1st International Conference (Central Laboratory For Aquaculture Research In Cooperation With Worldfish), Cairo, Egypt, 20-22 November, 2017, Vol. 1. *167-185*

PCR TARGETED THE VIRULENCE GENES OF *VIBRIO* SPECIES AFFECTING LONGNOSE PARROTFISH (*Hipposcarus harid*) INHABITING THE RED SEA BASIN AT HURGHADA, EGYPT

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Abstract

Vibriosis is a life-threatening septicemic bacterial disease affecting a wide range of fish species. In this study, several mortalities in the marine Longnose parrotfish, *Hipposcarus harid*, inhabiting the Red Sea basin in Hurghada, Egypt have been recorded. The clinicopathological picture of the examined fish was investigated, and the bacteriological examination was also done. The retrieved bacterial isolates were phenotypically identified. Further molecular identification of *Vibrio* spp using a multiplex PCR using different species-specific primers for *ToxR* (*V. alginolyticus*), and *Tlh* (*V. parahaemolyticus*) virulence genes. The examined fish displayed generalized septicemic signs; hemorrhages of skin and at the base of all fins with tail rot. Additionally, petechiae were found over the liver, congested spleen, intestine and gills, with petechial hemorrhages over the ovaries and peritoneum. Several *Vibrio* species were identified. It has been found that the prevalence of the identified *Vibrio spp* was 21.54, and 38.46 % for *V. alginolyticus*, and *V. parahaemolyticus* respectively. It is concluded that multiplex PCR helped the accurate detection of pathogenic *Vibrio* spp affecting the marine Longnose parrotfish, *Hipposcarus harid*.

Keywords: Vibriosis – IGS-PCR - Longnose parrotfish.

INTRODUCTION

Coral reef fishes, especially the marine Longnose parrotfish, *Hipposcarus harid* is widely located in coral reefs of Indian ocean and Red sea region especially at Hurghada, Egypt (Froese and Pauly, 2016). Several disease epizootics have been demonstrated to cause serious and potential effects of the coral reef fishes such as Viral (Rhabdoviruses, Iridoviral disease (Lymphocystis viral disease) (LVD), toxic dinoflagellates, ectoparasitic (metazoan and protozoan ectoparasites) as well as several fungal infections (Panek, 2006).

Studies of the bacterial pathologies encountered to cause disease problems in the red sea coral reef fishes were somewhat little; whereas, *Tenacibaculum maritimum*, was isolated and identified from diseased marine coral reef fishes as Broomtail wrasse, Picasso Trigger fish, and Black damsel fish (Abd El-Galil and Hashiem, 2011; Abd El-Galil and Hashem, 2012 and Haridy *et al*., 2015), *Photobacterium damselae* from Yellow tail surgeon (*Zebrasoma xanthurum*) (Hashem, 2015), *Vibrio harveyii* from Arabian Surgeon (*Acanthurus sohal*) (Hashem and El-Barbary, 2013), *V. alginolyticus* from ornamental Bird Wrasse (Abd El-Galil and Mohamed, 2012), and β-hemolytic *Carnobacterium maltaromaticum* from Longnose Parrot Fish (*Hipposcarus harid*) (Mohamed *et al*., 2017).

Vibrio spp (Family Vibrionaceae) can be defined as a halophilic (correlated to high salinities (30–35 ppt)), facultatively aerobic, gram-negative bacterium. Because it is considered as a normal component of the fish intestinal flora, under stress-related, immune suppressant conditions, they become pathogenic and cause devastating fish disease (Nagasawa and Cruz-Lacierda, 2004 and Haenen *et al*., 2014).

Vibriosis, a septicemic life-threatening worldwide bacterial disease (Ruwandeepika *et al*., 2012), have numerous pathogenic species (*Listonella anguillarum, V. harveyii, V. vulnificus, V. alginolyticus, V. fisherii, V. cholerae, V. ordalii, V. parahaemolyticus* and several others), that affect several fish species (cultured, wild, off-shore, and ornamental fish species), freshwater

fishes (Khalil and Abdel-Latif, 2013), marine fishes (Magariños *et al*., 2011; Moustafa *et al*., 2014), Shrimps (Indian prawn) (El far *et al*., 2015), and marine ornamental fishes (Sonia *et al*., 2012), causing high mortalities with significant economic impact (El-Bouhy *et al*., 2016).

