



Bacteriological and Histopathological Studies on *Vibrio* Species Isolated from Naturally Infected Freshwater Fish in Delta Region, Egypt

EL-SHARABY S. M. A¹, ABD-ELGABER M², TARABEES R^{3*}, KHALIL R. H⁴, ALI M. N.¹, EL-BALLAL S.⁵

¹Department of Fish Diseases, Animal Health Research Institute, Dokki, Giza, Egypt; ²Department of Pathology, Faculty of Veterinary Medicine, Menoufia University, Egypt; ³Department of Bacteriology, Mycology, and Immunology, Faculty of Veterinary Medicine, University of Sadat City, Egypt; ⁴Department of Poultry and Fish Diseases, Faculty of Veterinary Medicine, Alexandria University, Egypt. ⁵Department of Pathology, Faculty of Veterinary Medicine, University of Sadat City, Egypt.

Abstract | The study was aimed to elucidate the most predominant *vibrio* serovars isolated from naturally infected freshwater fish in the delta region, Egypt. The contamination of water with Copper and Iron was evaluated. Fishes (n=200) were examined clinically and upon post-mortem. Bacteriological identification was confirmed based on the cultural characteristics and biochemical activities of isolates. A total of 5 isolates of *Vibrio harveyi* and *Vibrio alginolyticus* were further examined for the presence of hemolysins (*vhh*) and collagenase encoding genes using PCR, respectively. The data revealed that *Vibrio* species were recovered from 39% (78/200) of the examined fishes, and the highest prevalence rate was observed for *Angilla angilla* (55%) while the lowest was observed for *Chrysichthys auratus* and *Oreochromis niloticus* (35%). The *V. harveyi* was the predominant isolate (29.48%, 23/78), followed by *V. anguillarum* (28.20%, 22/78), *V. vulnificus* (16.67%, 13/78), *V. alginolyticus* (15.38%, 12/78), and *V. fluvialis* (10.25%, 8/78). Among five examined *Vibrio harveyi* and *Vibrio alginolyticus* isolate, a total of 4 (80%) were found to harbor hemolysins (*vhh*) and collagenase, respectively. Areas where *vibrio* was recovered in a high percentage, water contamination with Copper and Iron was higher than the permissible limit. Taken together, the obtained information established the emergence of *V. harveyi* as a widespread *Vibrio* serovar warrants further studies to elucidate its public health impact.

Keywords | *Vibrio*, Freshwater fish, Virulence genes, Heavy metals, Egypt

Editor | Kuldeep Dhama, Indian Veterinary Research Institute, Uttar Pradesh, India.

Received | September 17, 2017; **Accepted** | December 11, 2017; **Published** | December 28, 2017

***Correspondence** | Reda Tarabees, Department of Bacteriology, Mycology, and Immunology, Faculty of Veterinary Medicine, University of Sadat City, Egypt;

Email: reda.tarabees@vet.usc.edu.eg

Citation | El-Sharaby SMA, Abd-Elgaber M, Tarabees R, Khalil RH, Ali MN, El-Ballal S (2018). Bacteriological and histopathological studies on vibrio species isolated from naturally infected freshwater fish in delta region, egypt. Adv. Anim. Vet. Sci. 6(1): 17-26.

DOI | <http://dx.doi.org/10.17582/journal.aavs/2018/6.1.17.26>

ISSN (Online) | 2307-8316; **ISSN (Print)** | 2309-3331

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INTRODUCTION

Fish is a food of high dietary importance providing a good source of high-quality protein, vitamins, and minerals (Hajeb and Jinap 2009). Aquaculture is the supplier of one-third of the entire fish supply worldwide (FAO 2003). Deprived ecological conditions are the main stresses adversely affect the fish immune status and make them immuno compromised and become frailer to bacterial infections (Hansen and Olafsen, 1999; Schulze et al., 2006). Bacterial infections of fish specifically infection with Gram-negative bacteria are still the foremost fret for fish industry (Toranzo et al., 2005). *Vibrio* species are

