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BACTERIAL INFECTIONS IN SOME RED SEA FISHES

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ABSTRACT

The present study was carried out to investigate the common bacterial infections of some saltwater fishes in Red Sea at Hurghada, Egypt. One hundred and twenty fishes of different species including *Hipposcarus harid, Scarus ferrugineus and Scarus niger* were subjected to bacteriological investigations. The bacteria isolated from the vital organs were phenotypically identified as *Vibrio logei*, *V. ichthyoenteri*, *V. fischeri*, *Moritella marina*, *Photobacterium damselae* subsp. *piscicida* and *Edwardsiella tarda*, in addition to two isolates of the genus *Streptococcus*. Experimental challenge with the dominant isolate, *V. logei*, proved its pathogenicity to *H. harid* with 86.7% mortality rate. Histopathological investigations of experimentally infected fish disclosed prominent pathological alterations in vital organs including liver and kidney. The antibiotic sensitivity of *V. logei*, revealed its sensitivity to Norfloxacin, Ciprofloxacin and Ofloxacin.

Key words: *Hipposcarus harid, Vibrio logei, pathogenicity, histopathological examination, antibiotic sensitivity*.

INTRODUCTION

Fisheries in the Red Sea are of considerable socio-economic importance to the Red Sea countries in terms of national food security and source of income for rural communities. In Egypt, the Red Sea has a major importance as a natural resource of fish (Sanders and Morgan, 1989; Azab *et al*., 1998). The marine environment encompasses a wide variety of biological, chemical and physical parameters, which if altered beyond acceptable limits, such as any stress inducer, may modulate the fish immunity leading to disease outbreaks (Roberts, 2012). Bacterial diseases represent the most influential category of disease problems that has a massive impact on fish health and production (Austin and Austin, 2016).

Many species belonging to genus *Vibrio* induce disease problems with subsequent mortalities in marine fish. Interestingly, *Vibrios* are present everywhere in marine environment, but clinical disease outbreaks only occur when stressed fish are exposed to the pathogenic infectious agent. Septicemia caused by vibriosis is characterized by hemorrhages at the bases of fins, exophthalmia, and ulcers on the body surface (Abdel-Aziz *et al*., 2013).

The objectives of current study were to investigate bacterial infections of some Red Sea fishes at the area of Hurghada, Egypt; to examine the pathogenicity of the dominant bacterial isolate; to study the histopathological alterations induced by the pathogen and to determine the most effective antibiotic(s) against the dominant isolate.

MATERIALS AND METHODS

Fish sampling:
A total of 120 marine fish (*Hipposcarus harid*, *Scarus ferrugineus* and *Scarus niger*; n=100, 15 and 5 respectively) with average body weight of 200 ± 20 g were collected from Red Sea at Hurghada, Red Sea governorate, Egypt. The collected fishes were transmitted immediately to the fish diseases laboratory at the National Institute of Oceanography and Fisheries (Hurghada branch, Red Sea governorate).

Bacterial isolation and identification:
Following their clinical examination, fish necropsy has been carried out as described by Noga (2010). Thereafter, fish were subjected to bacterial isolation and identification according to Austin and Austin

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(2016). Briefly, loopfuls from kidney, liver and/or spleen were inoculated onto trypticase soy agar (TSA; Oxoid, UK) supplemented with 1.5% (w/v) sodium chloride. The inoculated media were incubated at 25ºC for up to 72 hrs.

Phenotypic characterization of the isolates recovered was performed following the techniques previously reported by Buller (2004). Table 1 shows the different biophysical and biochemical characters examined. Motility has been examined using a semi solid TSA supplemented with of 1.5% (w/v) sodium chloride. Isolates were identified biochemically using API-20NE (Oxoid, UK) according to the instructions of manufacturer.

