to the lysate.

**2-**The solution was added to the column and centrifuged at 8000 rpm for 1 min. then the supernatant was discarded.

**3-**The sediment was washed using AW1 buffer (200 µl), the column was centrifuged at 8000 rpm / 1 min, and the filtrate was discarded.

**4-** The column was placed in a new clean tube then, 25-50 µl from the Elution buffer was added, centrifuged at 8000 rpm/1min. Then the column was discarded. The filtrate was put in clean tube containing the pure genomic DNA. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

##### **2. DNA amplification:**

**1-DNA amplification for detection of Vibrio species (Espeneira et al., 2010):**

The amplification was performed on Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany) using 20-µl reaction volumes. Each

reaction contained 2 units of AmpliTaq Gold, and final concentrations of each deoxynucleoside triphosphate (dNTP) and MgCl<sub>2</sub> of 0.2 mM and 1.5 mM, respectively, and primer concentrations ranged from 0.05 to 1 µM. Thus, a typical 20 µl reaction mixture contained 1.5 µl crude lysate, 0.2 µl of AmpliTaq Gold, 2 µl dNTP stock (2 mM each), 2 µl 10x buffer, 1.2 µl MgCl<sub>2</sub> stock (25 mM), 2.9 µl double distilled water and the various amounts of each 10 µM stock of the primers: 16S rRNA primers, 0.1 µl each and trh primers, 0.5 µl each. The thermal cycling profile was as follows: initial denaturation for 15 minutes at 93°C followed by 35 cycles at 92°C for 40 seconds, 57°C for 1 minute and 72°C for 1.5 minutes and a final soaking at 72°C for 7 minutes.

PCR amplicons were electrophoresed in 1.5% agarose for one hour at 100 V and then visualized by ethidium bromide staining and UV illumination. The 10 µl amplified products were detected by 2% of agarose gel electrophoresis stained with ethidium bromide (5 µg/ml) at 100 volts for 1 hour then visualized and captured on UV transilluminator. A 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes.

### **2-Amplification of virulence genes of *V. parahaemolyticus* (Khalil *et al.*, 2014):**

The final volume of the reaction mixture was adjusted to 50 µL with

sterile deionised distilled water. Amplification of DNA segment was performed) with the following temperature cycling parameters; initial denaturation at 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 sec, primer annealing at 57°C for 30 sec, primer extension at 72 °C for 1 min and a final extension at 72 °C for 2 min. Ten µL of each amplified product was loaded in 1.5% agarose gel in 1X Tris-boric acid-EDTA buffer [TBE: 0.089M Trisbase, 0.089M boric acid and 0.002 M EDTA (pH 8.0)] at 100 volts for 40 minutes.

### **3-Amplification of virulence genes of *V. alginolyticus* (Cai *et al.*, 2009):**

Polymerase chain reaction primers were designed to target the *toxR* and *ompK* genes of *V. alginolyticus*. All primers were custom synthesized by Shanghai Sangon Biologic Engineering and Technology and Service, China. The isolated bacteria were tested with a single PCR pair, and then by multiplex PCR respectively. The single-pair PCR reactions were performed in 50 µL of reaction mixture, which contained 2 µL crude DNA template, 1 × buffer (100 mmol L Tris-Cl pH 8.0, 50 mmol L KCl and 1.5 mmol L MgCl<sub>2</sub>), 1 µmol L of each primer, 0.2 mmol L (each) deoxynucleoside triphosphate (dNTP mix) and 1 U ExTaq™ DNA polymerase (all from Takara, Japan). A multiplex PCR reaction was performed in a

50 µL reaction mixture, which contained 2 µL crude DNA sample, 1 × buffer (100 mmol L Tris-Cl pH 8.0, 50 mmol L KCl, 1.5 mmol L MgCl<sub>2</sub>), 0.5 µmol L of each primer, 0.25 mmol L (each) dNTP and 2 U ExTaq<sup>TM</sup> DNA polymerase. The PCR reactions were performed in a DNA thermal cycler 9700 (Applied Biosystems, Foster City, CA, USA) with the following parameters: 94 °C for 5 min, followed by 30 cycles each at 94 °C for 1 min, 65 °C for 1 min and 72 °C for 1.5 min, with a final step of 72 °C for 10 min. All amplified DNAs (10 µL) were detected in 0.8% agarose with ethidium bromide by electrophoresis using 1 × TAE. The gels were run at 60 V for 60 min. and the DNAs were visualized and photographed using UV transilluminator.