Gram-negative bacteria commonly isolated from different ecosystems as well as aquaculture farms (Shruti and Haldar, 2012). Vibriosis is a disease of marine and aquaculture reared fishes caused mainly by several *Vibrio* species. The disease is widely accused of high mortality rate amongst fishes in marine and aquaculture system worldwide (Lavilla-Pitogo CR et al., 1998; Shruti and Haldar, 2012). *V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, and *V. vulnificus* are the major *Vibrio* species infecting marine and aquaculture fishes worldwide. Noteworthy mentioned, *V. anguillarum* and *V. vulnificus* are the common *Vibrio* species infecting fishes in aquaculture ecosystem (Austin and Austin, 2012; Shruti and Haldar, 2012).

The severity of any bacterial infection is mediated mainly via an array of virulence encoding genes that facilitate adhesion, colonization and toxin production and hence disease. The pathogenic *V. harveyi* is known to harbor many virulence encoding genes, including hemolysins (*vhb*) (Parvathi et al., 2009), and lots of literature of works established the use of *vhb* genes targeted PCR as a proof of pathogenic *V. harveyi* (Castroverde et al., 2006; Conejero and Hedreyda, 2004). Collagenase virulence encoding genes have been shown to be produced by various species of bacteria, including *Vibrioalginolyticus* for more details about the precise role of collagenase in the pathogenesis of bacterial diseases including *Vibriosis* see (Harrington, 1996).

Aquaculture systems are presented to various toxins that mainly discharged from effluents released from businesses, sewage treatment plants and seepage from urban and farming regions. These toxins cause genuine harm to fishes (Al – Masri et al., 2002; Karbassi et al., 2006). The contamination of water with heavy metals has turned into a matter of tremendous worry during the last two decades, not just as a result of the danger to open water supplies, additionally it significantly affect the aquatic life structures. The waterway frameworks might be too much polluted with heavy metals discharged from residential, mechanical, mining and horticultural effluents (Vander Oost et al., 2003). Contamination of water with heavy metals will likely modify the biochemical and physiological parameters of fish and hence will magnify the severity of bacterial infection (Vinodhini and Narayanan, 2009). The present study was undertaken to determine the common *Vibrio* species affect wild and cultured fishes collected from different localities in Menofia and Kafr El-Sheikh Provinces. In addition, the histopathological lesions of internal organs were recorded. Ultimately, the association between the contamination of the water with heavy metals and the incidence of *Vibriosis* was elucidated.

MATERIAL AND METHODS

FISH

A total 200 fishes (80 ± 20 gm) of six main species as follow; 80 of Nile tilapia (*Oreochromis niloticus*), 40 of African Sharp tooth catfish (*Clarias gariepinus*), 20 of European eel (*anguilla*), 20 of Bottle nose (*Mormyrus kannume*), 20 of Long blade catfish (*Chrysichthys auratus*) and 20 of Mullet (*Mugil cephalus*) were collected from various territories in Menofia Province (El Khadroyou area, Elatfa locale and Shabra bass district) and from the private fish farms at Kafr El Sheik region, Egypt (Table 1). Fishes were transported alive to the Fish Diseases Department at the Animal Health Research Institute and subjected to clinical examination and bacterial isolation.

Table 1: The fish species, location and number

Fish species	Location (Province)	No
Oreochromis niloticus	Kafr el-sheikh and Menofia	80
Clarias gariepinus	Kafr el-sheikh and Menofia	40
Anguilla anguilla	Kafr el-sheikh and Menofia	20
Mormyrus kannume	Menofia	20
Mugil cephalus	Kafr el-sheikh and Menofia	20
Total		200

CLINICAL EXAMINATION AND POSTMORTEM LESIONS

Clinical examination of the fishes was carried out according to (Austin and Austin, 2012; Noga, 2010), while the post-mortem examination was carried out according to (Amlacher, 1970).