Studies concerning the utilization of targeted the multiplex PCR, as a characteristic diagnostic tool for demonstration of various specific genes of *Vibrio* spp, were investigated (Maeda *et al*., 2000 and Xie *et al*., 2005). Data focused on studying the bacterial pathogens encountered in disease conditions and the mortalities of the coral reef fishes in Egypt was limited, therefore, in this context, the clinical picture caused by the defined *Vibrio* spp from diseased and moribund *Hipposcarus harid* was studied. Additionally, molecular diagnosis of the retrieved isolates through multiplex PCR was also demonstrated.

MATERIALS AND METHODS

Examined Fish:

A total of thirty (30) suspected diseased, moribund, and freshly dead coral reef seawater fish, *Hipposcarus harid*, were collected from the indoor rearing aquaria of the National Institute of Oceanography and Fisheries (NIOF) and the fisher port at Hurghada, Red Sea province (Egypt). Surveyed fish were examined clinically (recording the clinical signs and postmortem (PM) lesions) (Buller, 2004), and bacteriologically (Austin and Austin, 2012).

Bacteriological examination: Isolation and identification:

Samples were taken under aseptic conditions from the examined fish spleen, heart, kidney, and liver, and was enriched in trypticase soya broth has 2% NaCl, incubated under aerobic conditions at 37 °C for 24 hrs. The grown bacterial colonies were then cultured on TCBS (thiosulfate citrate bile salts sucrose) agar plates (Oxoid, UK), that were accordingly incubated at 28 °C for 18-24 hrs. The presumptive retrieved bacterial isolates were identified

biochemically using common traditional identification keys (Motility, Gram staining, traditional biochemical tests) (Alsina and Blanch, 1994).

As stated by the manufacturer's instructions, a commercial API 20E strips (BioMerieux, France) were further used for identification of the bacterial biochemical characters, and the isolates were then identified (Nicky, 2004). The purified bacterial strains were then stored in semisolid nutrient agar and kept in the refrigerator at - 20 °C for further molecular diagnosis.

Molecular diagnosis of the identified *Vibrio* spp:

Reference strains:

The reference *Vibrio* spp were gotten from the American Type Culture Collection (ATCC), that were kept, processed, and handled referring to the instructions of the producer [the reference strains for *V. parahaemolyticus* (ATCC-17802), and *V. alginolyticus* (ATCC-17749)] that were used as positive control samples.

Handling the identified strains:

The bacterial isolates that were classified as *Vibrio* spp biochemically, were then allowed to grow overnight in trypticase soya broth (Oxoid) containing 2% NaCl and left incubated at 37°C. The overnight grown broth cultures were then transferred into microcentrifuge tubes (1.5 ml tubes) and centrifuged at 8,000 rpm/15 minutes at room temperature. Discard the supernatant completely, and the bacterial pellets present at the sediment were then obtained for extraction of the bacterial nucleic acid.

Protocol of DNA extraction using QIAamp DNA Mini Kit:

The bacterial DNA was extracted, for the polymerase chain reaction (PCR), using the commercial kits (QIAamp DNA Mini Kit), (Qiagen, Hilden, Germany). The bacterial pellets were re-suspended in phosphate buffer saline (PBS) (Sigma Chemical Co., St. Louis, Mo.) and incubated for 30 min at 37°C. Then, add 20 µl of QIAGEN protease (Proteinase K), and 200 µl of the lysis

buffer (Qiagen) and were mixed using pulse-vortexing for 15 seconds, and the suspension was incubated at 56°C for 10 min.