**Primer sequences of Vibrio species used for PCR identification system:**

Application of PCR for identification and characterization of Vibrio species using different primers (Pharmacia Biotech) as shown in (Table 1).

**Primer sequences of V. parahaemolyticus for virulence factors:**

Molecular characterization of virulence factors including thermostable direct hemolysin (*tdh*) and thermolabile hemolysin (*tlh*) genes of *V. parahaemolyticus* was adopted by using the following primers as shown in (Table 2).

**Primer sequences of V. alginolyticus for virulence factors:**

Accurately, the molecular characterization of virulence genes for *V. alginolyticus* represented by exotoxin (*toxR*) and outer membrane protein (*ompK*) were adopted as shown in (Table 3).

**Table (1): Primer sequences of Vibrio species used for PCR assay:**

Vibrio Spp.	Primer	Oligonucleotide sequence (5' → 3')	Product size (bp)	References
All	<i>16S rRNA</i> (F)	5' CGGTGAAATGCCGTAGAGAT '3	663	Rao and Surendran (2013)
	<i>16S rRNA</i> (R)	5' TTACTAGCGATTCCGAGTTC '3		
<i>V. parahaemolyticus</i>	<i>flaE</i> (F)	5' GCAGCTGATCAAAACGTTGAGT'3	897	Tarr <i>et al.</i> , (2007)
	<i>flaE</i> (R)	5' ATTATCGATCGTGCCACTCAC '3		
<i>V. alginolyticus</i>	<i>gyrB</i> (F)	5'ATTGAGAACCCGACAGAAGCGAAG'3	340	Zhou <i>et al.</i> , (2007)
	<i>gyrB</i> (R)	5'CCTAATGCGGTGATCAGTGTTACT'3		

**Table (2):** Primer sequences of *V. parahaemolyticus* for virulence factors:

Target gene	Oligonucleotide sequence (5' → 3')	Product size (bp)	References
tdh (F)	5' TTGGAAACGGTTAAAACGAA'3	269	Bej <i>et al.</i> , (1999)
tdh (R)	5' GAACCTTCCCATCAAAAACA '3		
tlh (F)	5' TCGCATCAAACGACAAACG '3	450	
tlh (R)	5' GCGGTACTCTATAAGTGCC '3		

**Table (3):** Primer sequences of *V. alginolyticus* for virulence factors:

Target gene	Oligonucleotide sequence (5' → 3')	Product size (bp)	References
<i>toxR</i> (F)	5' CAGAAGAATCGGAAGAACA'3	173	Cai <i>et al.</i> ,(2009)
<i>toxR</i> (R)	5' TAGAATGACGCACAAAGG '3		
<i>ompK</i> (F)	5' GCGGGTCGCTCTGGTATT'3	319	
<i>ompK</i> (R)	5' TTGCCATCGTAAGTGCTGTA '3		

### Result and discussion

The clinical examination of the moribund shrimps showed swimming lethargically, stop feeding, general weakness, loss of balance and whirling movement with varied degree of shell lesions appeared as dark brown to black patches scattered all over the body surface including the cuticle of the carapace, abdominal segments, uropod, pereopod (walking legs) and pleopod (swimming legs), (photos 1&2). With necrotic foci most frequently located on the cuticle of the abdominal segments, (photo 3). Necrosis and destruction of the antennal flagellum and the eyes of some moribund shrimps were affected and became protruded and edematous (Exophthalmia), (photo 4). The drawn haemolymph changed their colour to reddish (bloody haemolymph), (photo 5). These results agreed with *El-bouhy et al. (2006)* who reported that the clinical examination of the naturally infected adult shrimp with vibriosis

showed black spots to brown spots on the carapace and the abdominal segments. Erosions and black spots on the uropod, pleopods and pereopods. Dirty appearance and total blackness of all body surfaces. Necrosis and destruction of the pereopods, pleopods and uropods, slow motion, loss of the appetite, swim around the pond wall, and the shell was attached by algae. the destruction of the exoskeleton and appendages may be attributed to the chitinolytic and proteolytic enzymes produced by vibrio sp.