BACTERIOLOGICAL IDENTIFICATION

Isolation and biochemical characterization of *Vibrio*

Samples were collected from fish's gills, kidney, liver, and spleen under full aseptic conditions. The inoculums were further spread over Tryptic soya broth (Difco, Detroit, MI, USA) supplemented with 3 % NaCl and then incubated at 25 °C for 24–48 hrs. For purification purpose, pure colonies were further sub-cultured into Tryptic soya agar supplemented with 3 % NaCl and 5% sheep blood agar media supplemented with 3 % NaCl and incubated for 18–24 hrs at 37 °C. Full identification scheme mainly includes colonial characteristics, Gram stain and biochemical activities of the isolates (Austin and Austin, 2012; Quinn et al., 2002).

API 20E and API 20 NE

The biochemical characters of the isolates were determined by using the API-20E and API-20NE system. These strips included the following reactions: ONPG 2-nitrophenyl-β-D-galactopyranoside, ADH L-arginine, LDC L-lysine, CIT trisodium citrate, H₂S sodium thiosulfate, URE urea, TDA L-tryptophane, IND L-tryptophane, VP sodium pyruvate, GEL Gelatin, GLU D-glucose, MAN D-mannitol, INO inositol, SOR D-sorbitol, RHA L-rhamnose, SAC D-sucrose, MEL D-melibiose, AMY amygdalin, ARA L-arabinose.

VITEK® 2 compact (BioMérieux) biochemical identification

It is an automated microbiological system used for biochemical identification of isolates according to the manufacturer's procedure (Malainine et al., 2013).

GENOTYPIC CHARACTERIZATION OF SOME VIRULENCE ENCODING GENES IN *V. HARVEYI* AND *V. ALGINOLYTICUS*

Genotypic characterization of the hemolysins encoding gene (*vvh*) and collagenase encoding gene in *V. harveyi* and *V. alginolyticus* respectively was carried out according to the

protocol previously established (Di Pinto et al., 2005; Maiti et al., 2009). Briefly, Genomic DNA extraction was carried out using 24 hrs cultures by boiling at 100 °C for 10 minutes (De Medici et al., 2003). PCR reactions were conducted in a total volume of 50 µl containing 25 µl Green Master Mix, 10 µl DNA template, 5 µl upstream primers, 5 µl downstream primers (Table 2) and 5 µl nuclease free water. For the amplification of hemolysin encoding gene (*vvh*) the PCR condition was 94°C/ 5 min; 30 cycles of (95 °C/ 1 min, 50 °C/ 1 min, 72 °C /5 min) followed by 72 °C /7 min. While, in the case of collagenase encoding gene, the PCR condition was 94° C/ 5 min; 35 cycles of (95 °C/ 1 min, 50 °C/ 1 min, 72 °C /10 min) followed by 72 °C /7 min. The products of PCR were separated by electrophoresis on 1.5% agarose gel (AppliChem, Germany) to define the fragment sizes.

Table 2: Nucleotides sequences of *vvh* and collagenase encoding genes primers sets

Bacteria	Target gene	Nucleotide sequences	Size (bp)
V. harveyi	vvh	F: CTTACAGCTT GATGGCTACTG R: GTCACCCAAT G CTACGACCT	235
V. alginolyticus	collagenase	F: CGAGTACAGT CACTTGAAAGCC R: CACAA- CAGAAC TCGCGTTACC	737

HISTOPATHOLOGICAL EXAMINATION

Samples collected from internal organs (gills, liver, spleen, and kidney) were immediately fixed at 10% neutral buffered formalin solution, dehydrated in ascending grades of ethyl alcohol, cleared in methyl benzoate and embedded in paraffin wax. Paraffin sections of 3-5 microns thickness were obtained and stained with hematoxylin and eosin dyes (Bancroft and Gamble, 2002).

DETERMINATION OF HEAVY METAL CONTAMINATION LEVEL (MG/L)

Water samples were collected from different vicinities in Menofiya Province (ElKhadroyou region, Elatfa region and Shabra bass region) and from a private fish farm in Kafr El-Sheikh province, Egypt. The samples were acidified by nitric acid and chilled on icebox for transport to the laboratory for heavy metals determination according to the American Public Health Association (APHA, 1995).