A 200 µl of ethanol (96-100 %) was added, and mix again by pulse-vortexing for 15 seconds, the resulting mixture was applied carefully to the QIAamp DNA spin column (Qiagen). The DNA that bound to the column was washed in two centrifugation steps (8000 rpm / 1min for the 1st step and 14000 rpm / 3 min for the 2nd step of the centrifugation) using two different wash buffers (Buffer AW1 and AW2) to improve the purity of the eluted DNA. The purified DNA was then eluted carefully from the column in 100-200 l Buffer AE. The concentration of the eluate DNA was then measured by absorbance at 280 nm wavelength using a spectrophotometer. The resulted eluted DNA was finally served as a template in the PCR protocol.

Multiplex PCR targeting the virulence genes of *Vibrio* spp:

The primers used for amplification of virulence genes of *Vibrio spp* were demonstrated in Table (1).

Table 1. Primers used for the PCR of the targeted *Vibrio* spp.

Targeted *Vibrio* spp	**Genes**	**sequence (5' to 3')**	**Size (bp)**	**References**
V. alginolyticus	*ToxR* gene	F-TOXR: 5´- GATTAGGAAGCAACGAAAG-3´ R-TOXR: 5´- GCAATCACTTCCACTGGTAAC-3´	658	(**Xie *et al.*, 2005**)
V. parahaemolyticus	*Tlh* gene	F-TLH: 5´-AAAGCGGATTATGCAGAAGCACTG-3´ R-TLH: 5´B-GCTACTTTCTAGCATTTTCTCTGC-3´	450	(**Bej *et al.*, 1999**)

(F) Forward primer, (R) Reverse primer, (bp) base pair.

The PCR assay setup:

It was done in a total volume of 50 µl using 25 µl of HotStarTaq Master Mix (Qiagen) (2.5 units per reaction of DNA polymerase, 0.2 mM of each deoxynucleotide triphosphate dNTPs, and buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM MgCl2), 20 pmol of each primer, and 50 ng of the DNA template.

Processing the mixture in a Master cycler (Eppendorf, Milan, Italy). The thermocycling program adjusted according to the Table (2).

Detection of the amplified product:

The amplified products were analyzed by electrophoresis on a 1.5% agarose NA gel (Pharmacia, Uppsala, Sweden) in Tris-borate-EDTA (TBE) buffer (its composition is; 0.89 M Tris, 0.89 M boric acid, 0.02 M EDTA, pH 8.0), stained using ethidium bromide and then visualized using a UV light transilluminator. A 100-1000-bp DNA Ladder (Gene Ruler ^{TM}) (Biotools; B & M Laboratories S.A., Madrid, Spain), obtained by Lab Service Co. Egypt, was used as a molecular weight marker.

Table 2. Programming of thermal cycler of the PCR assay.

No. of cycles	Temperature (°C)	Time	Target
1 Cycle	95	3 min	Initial denaturation
35 cycles	a) 95	30 secs	Denaturation
	b) 64	30 secs	Annealing
	c) 72	1 min	Extension
1 cycle	72	5 min	Final extension
1 cycle	- 20	Until used	Preservation

The template-free reactions were included in the PCR setup as negative controls.

RESULTS

Results of clinical examination:

The clinical signs and necropsy findings of the diseased *Hipposcarus harid* were demonstrated in figures 1 and 2, whereas, the external signs were detached scales, congestion of the gills, and several areas of hemorrhages over the whole body of the fish, particularly at fins bases, mouth region, and abdomen (Fig. 1). Additionally, the PM lesions were found in the form of generalized septicemic signs, congestion of the liver, spleen, intestine, with hemorrhages over the ovaries and peritoneum and with several petechial hemorrhages were found over the liver (Fig. 2).

Bacterial identification and characterization:

The morphological and phenotypic characterization of the retrieved bacterial isolates was demonstrated in Table 3.