Experimental infection (Challenge):
Twenty five apparently healthy longnose parrotfish, *H. harid*, with an average body weight of 100 ± 12 g were used for challenge test. Fish were acclimated in 200-liters glass aquaria, for 2 weeks and feed was discontinued two days before injection as previously described (Ellsaesser and Clem, 1986). The dominant isolate (*V. logei*) was grown on trypticase soy broth (TSB; Oxoid, UK) supplemented with 1.5% (w/v) sodium chloride. Fishes were divided into 5 groups with 5 fish per group. The first 3 groups (representing 3 replicates of challenge) were intraperitoneally injected (I/P) with 0.3 ml bacterial culture suspension in saline (1×10^7 CFU/ ml), according to our preliminary challenge trials. The fourth (sham control) group was injected with 0.3 ml sterile normal saline. The fifth group was kept un-injected as an absolute control. Fish were observed daily for 15 days to record the clinical signs and mortalities. Moribund fish demonstrating lesions were subjected to *V. logei* re-isolation on a *Vibrio* spp. selective medium; Thiosulfate Citrate Bile Salts (TCBS) agar (Oxoid, UK). After 15 days, cumulative mortalities were recorded, then the surviving fish were sacrificed, post-mortem findings were recorded and the pathogen was isolated from kidney, liver and spleen on TCBS.

Antibiotic sensitivity:
The antibiotic sensitivity of the dominant isolate (*V. logei*) was examined following the Kirby Bauer disk diffusion method (Bauer *et al.*, 1966) using Mueller-Hinton Agar (MHA; Oxoid, UK) supplemented with 1.5% (w/v) sodium chloride. Antibiotics used are listed in Table 2. At the end of incubation, inhibition zones were measured using measuring caliber. The interpretation of the results as sensitive, moderately sensitive and resistant was done according to the interpretive standards of Clinical and Laboratory Standards Institute (2007).

Histopathological examination:
Specimens from kidney, liver and spleen of experimentally infected fish were trimmed, fixed in 10% phosphate-buffered formalin and processed for histopathological examination as described by Drury and Wallington (1980).

RESULTS

Bacterial isolation and identification:
No apparent specific clinical signs were observed on the fish samples either externally or internally. The bacterial isolation resulted in recovery of 35 isolates from kidney, liver and spleen of the examined fish. Phenotypic (biophysical and biochemical) characterization and identification of the different Gram negative isolates are shown in Table 1. The identification resulted in 33 Gram negative isolates representing 94.3% of the total isolates. Two isolates were Gram positive (5.7%) and displayed small and colorless colonies on TSA, alpha hemolysis on blood agar (containing 5% sheep blood) and cocci arranged in chains when examined microscopically after staining with Gram stain. Of the Gram negative, 30 isolates belonged to genus *Vibrio* (~ 91.0%), 1 isolate of *Moritella marina*, *Photobacterium damselae* subsp. *piscicida*, and *Edwardsiella tarda* (~ 3.0% each). On the other hand, the two Gram positive isolates obtained were of the genus Streptococcus (5.7%). *V. logei* was the dominant pathogen with 28 isolates representing 80% of total isolated pathogens; 84.8% of the Gram negative isolates and 93.3% among the isolated *Vibrio* spp. The other species of *Vibrio* in the present study were *V. ichthyoenteri* and *V. fischeri* (1 isolate for each species; approx. 2.9%). It is noteworthy that out of the 30 *Vibrio* spp. isolates, 18 were isolated from liver; 8 from kidney and 4 from spleen (60%, 26.7% and 13.3% isolation rates respectively).

Antibiotic sensitivity:
V. logei, as the dominant isolate in the current study, was sensitive to Norfloxacin, Ciprofloxacin and Ofloxacin, moderately sensitive to Amikacin and Gentamicin and resistant to Cefotaxime (Table 2).

Experimental infection (Challenge):
Challenge confirmed *V. logei* pathogenicity to *H. harid* with cumulative mortality of 86.7% ± 11.5 (Mean ± standard deviation). No mortalities have been recorded in control group. Data are shown in Fig. 1. The external signs appeared as haemorrhagic areas on skin and at the base of fins, skin ulceration and abdominal distension (Fig. 2 and 3) and corneal opacity (Fig. 4). Meanwhile, internal examination revealed paleness of the liver with peticheal hemorrhages, fibrinous gas bladder, intestine filled with white serous fluid, presence of caseous material in the abdominal cavity (Fig. 4), congestion and enlargement of spleen and kidney. Bacteria were recovered from kidney, liver and spleen of the experimentally infected fish and confirmed as *V. logei.*

Histopathological examination:
Liver displayed dissociation and vacuolation of hepatocytes around central vein. Focal areas of coagulative necrosis and fatty degeneration of hepatocytes were also observed. Large fat cells replaced the exocrine pancreas accompanied by congestion of the portal vein. Necrosis of pancreatic acini was also recorded (Fig.5 to Fig. 8).