RESULTS

CLINICAL AND POST-MORTEM EXAMINATION OF FISH

The clinical examination of the infected fishes revealed skin darkness, hemorrhages all over the fish body especially at

the base of fins, tail, and fins rot, abdominal dropsy, hemorrhagic ulceration and detachment of scales in scaly fishes. While, the post-mortem examination of infected fishes revealed congestion of gills and visceral organs, enlargement of different organs (liver, spleen, and kidney) and some cases showed that paleness of liver (Figure 1, 2 and 3).



Figure 1: (A) Naturally infected *Oreochromis niloticus* fish showing skin darkness and fin and tail rot, (B): Naturally infected *Oreochromis niloticus* fish showing congested gills, pale and enlarged liver, (C): Naturally infected *Clarias gariepinus* fish showing hemorrhagic ulceration as well as fin and tail rot, and (D): Naturally infected *Clarias gariepinus* fish showing pale and enlarged liver and hemorrhages all over the body.



Figure 2: (A) Naturally infected *Anguilla anguilla* fish showing skin darkness and hemorrhages., (B): Naturally infected *Anguilla anguilla* fish showing pale and enlarged liver and congested spleen, (C): Naturally infected *Mormyrus kannume* fish showing hemorrhages all over the body., and (D): Naturally infected *Mormyrus kannume* fish showing congestion in different organs (gills, liver and kidney)

RESULTS OF BACTERIOLOGICAL EXAMINATIONS

A- Morphological, culture characters, and Biochemical identification

The bacterial colonies isolated from gills, liver, spleen and kidney of naturally infected fishes on Tryptic soya agar supplemented with NaCl (3 %) and on TCBS media at 25°C

Table 3: Morphological, culture characteristics and biochemical identification of suspected *Vibrio* species isolated from naturally infected fishes.

Criteria	<i>V.anguillarum</i>	<i>V. harveyi</i>	<i>V.vulnificus</i>	<i>V.alginolyticus</i>	<i>V.fluvialis</i>
Gram stain	-ve	-ve	-ve	-ve	-ve
Growth on TCBS	Yellow colonies	Yellow colonies	Green colonies	Yellow colonies	Yellow colonies
Swarming on solid media	-ve	-ve	-ve	+ve	-ve
Motility	+ve	+ve	+ve	+ve	+ve
Oxidase	+ve	+ve	+ve	+ve	+ve
Catalase	+ve	+ve	+ve	+ve	+ve
H ₂ S production	-ve	-ve	-ve	+ve	-ve
Urease	-ve	+ve	-ve	-ve	-ve
Citrate	-ve	+ve	+ve	-ve	+ve
Indol production	+ve	+ve	-ve	+ve	+ve
VP	+ve	-ve	-ve	+ve	-ve
Methyl red	(Weak)+ve	+ve	+ve	+ve	+ve
Glucose	+ve	+ve	+ve	+ve	+ve
Sucrose	+ve	+ve	-ve	+ve	+ve
Mannitol	+ve	+ve	-ve	+ve	+ve
Lactose	+ve	-ve	+ve	-ve	-ve

for 24-48 hours. *V.anguillarum*, *V. harveyi*, *V. alginolyticus* and *V.fluvialis* produced yellow colonies on TCBS media. *V.vulnificus* produced green colonies on TCBS media. All isolated *Vibrio* spp. were Oxidase positive. *V.alginolyticus* produced swarming colonies on solid media. Most *Vibrio* spp. showed curved or bent bacilli when examined by Gram stain. The data presented in Table 3 demonstrated the full identification scheme of isolated *Vibrio* serotypes.

Table 4: Prevalence of *Vibrio* species isolated from naturally infected fishes.

Fish species	No of examined fishes	Positive cases for <i>Vibrio</i> spp.	
		NO.	%
Oreochromis niloticus	80	28	35
Clarias gariepinus	40	15	37.5
Anguilla anguilla	20	11	55
Mormyrus kannume	20	8	40
Chrysichthys auratus	20	7	35
Mugil cephalus	20	9	45
Total	200	78	39

B- Results of Vitek2 system:-

Five isolates were identified using Vitek2 compact. Five isolates were identified as *V. anguillarum*, *V. harveyi*, *V.vulnificus*, *V. alginolyticus* and *V. fluvialis*, with a probability of 99%.