Then by *Sharshar and Azab (2008)* who described shrimps suffered from Vibriosis showed dark brown focal lesions and necrosis of appendage tips. Moribund prawns assembled at the edges of ponds and swim slowly near the surface.

Similar finding was previously obtained by *Zhang et al. (2014)* who recorded that infected shrimp showed lethargy, swimming near the water surface and close to pond edges, breaking of antenna, and

reduction in food consumption. These results concomitants with the previously reported by **Kumaran and Citarasu (2016)** who recorded that the diseased shrimps with vibriosis showed symptoms such as lethargy, loss of balance, whirling movement and general weakness. The common postmortem findings in the moribund shrimps revealed that the gill lamellae adhered each other and changed their colour to black, (photo 6) and there were swelling and congestion of hepatopancreas and heart, (photo 7). These results agreed with **El-bouhy et al. (2006)** who mentioned that the naturally infected adult shrimp revealed that black spots of the gills. Hepatopancreas appears congested, swollen, soft and surrounded by congested fluid. Congestion of the heart and the intestine free from any food particles.

The isolates were identified according to the morphological and biochemical tests to *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, as shown in (Tables 4&5) and (Photos 8 & 9). The obtained results were nearly similar to that recorded by several studies including **Baticados et al. (1986)** who isolated chitinolytic bacteria from tiger prawn (*penaeus monodon*) from brackish water pond suffered from soft shell, the isolates were *Vibrio* and *Aeromonas* species, **Hung et al. (1991)**. **Thakur et al. (2003)** identified 4 species of *Vibriosis* from

the hepatopancreas of moribund shrimp with vibriosis namely *Vibrio parahaemolyticus*, *Vibrio alginolyticus* and others *vibrio* sp. based on their cultural, morphological, biochemical characteristics, **Ferrini et al. (2008)** identified 92 *Vibrio* strains which isolated over a period of 9 years from different sources (national and imported fishery products, shellfish, seawater from aquaculture settings) and belonging to 2 species relevant for human health and fish pathology, *V.alginolyticus* and *V.parahaemolyticus* and many *Vibrio* species have which described as important fish and shellfish pathogens, **Deviet al. (2009)** who identified *Vibrio parahaemolyticus* isolates from shrimp farms along the southwest coast of India, **Abraham et al. (2013)** identified the isolated bacteria to *Vibrio* spp. from the hepatopancreas, hemolymph, intestine, gills and eroded portion of the exoskeleton of the cultured shrimp, **Prakash and Karmagam (2013)** recorded during a study on bacterial flora associated with fresh water prawn, *Macrobrachium rosenbergii* that the majority of the bacteria isolated were Gram-negative. In prawn samples *Vibrio* sp. And *Aeromonas* sp., represented the dominant flora, **Heenatigala and Fernando (2016)** identified 24 isolates belonged to *Vibrio* species which are responsible for vibriosis in shrimps. Those were *Vibrio alginolyticus*, *V. parahaemolyticus*

and others *Vibrio*. Most frequently isolated species was *V. parahaemolyticus* during a bacteriological study which was undertaken in semi intensive shrimp (*Penaeus monodon*) culture ponds, **Kumaran and Citarasu (2016)** could isolate *V. parahaemolyticus* from the infected shrimp farms at Marakkanam, Kancheepuram district of Tamilnadu and *Artemia franciscana* culture tank at CMST campus, **Mastan and Begum (2016)** isolated five species of *Vibrio* bacteria from diseased shrimp, *Litopenaeus vannamei* with vibriosis, collected from commercial shrimp cultured ponds. The isolated bacterial species were identified as *Vibrio parahaemolyticus*, *Vibrio alginolyticus* and other vibrio species.