Kidney exhibited degeneration and necrosis of renal tubules. Also, shrinkage of renal corpuscles, hemorrhage and necrosis of hematopoietic tissue associated with inflammatory cell infiltration had been noticed. Melanomacrophage centers were atrophied (Figs. 9 &10).

Table 1: Phenotypic (biophysical and biochemical) characterization of Gram negative isolated bacteria.

Characteristic	*V. logei*	*V. ichthyoenteri*	*V. fischeri*	*M. marina*	*P. damselae* subsp. *piscicida*	*E. tarda*
Colonies morphology on TSA	Round, smooth and creamy			Round and colorless	Regular, round and convex	Small and round
Growth on TCBS	Yellow colonies	Green colonies	Yellow colonies	No growth	No growth	No growth
Gram staining	Gram negative, short and straight or slightly curved rods			Gram negative, short rods	Gram negative rods with bipolarity	Gram negative, straight and short rods
Cytochrome oxidase	+	+	+	+	+	-
Motility test	Motile	Motile	Motile	Motile	Non motile	Motile
API 20 test:						
Nitrate	+	+	+	+	-	-
Lysine	-	-	+	-	-	-
Ornithine	-	-	-	-	-	-
H_2S	-	-	-	-	-	+
Glucose	-	-	-	-	+	-
Mannitol	-	-	-	-	+	-
Xylose	+	+	+	+	-	+
ONPG	+	-	+	+	-	-
Indole	-	-	-	-	-	+
Urease	+	-	-	+	+	-
V-P	-	-	+	-	-	-
Citrate	-	-	-	-	-	-
TDA	-	-	-	-	-	-
Gelatin	-	-	-	-	-	-
Malonate	-	-	-	-	-	-
Inositol	-	-	-	-	+	+
Sorbitol	-	-	-	-	+	+
Rhamnose	-	-	-	-	+	-
Sucrose	-	-	+	-	+	+
Lactose	-	-	-	-	+	+
Arabinose	+	+	+	+	+	-
Adonitol	-	-	-	-	+	+
Raffinose	-	-	-	-	+	+
Salicin	-	-	-	-	+	+
Arginine	-	-	-	-	-	-
NaCl tolerance:						
0%	+	+	+	+	+	+
2%	+	+	+	+	+	+
4%	-	-	-	-	-	+
6%	-	-	-	-	-	-
8%	-	-	-	-	-	-
10%	-	-	-	-	-	-

ONPG, o-nitrophenyl-b-d-galactopyranoside; V-P, Voges-Proskauer; TDA, Tryptophane deaminase.

Table 2: Antibiotic sensitivity of *V. logei.*

Antibiotic	Sensitivity
Norfloxacin	Sensitive
Ciprofloxacin	Sensitive
Ofloxacin	Sensitive
Amikacin	Moderately sensitive
Gentamicin	Moderately sensitive
Cefotaxime	Resistant

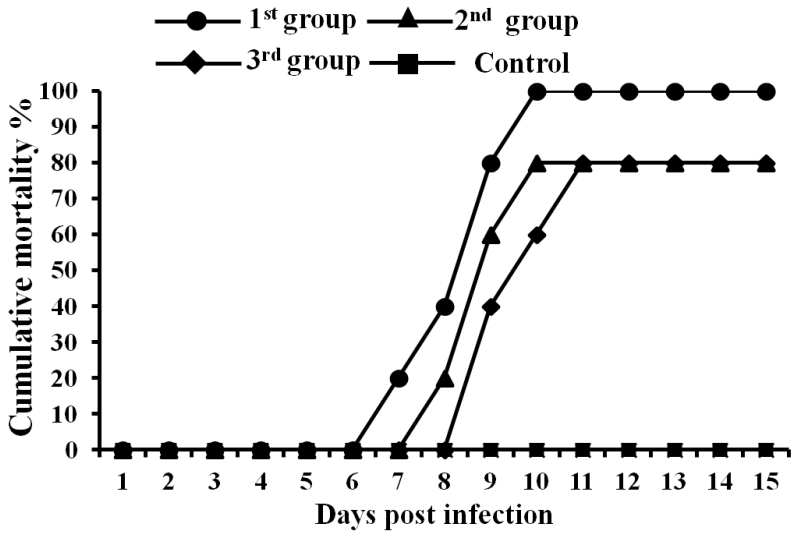

Fig. 1: Mortalities in longnose parrotfish, *Hipposcarus harid*, challenged with *Vibrio logei*. 1st., 2nd. and 3rd. groups represent the 3 replicates of infected fish. Control is the group of fish injected with sterile normal saline only.