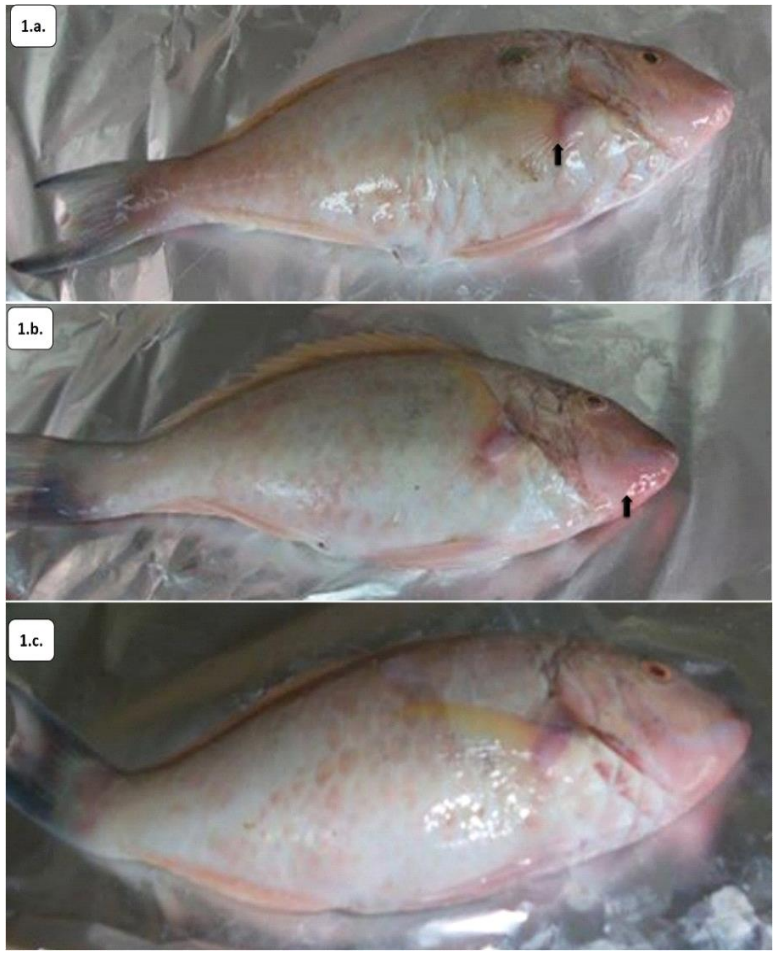

Fig. 1. The external signs of *Hipposcarus harid* showed generalized septicemic signs; skin hemorrhages, and hemorrhages at the base of fins (arrow at pectorals) (Photo 1.a.), at isthmus (Photo 1.b.) with tail rot.