In this study the total of 1050 Samples were collected from 210 naturally infected shrimp organs, 5 organs from each shrimp, as following, muscles, cuticle, gills, haemolymph and hepatopancreas, and the number of positive samples were 732 with rate of infection 69.71%. The number of isolates for *Vibrio alginolyticus* was 568 isolates by incidence of 77.59%, *Vibrio parahaemolyticus* was 131 isolates by incidence of 17.89%. The result obtained showed that the highest prevalence of isolated bacteria was *Vibrio* species especially *V. alginolyticus* which explained by **Baffone et al. (2001)** and **Saifedden et al. (2016)** who

mentioned that *Vibrio alginolyticus*, *V. parahaemolyticus* were a halophilic gram negative bacteria, widely spread geographically in marine and estuarine waters. And coincided with that obtained previously by **Eduardo et al. (1998)** who isolated 172 bacterial isolates from the hepatopancreas of *Penaeus monodon* and found that most 90.12% were *Vibrio* species, moreover **Sudheesh et al. (2002)** recorded that *V. alginolyticus* and *V. parahaemolyticus* are two important pathogenic species. They considered opportunistic pathogens and isolated from shrimps suffering from vibriosis, meanwhile **El-bouhy et al. (2006)** isolated about 173 bacterial isolates from the 135 samples (larvae, adults and water samples) of diseased shrimp with vibriosis; all of them are belonging to the *Vibrio* species and represented by 31.8% *Vibrio alginolyticus*, 23.7% *Vibrio parahemolyticus*, 27.7% *Vibrio harveyi*, 8.1% *Vibrio anguillarum* and 8.6% *Vibrio campbelli*. *Vibrio alginolyticus* was isolated in 50%, 40%, 30% and 30% from the examined *P. japonicus*, *P. kerathurus*, *P. semisulcatus* and larvae respectively. *Vibrio parahemolyticus* was isolated in 43.3%, 26.7%, 30% and 25% from the examined *P. japonicus*, *P. kerathurus*, *P. semisulcatus* and larvae, respectively, finally by **Abraham et al. (2013)** Isolated *Vibrio* spp., *Aeromonas* spp. and *Pseudomonas* spp. from the



hepatopancreas, hemolymph, intestine, gills and eroded portion of the exoskeleton of the cultured shrimp *Penaeus monodon*. *Vibrio* species were the dominant bacterial flora in the affected organs.

PCR protocol used for amplification and detection of *16S rRNA* gene for all *Vibrio* isolates and *gyrB* gene, *toxR* and *ompK* virulence genes for *V. alginolyticus* and *flaE* gene, *tdh* and *tlh* virulence genes for *V. parahaemolyticus* isolates to confirm the diagnosis of these isolates, the positive amplification of *16S rRNA* gene showed at 663bp fragment of the extracted DNA of all *vibrio* isolates, the positive amplification of *gyrB* gene, *toxR* gene and *ompK* gene showed at 340bp, 173bp and 319bp fragment respectively of the extracted DNA of *V. alginolyticus* and the positive amplification of *flaE* gene, *tdh* gene and *tlh* gene showed at 897bp, 269bp and 450bp fragment respectively of the extracted DNA of *V. parahaemolyticus*, these result harmonies with the previously

mentioned by several studies including *Taniguchi et al. (1985)* who found that *Vibrio parahaemolyticus* strains produce a thermolabile direct haemolysin (*tlh*) that is species-specific, *Robert-Pillotet et al. (2004)* who recorded that the occurrence of the hemolysin gene, *tdh* in *Vibrio parahaemolyticus* strains isolated from environmental samples collected in two French coastal areas, clinical samples, and seafood products imported into France and mentioned that The *tdh* gene plays an important roles in virulence, *Gopal et al. (2005)* who mentioned that *V. parahaemolyticus* was confirmed by PCR targeting the *toxR* gene also the presence of the virulence associated *tdh* (thermostable direct haemolysine) gene in the *Vibrio parahaemolyticus* isolates was also detected by PCR, *Gargout et al. (2015)* who recorded that the *Vibrio alginolyticus* possessed *toxR* (toxic) gene.