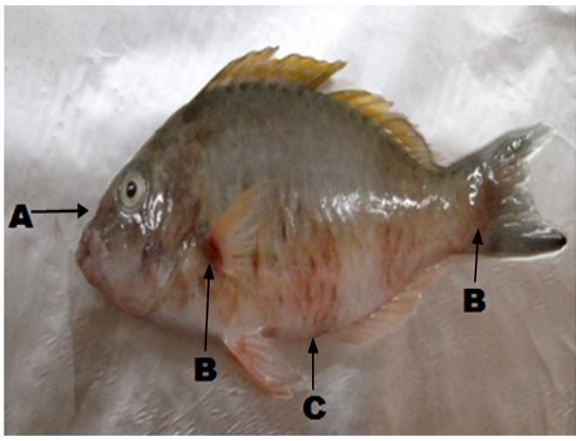

Fig. 2: Longnose parrotfish, *Hipposcarus harid*, challenged with *Vibrio logei* showing ulceration (A), hemorrhages at the base of fins (B) and abdominal distension (C).

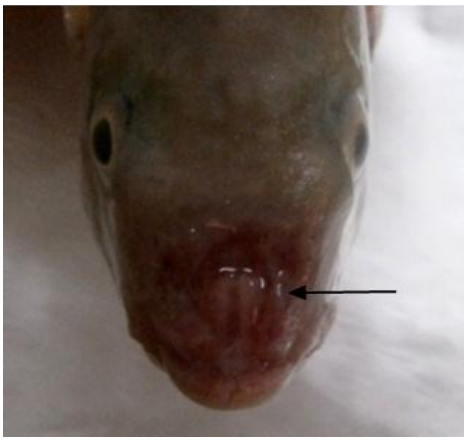

Fig. 3: Longnose parrotfish, *Hipposcarus harid*, challenged with *Vibrio logei* showing skin ulceration and hemorrhages (arrow).

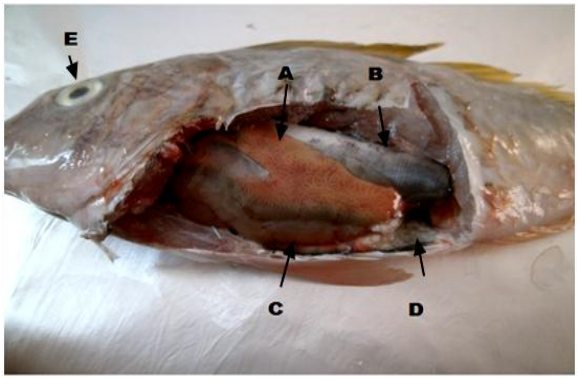

Fig. 4: Longnose parrotfish, *Hipposcarus harid*, challenged with *Vibrio logei* showing paleness of the liver with peticheal hemorrhages (A), fibrinous gas bladder (B), intestine filled with white serous fluid (C), presence of caseous material in the abdominal cavity (D) and corneal opacity (E).

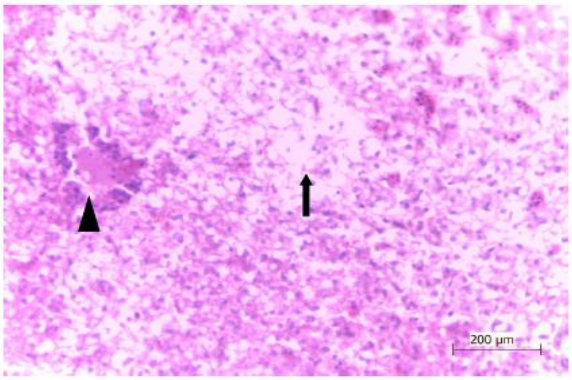

Fig. 5: Liver of longnose parrotfish, *Hipposcarus harid*, challenged with *Vibrio logei* exhibits coagulative necrosis and vacuolation of hepatocytes (arrow) and necrosis of exocrine pancreas (arrow head). (H&E, bar = 200µm)