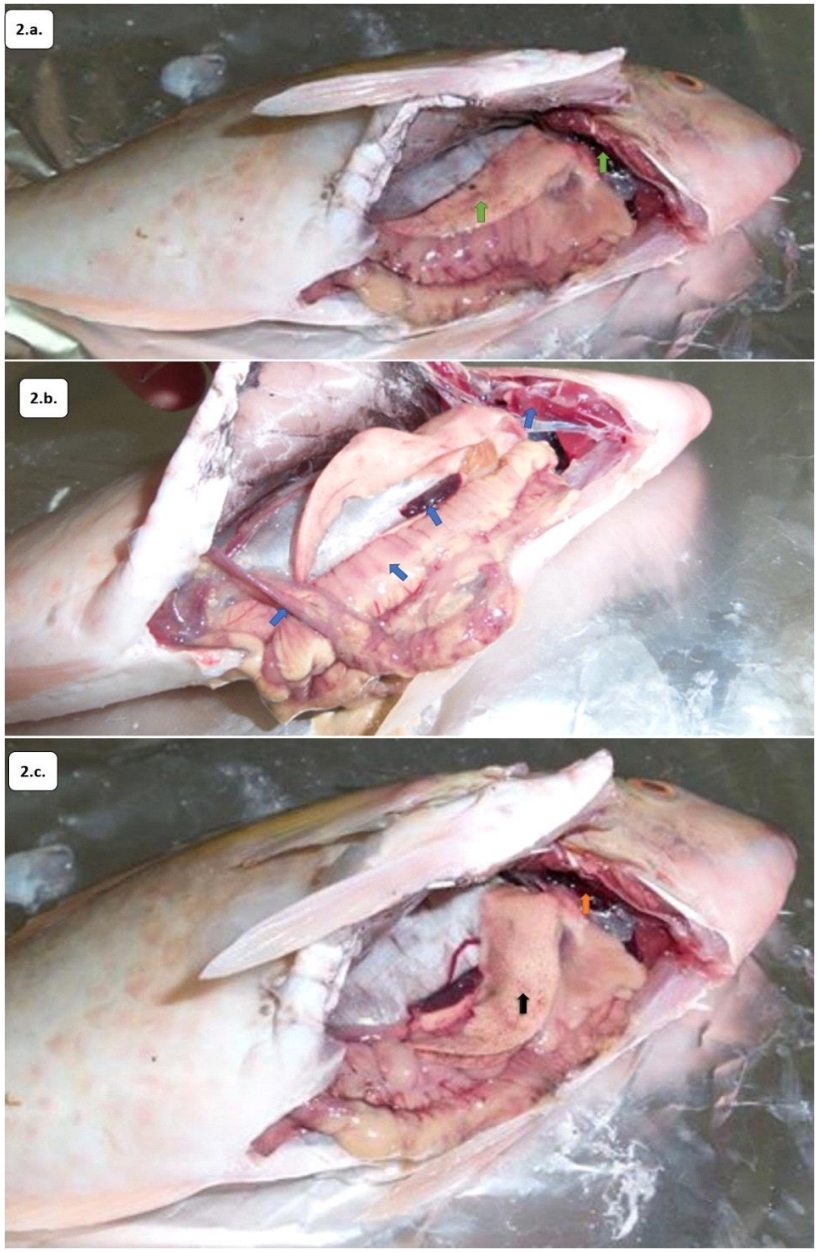

Fig. 2. The PM lesions of *Hipposcarus harid* showed congestion of the gills and liver (green arrows) (Photo 2.a.), congested spleen, intestine, and gills, with hemorrhages over the ovaries and peritoneum (blue arrows) (Photo 2.b.), and with several petechiae were found over the liver (black arrow) (Photo 2.c.).

Table 3. Colony morphology, cultural and biochemical characteristics of the retrieved *Vibrio* spp from diseased *Hipposcarus harid*.

Items	*V. alginolyticus*	*V. parahaemolyticus*
Gram stain reaction	Gram-negative	Gram-negative
Cell morphology	Rod-shaped bacilli	Straight Rods
Motility	+	+
Catalase test	+	+
Cytochrome oxidase test	+	+
Vogues Proskauer test	+	-
Growth on TSA+2 % NaCl	Small, white, swarming colonies	Smooth, circular, buff cream colored, 2-5 mm in diameter with entire margins.
Growth on 8% NaCl	-	+
Colonies on TCBS	Large green colonies	Pinpointed green colonies
Hemolysis of Sheep RBCs	Non-hemolytic	hemolytic
Sensitivity to 0/129 disc	+	+
Aesculin hydrolysis	+	-
Simon's citrate	+	+
Production of: -		
H_2S	-	-
Indole	+	+
Urease	-	-
Utilization of: -		
D-Mannitol	+	+
Glucose	+	+
Lactose	-	-
L-Arabinose	-	+
Maltose	+	+
Sorbitol	+	-
Sucrose	+	-
Mannose	+	+

Molecular identification of the identified *Vibrio* spp:

The primers selected for multiplex PCR amplification of the biochemically identified *Vibrio* spp were found to be specific for their respective targeted gene sequence with identity with their reference strains; *ToxR*, and *Tlh* genes of *V. alginolyticus*, and *V. parahaemolyticus* that were observed at 658 bp, and 450 bp respectively (**Fig. 3**).