**Photo (1):** Diseased adult *penaeus japonicas* shrimp showed black patches scattered all over the body surface including the cuticle of the carapace, abdominal segments, uropod, periopod and pleopod.



**Photo (2):** *Shell diseased adult penaeus japonicas shrimp showed black discolouration spot area on the carapace.*



**Photo (3):** *Shell diseased adult penaeus japonicas shrimp showed necrotic foci on the cuticle of the abdominal segments.*



**Photo (4):** *Diseased adult penaeus japonicas shrimp showed destruction of the antennal flagellum and edematous eyes.*



**Photo (5):** Diseased adult *penaeus japonicas* shrimps showed reddish colour of drawn haemolymph (bloody haemolymph).



**Photo (6):** Shell diseased adult *penaeus japonicas* shrimp showed black colored gills with adhesion of gills lamellae.



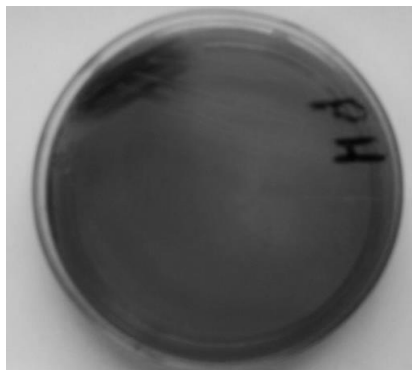
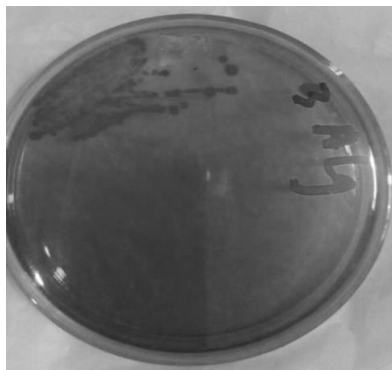
**Photo (7):** Diseased adult *penaeus japonicas* shrimp showed swelling and congestion of the hepatopancreas and the heart.

**Table (4):** Morphological and biochemical characters of suspected *Vibrio alginolticus* isolated from naturally infected shrimps (*Penaeus japonicus*):

Items	<i>Vibrio alginolticus</i>
Gram stain	Negative
Motility test	+
shape	Curved rods
Cytochrome Oxidase	+
Catalase	+
Growth on TCBS	+Yellow colonies
Growth at 43° c	+
<b>Growth on media contain sodium chloride weight per volume:</b>	
Growth at 0.0% NaCl	-
Growth at 3.0 %NaCl	+
Growth at 6 .0% NaCl	+
Growth at 8.0 %NaCl:	+
Growth at 10.0% NaCl	+
Lysin decarboxylase	+
Arginin dihydrolase	+
Ornithin decarboxylase	+
Esculin hydrolysis	-
Voges-proskauer test	+
ONPG hydrolysis	-
Nitrate reduction	+
Gas from glucose	-
Acid from L-Arabinose	-
Acid from inositol	-
Acid from sucrose	+
Acid from salicin	-

**Table (5):** Morphological and biochemical characters of suspected *Vibrio parahemolyticus* isolated from naturally infected shrimps (*Penaeus japonicus*):