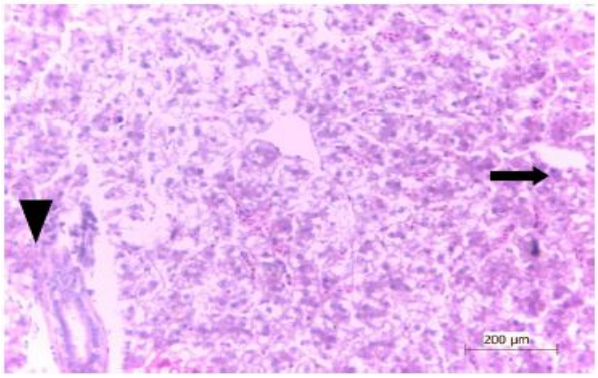

Fig. 6: Liver of longnose parrotfish, *Hipposcarus harid*, challenged with *Vibrio logei* reveals dissociation and vacuolation of hepatocytes around central vein (arrow) with necrosis of exocrine pancreatic acini (arrow head). (H&E, bar = 200µm).

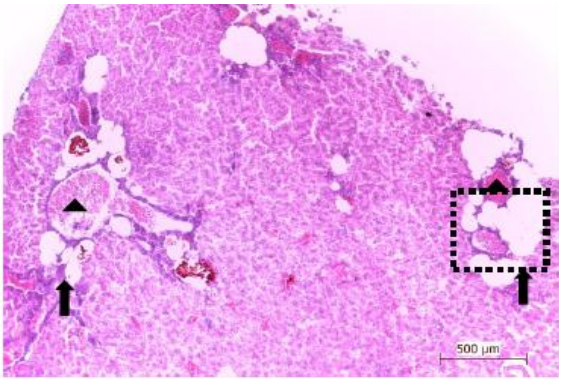

Fig. 7: Liver of longnose parrotfish, *Hipposcarus harid*, challenged with *Vibrio logei* shows fatty degeneration in hepatocytes and replacement of exocrine pancreatic acini with fat cells (arrows) associated with congestion of portal vein (arrow head). (H&E, bar = 500µm).

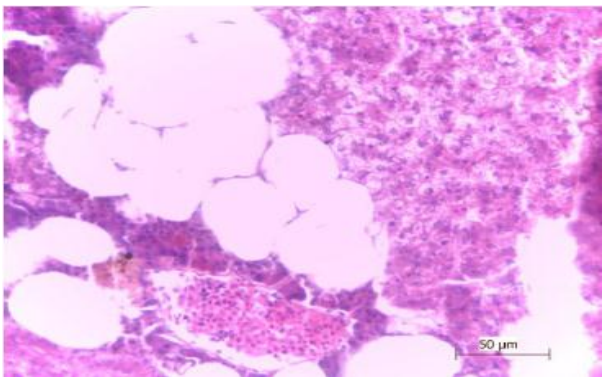

Fig. 8: Higher magnification of Fig. 7 revealed atrophy of the exocrine pancreatic acini with fat cell replacement associated with congestion of portal vein. (H&E, bar= 50µm)

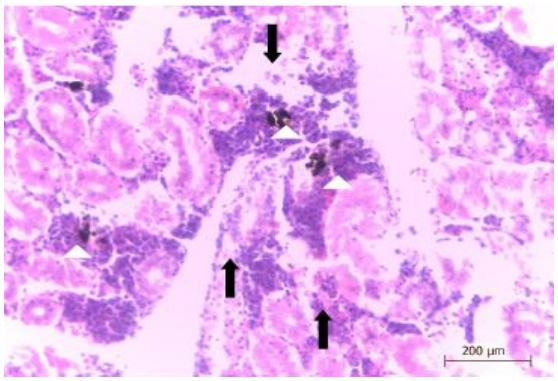

Fig. 9: Kidney of longnose parrotfish, *Hipposcarus harid*, challenged with *Vibrio logei* reveals degeneration of the renal tubules, hemorrhage and necrosis of hematopoietic tissue (arrows) and atrophy of melanomacrophage centers (arrow head). (H&E, bar = 200µm)