PREVALENCE OF *VIBRIO* SPECIES ISOLATED FROM EXAMINED FISHES

The prevalence of *Vibrio* species in the naturally infected

Table 5: Prevalence of *Vibrio* spp. isolated from naturally infected fishes.

Fish species	+ve cases	<i>Vibrio</i> spp.	+ve cases	
			NO.	%
Oreochromis niloticus	28	<i>V.anguillarum</i>	11	39.3
		<i>V. harveyi</i>	9	32.1
		<i>V. vulnificus</i>	6	21.5
		<i>V. fluvialis</i>	2	7.1
Clarias gariepinus	15	<i>V.anguillarum</i>	9	60
		<i>V. harveyi</i>	6	40
Anguilla anguilla	11	<i>V.alginolyticus</i>	7	63.6
		<i>V.vulnificus</i>	4	36.4
Mormyrus kannume	8	<i>V. harveyi</i>	5	62.5
		<i>V.vulnificus</i>	3	37.5
Chrysichthys auratus	7	<i>V. fluvialis</i>	4	57.1
		<i>V. harveyi</i>	3	42.9
Mugil cephalus	9	<i>V.anguillarum</i>	2	22.2
		<i>V. fluvialis</i>	2	22.2
		<i>V. alginolyticus</i>	5	55.6



Figure 3: (A) : Naturally infected Mugil cephalus fish showing hemorrhages all over the body, abdominal dropsy and scales detachment, (B): Naturally infected Mugil cephalus fish showing congested gills, pale liver and paleness of intestine.

Table 6: Heavy metal concentrations (mg/L) in water samples from different localities in comparison with the permissible levels.

Element	Kafr-Elsheikh (farm)	Elkhadroyou (region)	Elatfa (region)	Shabra- bass (region)	Permissible level
Copper	0.24	0.19	0.30	0.38	0.2
Iron (Fe)	1.12	0.89	1.27	0.87	1.00

fishes was shown in Table . The data demonstrated that only 39% (78/200) of the examined fishes were infected with *Vibrio* species, and the highest prevalence rate was observed in case of *Oreochromis niloticus* (35%) while the lowest prevalence rate was recorded in the case of *Chrysichthys auratus* (7%). Regarding the incidence rates of different serotypes, the predominated serotypes in the case of *Oreochromis niloticus* were *V. anguillarum* (39.3%) *V. harveyi* (32.1%). While in the case of *Clarias gariepinus* *V. anguillarum* (60%) *V. harveyi* (40%). Conversely, in case of *Anguilla anguilla*, only *V. alginolyticus* (63.6%) and *V. vulnificus* (36.4%) were successfully recovered. While in the case of *Mormyrus kannume* only *V. harveyi* (62.5%) and *V. vulnificus* (37.5%) were successfully confirmed. In the case of *Chrysichthys auratus* only *V. fluvialis* (57.1%) and *V. harveyi* (42.9%) were confirmed. Finally, in the case *Mugil cephalus* the predominated isolated serotype was *V. alginolyticus* (55.6%) (Table 4 and 5).

GENOTYPING CHARACTERIZATION OF SOME *VIBRIO* SEROTYPES VIRULENCE GENES USING PCR

Genotypic characterization of some virulence determinants in particular hemolysins (*vhh*) and collagenase virulence encoding genes was carried out using specific primers set for *Vibrio harveyi* and *Vibrio alginolyticus* respectively. The data showed that the isolated serotypes exhibited a specific band of 737 bp and 235 bp respectively Figure 4A-B.