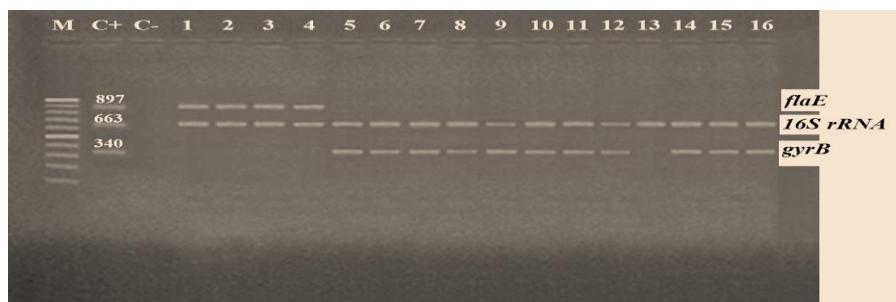
Items	<i>Vibrio parahemolyticus</i>
Gram stain	Negative
Motility test	+
shape	Curved rods
Cytochrome Oxidase	+
Catalase	+
Growth on TCBS	Green colonies
Growth at 43° c	+
<b>Growth on media contain sodium chloride weight per volume:</b>	
Growth at 0.0% NaCl	+
Growth at 3.0 %NaCl	-
Growth at 6 .0% NaCl	+
Growth at 8.0 %NaCl:	+
Growth at 10.0% NaCl	-
Lysin decarboxylase	+
Arginin dihydrolase	+
Ornithin decarboxylase	+
Esculin hydrolysis	-
Voges-proskauer test	-
ONPG hydrolysis	-
Nitrate reduction	+
Gas from glucose	-
Acid from L-Arabinose	Different reaction
Acid from inositol	-
Acid from sucrose	-
Acid from salicin	-



**Photo (8):** *Vibrio alginolyticus* on TCBS

**photo (9):** *Vibrio*

*parahemolyticus* on TCBS



**Photo (10):** Agarose gel electrophoresis of multiplex PCR for characterization of 16S rRNA (663bp) for all *Vibrio* Spp., *gyrB* (340 bp) for *V.alginolyticus* and *flaE* (897 bp) for *V.parahaemolyticus*.

**Lane M:** 100 bp ladder as molecular size DNA marker.

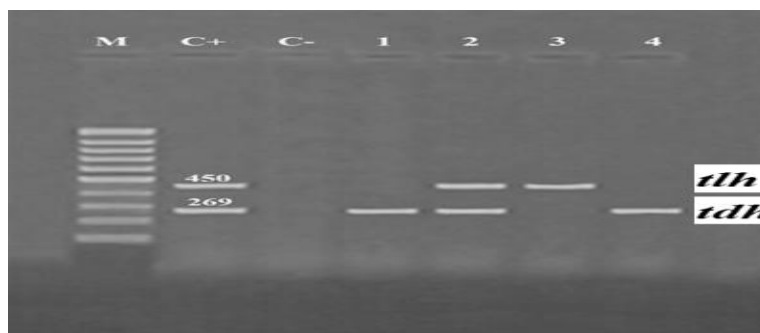
**Lane C+:** Control positive for 16S rRNA, *gyrB* and *flaE* genes.

**Lane C-:** Control negative.

**Lanes from 1 to 4:** Positive *V.parahaemolyticus* for 16S rRNA and *flaE* genes.

**Lanes 5 to 12 & 14 to 16:** Positive *V.alginolyticus* for 16S rRNA and *gyrB* genes.

**Lane 13:** Positive *Vibrio* Spp. for 16S rRNA gene.



**Photo (11):** Agarose gel electrophoresis of multiplex PCR of *tdh* (269 bp) and *tlh* (450 bp) virulence genes for characterization of *V. parahaemolyticus*  
**Lane M:** 100 bp ladder as molecular size DNA marker.

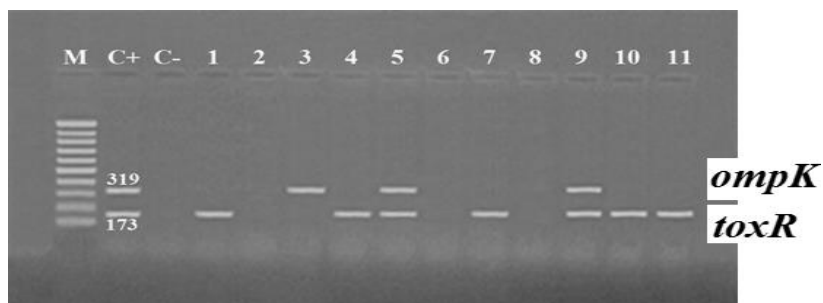
**Lane C+:** Control positive *V. parahaemolyticus* for *tdh* and *tlh* genes.