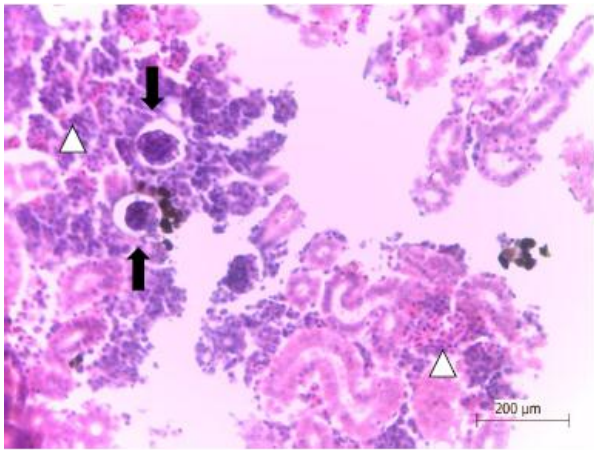

Fig. 10: Kidney of longnose parrotfish, *Hipposcarus harid*, challenged with *Vibrio logei* exhibits shrinkage of renal corpuscles (arrow) with hemorrhage in hematopoietic tissue and inflammatory cell infiltration (arrow head). (H&E, bar = 200µm)

DISCUSSION

Red Sea represents a major source of marine fish in Egypt, however, little is still known about the map of diseases affecting fishes in this area. The current study draws attention to the bacterial affections of some economic and famous fish species in the area of Hurghada, Red Sea governorate.

Throughout the period from March 2013 to February 2014, a total of 120 marine fish of different species were collected from Red Sea at Hurghada. The bacteriological investigations resulted in recovery of 35 isolates, only 2 of which were Gram positive (*Streptococcus* spp.; 5.7% of total isolated bacteria), while the majority were Gram negative (94.3% of total isolated pathogens). The dominant isolates were *Vibrio* spp., and other Gram negative bacteria as *M. marina*, *P. damselae* (subsp. *piscicida*) *and E. tarda* were also isolated. Balebona *et al*. (1998) declared that the main pathogenic microorganisms isolated from diseased gilt-head sea bream in the marine environment were; *Vibrio* spp., *Pseudomonas* spp., *Photobacterium piscicida*, *Tenacibaculum maritimum* (= *Flexibacter maritimus*), *Aeromonas* spp. and Gram positive bacteria were also isolated but in low prevalence. Also, Moustafa *et al*. (2010) isolated *V. anguillarum*, *V. alginolyticus*, *P. damselae* subsp. *piscicida* (Formerly *Pasteurella piscicida*), *P. fluorescens*, *S. fecalis*, *A. hydrophila*, *A. sobria* and *Staphylococcus aureus* from fish samples from areas of Qarun Lake and Suez Gulf, Egypt.

Results of present study revealed that *V. logei*, *V. ichthyoenteri* and *V. fischeri* were identical in morphology, motility test, and cultural characteristics on TCBS, oxidase test, nitrate test and sodium chloride tolerance. However, they displayed different characters in utilization of carbohydrates and carbon sources. In the same way, many authors reported similar characteristics of *Vibrio* spp. (Matte *et al.*, 2007; Korun and Timur, 2008; Austin, 2009; Al-Sunaiher *et al.*, 2010 and Mahbub *et al.*, 2011). While, Sankar *et al.* (2012) reported different characteristics among various *Vibrio* spp. examined. This variation was reported in Bergey's Manual of Systemic Bacteriology (Johannes, 2005) as *Vibrios* can ferment and utilize a wide variety of simple and complex carbohydrates and utilize a wide variety of carbon sources. Also, the difference in characters among various strains may be attributed to wide distribution of *Vibrios* around the world and the difference of characteristics of each location from others in the availability of carbohydrates and carbon sources.

Vibrios represented 85.7% from the total recovered isolates as the most prevalent bacterial species identified in our study. This may be because *Vibrios* are very common in marine environments due to their halophilic nature (Asplund, 2013). Also, Tanekhy (2013) found that the highest percentage from all bacteria isolated in his study was *Vibrio* species (41.2 %) in marine fish species raised in cage culture. Among the *Vibrios* isolated in the present study, *V. logei* was the dominant isolate which may be due to the host specificity and availability of bacterial sources at the site of fish collection.

Regarding the incidence rate of bacterial isolation from the vital organs of the fish examined, 60% of isolates were from the liver followed by 26.7% from kidney and 13.3% from the spleen. Similarly, Tanekhy (2013) recorded the highest incidence rate of bacteria in liver (40.0%), followed by kidney (24.8 %) then heart (19.6 %) and the lowest incidence was found in spleen (15.6 %). However, Balebona *et al*. (1998) stated that the bacterial isolation rate from sea bream was higher in spleen (49.5%), followed by liver (29.1%), then kidney (11.7%) and other organs (9.7%). This may be attributed to the difference in fish species, stage of infection and bacterial species.