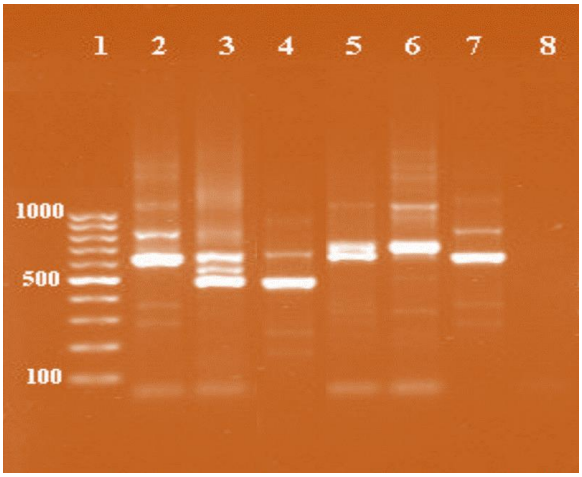

Fig. 3. The amplified products determined by the agarose gel electrophoresis as following; Lane 1: 100-1000 bp DNA ladder, lanes 2, 5 & 6 are 658 bp of *V. alginolyticus*, lane 7 is a control positive for *V. alginolyticus*, lane 3: 450 bp of *V. parahaemolyticus*, lane 4: is served as the control positive of *V. parahaemolyticus*, and lane 8: has no template, and served as a negative control.

Prevalence of the retrieved *Vibrio* strains:

Prevalence of the retrieved *Vibrio* strains showed in table (4). It has been demonstrated that the total number of identified isolates were (65), from them (25) were for *V. parahaemolyticus* and (21) were for *V. alginolyticus*. It was found that prevalence of the identified *Vibrio* spp were 38.46 and 21.54 % for *V. parahaemolyticus*, and *V. vulnificus* respectively.

Table 4. Prevalence of the retrieved bacterial strains isolated from diseased *Hipposcarus harid*.

Bacterial Isolates	No. of isolates	Prevalence %
V. parahaemolyticus	25	38.46
V. alginolyticus	14	21.54
Other bacterial strains	26	40.0
Total No. of isolates	A total of 65 isolates	

DISCUSSION

Longnose parrotfish, *Hipposcarus harid* is one of the thousands of reef fish species of great ecological importance. Their importance was focused mainly on protecting the sea coral reefs from the overgrowing with sponges and seaweeds (Loh *et al*., 2015). This is due to their scrapings and cleaning them, so, we should pay great attention to protecting them from the invading diseases. Vibriosis is a serious septicemic, and devastating bacterial disease infecting a wide range of wild and cultured fishes worldwide causing serious economic impacts (Balebona *et al*., 1998 a,b).

The reviews on the bacterial diseases affecting the coral reef fishes, generally, and their ecological and economic impacts in Egypt, were little. Therefore, this study was focused mainly to study the bacterial fish pathologies encountered in mortalities of the coral reef fishes. In this investigation, *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus* were identified to cause mortalities of the coral reef fish, *Hipposcarus harid*, inhabiting the Red Sea basin, at Hurghada, Egypt. Several other *Vibrio* spp have been identified from diseased coral reef fishes, *V. alginolyticus* from Ornamental Bird Wras (Abd El-Galil and Mohamed, 2012), and *Vibrio harveyii* from Arabian Surgeon (*Acanthurus sohal*) (Hashem and El-Barbary, 2013).

The clinical findings (signs and PM lesions) of *Hipposcarus harid* showed the typical signs of septicemia, which were in the concordance of the previous findings of Vibriosis infecting fishes (Khalil and Abd El-Latif, 2013; Hashem and El-Barbary, 2013; El-Bouhy *et al*., 2016).

The scenario of the pathogenicity mechanisms of *Vibrio* spp affecting fishes were built specifically upon the mechanism of iron sequestration from the iron binding protein cites (Ferritin or transferrin) (known as iron sequesters or Siderophore-mediated pathogenicity), together with the production of various types of exotoxins, that promote favorable conditions for the induction

of hemorrhagic septicemia phenomenon (Toranzo *et al*., 2005; Naka *et al*., 2013).