**Lane C-:** Control negative.

**Lanes 1 & 4:** positive *V. parahaemolyticus* strains for *tdh* gene.

**Lane 3:** positive *V. parahaemolyticus* strain for *tlh* gene.

**Lane 2:** Positive *V. parahaemolyticus* strain for *tdh* and *tlh* genes.



**Photo (12):** Agarose gel electrophoresis of multiplex PCR of *toxR* (173 bp) and *ompK* (319 bp) virulence genes for characterization of *V. alginolyticus*.

**Lane M:** 100 bp ladder as molecular size DNA marker.

**Lane C+:** Control positive *V. alginolyticus* for *toxR* and *ompK* genes.

**Lane C-:** Control negative.

**Lanes 1, 4, 7, 10 & 11:** Positive *V. alginolyticus* strains for *toxR* gene.

**Lane 3:** Positive *V. alginolyticus* strain for *ompK* gene.

**Lanes 5 & 9:** Positive *V. alginolyticus* strains for *toxR* and *ompK* genes.

**Lanes 2, 6 & 8:** Negative *V. alginolyticus* strains *toxR* and *ompK* genes.

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## الملخص العربي

في هذه الدراسة تم عمل التوصيف الظاهري والجيني للبكتريا من الجمبري في قناة السويس فقد تم جمع 210 عينة من الجمبري المريض وأخذ وهو حي من محافظة بورسعيد في الفترة من شهر يناير الي أكتوبر 2016 وتم نقلها الى معمل البكتريولوجي بقسم أمراض الأسماك بالمعمل المركزي لبحوث الثروة السمكية بالعباسة – أبوحماد- بمحافظة الشرقية وتم فحصها اكلينيكيًا وتم عمل الصفة التشريحية والفحوص البكتريولوجية وقد تم التوصل في هذه الدراسة الى النتائج التالية: تم تصنيف انواع البكتريا التي تم عزلها من الجمبري من العضلات، الغطاء الكيتيني، الخياشيم، الهيموليمف والهيئاتونكرياس كالاتي: فيبريو الجينوليتكس و فيبريو باراهيموليتيكس وأخرى وكانت عدد العزلات البكتيرية بالنسبة للعدد الكلي (732) من العزلات كالاتي وبالفحص الاكلينيكي للجمبري المصاب وجد أنه كان يسبح ببطء مع انتشار بعض العلامات المرضية مثل بقع بنية الي سوداء اللون على جميع أنحاء جسم الجمبري غالبا على الصدفة و البطن والذيل وارجل المشي والعموم مع هشاشة ونخر و في الغطاء الكيتيني للبطن وأيضا نخر وفقد في الأرجل وقرون الاستشعار. وفي بعض الأحيان وجد فقد في أحد الأرجل وأيضا جحوظ العين. أما الصفة التشريحية فكانت التصاق بالخيوط الخيشومية وتغير لونها الي الأسود وأيضا تورم وإحتقان في الهيئاتونكرياس والقلب. وتغير في لون الهيموليمف الي البرتقالي. وقد تم في هذا البحث استخدام اختبار البلمرة المسلسل في تأكيد وتصنيف وضراوة ميكروبات فيبريو الجينوليتكس و فيبريو باراهيموليتيكس وذلك عن طريق تحديد *16S rRNA* الجين الخاص بميكروب الفيبريو وكذلك *tdh* , *tlh* الجينات المعبرة عن ضراوة الفيبريو باراهيموليتيكس وجينات *toxR* , *ompK* المعبرة عن ضراوة الفيبريو الجينوليتكس وقد أعطيت كل العترات المستخدمة في الاختبار نتائج موجبة مع الجينات الخاصة بكل منهم.