HISTOPATHOLOGICAL EXAMINATION OF THE EXAMINED FISHES

The data presented in Figure (5,6 and 7) demonstrated the histopathological changes of the examined internal organs (Liver, spleen, kidney, and gill). Histopathological examination of spleen tissue collected from *Clarias gariepinus*

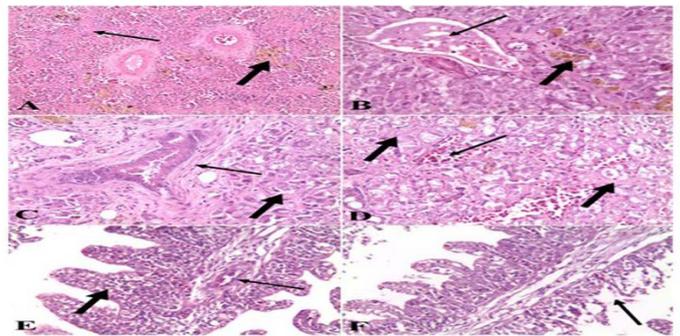


Figure 5: A). Spleen of naturally infected *Clarias gariepinus* with *Vibrio* species showed severe fibrosis (thin arrow) and hemosiderin-phages (thick arrow) (H&E x 10); B). Liver of naturally infected *Clarias gariepinus* with *Vibrio* species Showed congested portal vein (thin arrow), accumulation of bile pigment (thick arrow) and coagulative necrosis of hepatocytes (H&E x 20); C) Liver of naturally infected *Clarias gariepinus* with *Vibrio* species, showed bile duct hyperplasia, increased connective tissue around the bile duct (thin arrow) and coagulative necrosis of hepatocytes (thick arrow) (H&E x 20); D). Liver of naturally infected *Clarias gariepinus* with *Vibrio* species showed severe hemorrhages (thin arrow) and proliferation of connective tissue in between hepatocytes (thick arrow) (H&E x 20); E). Gills of naturally infected *Clarias gariepinus* with *Vibrio* species showed congested blood vessels (thin arrow), mononuclear cell infiltrations and thickening of secondary lamellae (thick arrow) (H&E x 20); F): Gills of naturally infected *Clarias gariepinus* with *Vibrio* species, showed necrosis and desquamation of the secondary lamellae epithelium (arrow) (H&E x 20).

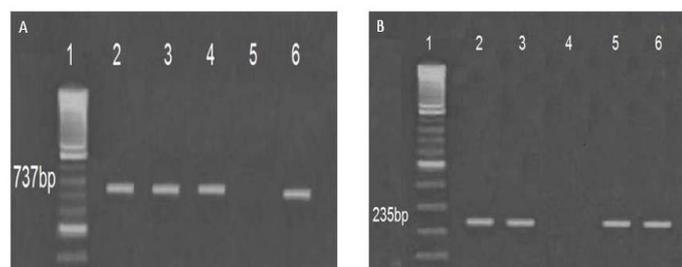


Figure 4: Genotyping characterization of (A) *Vibrio alginolyticus* lanes (2-6), (737 bp), Lane (1) Ladder (100 bp) and (B) *Vibrio harveyi* lanes (2-6) (235 bp), Lane (1) Ladder (100 bp) using PCR specific primers targeting and collagenase and hemolysins (*vhh*) virulence encoding genes respectively.

infected with *Vibrio* species showed severe fibrosis and proliferation of hemosiderin-phages (Figure 1 A). While in the case of liver samples the data showed congested blood vessels and hepatic sinusoids, severe hemorrhages in the hepatic parenchyma, the hepatocytes showed degenerative changes and coagulative necrosis with an accumulation of bile pigment inside the cells. There is a proliferation of connective tissue fibers between hepatocytes and also in the portal areas hyperplasia of bile duct epithelium (Figure 1 B, C & D). Examination of gill samples collected from *Clarias gariepinus* infected with *Vibrio* Species demonstrated severe congestion of blood vessels, mononuclear cell infiltration, thickening and adhesion of secondary lamellae with necrosis and desquamation of epithelium lining some secondary lamellae (Figure 1 E & F). Concerning the histopathological examination of tissue samples collected from *Oreochromis niloticus* infected with *Vibrio* Species, the data