Antibiogram of the dominant isolate (*V. logei)* has declared its susceptibility to quinolones (Norfloxacin, Ofloxacin and Ciprofloxacin) and moderate susceptibility to aminoglycosides (Amikacin and Gentamicin) while resistance to beta-lactams (Cefotaxime). In a previous study, Laganà *et al.* (2011) reported susceptibility of the majority of *Vibrio* strains examined to aminoglycosides and other quinolones such as enoxacin, ofloxacin, ciprofloxacin and norfloxacin. However, Han *et al.* (2007) reported that tetracycline, cefotaxime, ceftazidime, and fluoroquinolones were highly effective against *V. parahaemolyticus* strains recovered from Louisiana gulf and retail oysters. The difference in susceptibility to antibiotics may be attributed to variance of the characters among different *Vibrio* strains investigated in the different studies.

Since *H. harid* represents an economic and important species among the fish examined in the current study, we used it in the challenge test. Intraperitoneal injection of the dominant isolate, *V. logei*, induced mortalities in the experimentally infected fish reaching 86.7% as a mean cumulative mortality among the challenged groups. Clinical signs observed on challenged fish were typical to signs of vibriosis reported by Actis *et al*. (2011). The external signs appeared as haemorrhagic areas on skin and at the base of fins, skin ulceration, abdominal distension and corneal opacity. Meanwhile, internal examination revealed paleness of the liver with peticheal hemorrhages, fibrinous gas bladder, intestine filled with white serous fluid, presence of caseous material in the abdominal cavity, congestion and enlargement of spleen and kidney. Similar lesions were recorded in *V. ordalii* infection in cultured fishes (El-Bassiony, 2001).

Histopathological examination of Liver displayed dissociation and vacuolation of hepatocytes around central vein, focal areas of coagulative necrosis and fatty degeneration of hepatocytes (El-Bassiony, 2001; Roberts, 2012). While, Enany *et al.* (2011) reported vacuolar degeneration of hepatocytes, hyperplasia in bile epithelial lining and budding of newly formed bile ductules in cultured *Mugil capito* infected with *Vibrio* species. Fatty degeneration in hepatic tissue has been recorded in acute and chronic infection of *M. capito* infected with *V. alginolyticus* (Khalil and Abd El-Latif, 2013). Kidneys exhibited degeneration and necrosis of renal tubules, shrinkage of renal corpuscles, hemorrhage and necrosis of hematopoietic tissue and atrophy of melanomacrophage centers (Roberts, 2012; Korun and Timur, 2008; Enany *et al.*, 2011).

Though fish samples used in present work did not show any specific disease signs, many bacterial spp. have been isolated indicating the subclinical infection (or carrier state) of fish. These fish may act as a source of infection to other aquatic animals and human. Further studies are still needed to investigate other microbial pathogens inhabiting fish in the Red Sea.

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الإصابات البكتيرية فى بعض أسماك البحر الأحمر

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أجريت هذه الدراسة لتحديد العدوى البكتيرية فى بعض أسماك المياه المالحة فى مدينة الغردقة بمحافظة البحر الأحمر. تم استخدام مائة وعشرون سمكة من أسماك الحريد والغبان المستخرجة من البحر الأحمر، حيث تم عزل البكتيريا من الكلى والكبد والطحال. أسفر تصنيف البكتيريا بواسطة الخصائص المورفولوجية والكيميائية عن الحصول على عترات من ميكروبات الفيبريو والموريتلا والفوتوبكتيريم والإدورادسيللا تاردا بالإضافة إلى عترتين من ميكروب الستربتوكوكس. وعند عمل عدوي إصطناعية باستخدام ميكروب الفيبريو لوجياي (الميكروب الأكثر عزلاً)، أثبتت النتائج قدرته على إحداث المرض بأسماك الحريد، حيث كانت نسبة الوفيات الناتجة ٨٦,٧ %. أوضحت الفحوصات الهستوباثولوجية فى الأسماك الممرضة إصطناعياً وجود تغيرات مرضية جسيمة فى الكبد والكلى وغيرهما. أيضاً تم عمل اختبار الحساسية لمضادات حيوية متعددة باستخدام ميكروب الفيبريو لوجياي، وأظهرت النتائج حساسية هذا الميكروب للنورفلوكساسين والسيبروفلوكساسين والأوفلوكساسين.