The bacteriological studies (colony morphology, and cultural characteristics) of isolated *Vibrio* spp were agreed with the findings of Zorrilla *et al*. (2003); Liu *et al*. (2004) and Zulkifli *et al*. (2009). Additionally, the results of the biochemical identification for the differentiation of the *Vibrio* spp retrieved were like that of Fouz and Amaro (2003); Yiagnisis and Athanassopoulou (2011). The molecular diagnostics that were concerned with the identification of the potentially pathogenic *Vibrio* spp using multiplex PCR, it is an essential need to target several multiple species-specific genes for the amplification (Panicker *et al*., 2004a). In this context, a species-specific *ToxR* (*V. alginolyticus*) (Rivera *et al*., 2001), *Tlh* (*V. parahaemolyticus*) (Bej *et al*., 1999 and Myers *et al*., 2003) genes were used.

It has been found that the prevalence of the identified *Vibrio* spp infecting *Hipposcarus harid*, that inhabiting the Red Sea basin at Hurghada (Egypt) were 38.46 and 21.54 % for *V. parahaemolyticus*, and *V. vulnificus* respectively. The results were not parallel to that of Abdelaziz *et al*. (2017) who recorded a prevalence of 64 %, and 29 % of Vibriosis infecting fishes at Lake Qarun and Gulf of Suez respectively. Similarly, the results were also not agreed with the preceding findings of Eissa *et al*. (2013); Adebayo-Tayo *et al*. (2011). These differences were attributed to several factors (Fish species and age differences, water parameters, environmental ecology, culture facilities, and others).

CONCLUSIONS

From this investigation, it can be concluded that the protection of coral reef fishes as *Hipposcarus harid*, is of high ecological and economic importance, to save the coral reefs especially at Hurghada from different weeds and overgrowing sponges. Additionally, *Vibrio* isolates especially *V. vulnificus*, *V. parahaemolyticus*, and *V. cholerae* can produce critical septicemic disease

problems to *Hipposcarus harid*, and the use of multiplex PCR can confirm the targeted species-specific genes of these bacterial strains.

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إستخدام تفاعل البلمرة المتسلسل في إستكشاف چينات أنواع الفيبريو التي تصيب أسماك الحريد الأبيض التي تقطن الشعب المرجانيه بالغردقه، البحر الأحمر، مصر

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الملخص العربي

مرض الفيبريوزيس من الأمراض البكتيريه الخطيره التي تصيب أنواع كثيره من الأسماك حول العالم. في هذه الدراسه قد لوحظ وجود نافق بأسماك الحريد الأبيض التي تقطن الشعب المرجانيه بالغردقه بالبحر الأحمر. تم فحص الأسماك إكلينيكيا وكذلك تم عمل فحص بكتريولوجي كامل من حيث عزل وتصنيف البكتريا المسببه. تم عمل الفحص الجزيئي للمعزولات بإستخدام إختبار البلمره الجزيئي المتعدد. أوضحت النتائج أن الأسماك المصابه يوجد عليها أعراض التسمم الدموي مثل وجود أنزفه على جلد السمك وعلى نهايات الزعانف مع وجود تأكل في الزيل. بالفحص الداخلي لوحظ وجود نقط نزفيه على الكبد مع وجود إحتقان في الطحال والخياشيم والأمعاء وبعض الأنزفه على المبايض والغشاء البريتوني. أوضحت نتائج الفحص البكتريولوجي أن نسب العدوى بمعزولات الفيبريو بمعدل (٣٨.٤٦ %) و(٢١.٥٤ %) لمعزولات الفيبريو باراهيموليتكس والفيبريو ألجيوليتكس. من هذه الدراسه نستنتج أن تفاعل البلمره الجزيئي المتعدد يعد وسيله فعاله لتأكيد تشخيص الأصابه بمعزولات الفيبريو التي تصيب أسماك الحريد الأبيض التي تقطن الشعب المرجانيه بالبحر الأحمر.