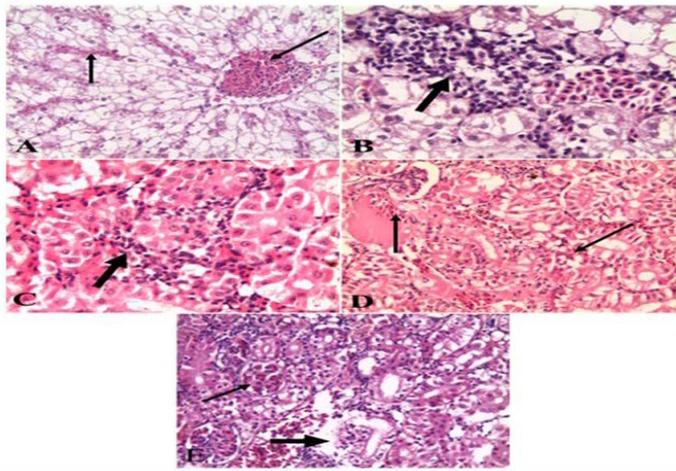


Figure 6: A). Liver of naturally infected *Oreochromis niloticus* infected with *Vibrio* Species Showed severely congested blood vessels and hepatic sinusoids (arrow) (H&E x 20); B). Liver of naturally infected *Oreochromis niloticus* infected with *Vibrio* Species Showed mononuclear cell infiltrations (arrow) (H&E x 40); C). Kidney of naturally infected *Oreochromis niloticus* infected with *Vibrio* Species, showed mononuclear cell infiltrations around renal tubules (arrow) (H&E x 40); D). Kidney of naturally infected *Oreochromis niloticus* infected with *Vibrio* Species showed severe hemorrhages (arrow) (H&E x 20); E). Kidney of naturally infected *Oreochromis niloticus* infected with *Vibrio* Species, showed congested glomerular capillaries (thin arrow), degeneration and necrosis of renal tubular epithelium (thick arrow) (H&E x 20).

showed that liver showed severely congested central veins and hepatic sinusoids. The hepatic parenchyma showed swelling and vacuolation of hepatocytes with focal and diffuse mononuclear cell infiltrations (Figure 2A & B). While examination of the kidneys revealed severe hemorrhages in the interstitial tissue with mononuclear cell infiltrations around renal tubules. The renal tubular epithelium showed vacuolation, necrosis, and desquamation. The glomerular capillaries appeared congested (Figure 2 C, D & E). As for the gills, the data presented showed severely congested blood vessels and mononuclear cell infiltrations. thickening and adhesion of secondary lamellae with vacuolation, necrosis, and desquamation of epithelium lining some secondary lamellae (Figure 3 A & B). Lastly, histopathological examination of spleen tissue samples showed severe hemorrhages with the presence of high number hemosiderin-phages in between necrotic tissue (Figure 3 C & D).

HEAVY METALS CONCENTRATION IN WATER SAMPLES

The level of heavy metal pollution in the water samples where the fishes have gathered specifically the levels of Copper and Iron was outlined in the (Table 6). The information displayed demonstrated that water samples gathered from Kafr Elsheim, Elatfa locale, and Shabrabass area

recorded a most noteworthy higher level of copper 0.24, 0.30 and 0.38 mg/ml respectively. Interestingly, the water samples gathered from Kafr Elsheim and El atfa district recorded the most elevated amount of Iron sullyng 1.12 and 1.27 mg/ml in a specific order. Altogether, the water samples gathered from Kafr Elsheim and El atfa district are considered exceptionally contaminated with higher levels of Copper and Iron contrasting the other areas.

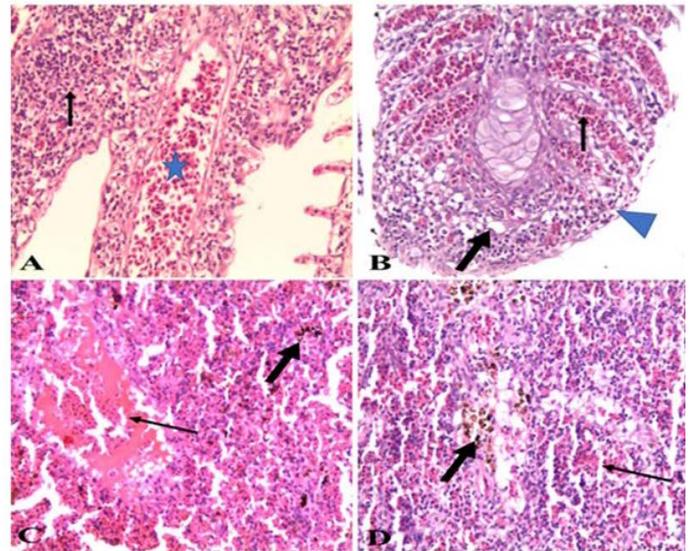


Figure 7: A). Gills of naturally infected *Oreochromis niloticus* with *Vibrio* Species showed severely congested blood vessels and mononuclear cell infiltrations (arrow) (H&E x 20); B). Gills of naturally infected *Oreochromis niloticus* with *Vibrio* Species, showed severely congested blood vessels (thin arrow), mononuclear cell infiltrations, vacuolation of epithelium (thick arrow) and adhesion between secondary lamellae (arrow head) (H&E x 20); C). Spleen of naturally infected *Oreochromis niloticus* with *Vibrio* Species showed severe hemorrhages (thin arrow) and hemosiderin-phages (thick arrow) (H&E x20); D). Spleen of naturally infected *Oreochromis niloticus* with *Vibrio* Species showed hemorrhages (thin arrow) and hemosiderin-phages (thick arrow) (H&E x 40).

DISCUSSION

Fisheries in particular aquaculture represent a vital sector in the Egyptian national income. Aquaculture is subject to a variable number of ecological stressors including bacterial infections particularly infection with *Vibrio* spp. In the present study, a total of 200 freshwater fishes were collected from different localities in Minufya and Kafr El-sheikh governorates and examined clinically and bacteriologically for the presence of *Vibrio* species. In addition, the genotypic characterization of *Vibrio* species was carried out using specific primers was assessed by PCR. Moreover, the association between the infection and the contamination of the water with the heavy metals was assessed. The clinical

examination of the fishes showed hemorrhages all over the fish body, at the base of fins, tail, and fins rot, abdominal dropsy, hemorrhagic ulceration, detachment of scales in scaly fishes, redness in the eye and skin darkness. These outcomes are consistent with those previously described (Abd El-Aziz et al., 2013; Ali, 2005; El-Hady et al., 2015; Minami et al., 2016; Younes et al., 2016). The fishes were further examined for postmortem lesions of the visceral lesions. The collected data showed that in some cases, congestion of gills, enlargement of liver, spleen, and kidney and in some cases paleness of liver was recorded. These results are in agreement with that formerly obtained in many studies (Abd El-Aziz et al., 2013; Ali, 2005; Rameshkumar et al., 2014; Reham, 2009; Stephens et al., 2006; Younes et al., 2016). Although, *Vibrio* infection in fish can be assumed by clinical signs, however, proper verification of the infection necessitates isolation and identification of the causative agent. Isolation and identification of *Vibrio* species based on the cultural morphology on selective media and the results of the biochemical tests. The obtained data showed that *V. anguillarum*, *V. harveyi*, *V. alginolyticus* and *V. fluvialis* produced yellow colonies and *V. vulnificus* produced green colonies when cultured on TCBS and *V. alginolyticus* produced swarming colonies on solid media. The isolated *Vibrio* spp. were Oxidase positive, and most of the isolates showed curved or bent bacilli on Gram stained film. These results are in agreement with that obtained previously (Chang et al., 2011; Rim et al., 2012; Sabir et al., 2013; Younes et al., 2016). The present study confirmed that the prevalence of *Vibrio* species amongst naturally infected fishes was 39%. This outcome is lower than that acquired by Adebayo-Tayo and coauthors who demonstrated that *Vibrio* species were successfully recovered from 44.2% of inspected seafood fish collected from Oron brook (Adebayo-Tayo et al., 2011). Conversely, this result is higher than that obtained by Moustafa and his companions who found that the pervasiveness of *Vibrio* spp among the tested fishes collected from Qarun Lake at EL-Fayoum region and Suez inlet at Suez governorate was 34% (Moustafa et al., 2010). The discrepancy in results between these studies and the study herein can be attributed to many factors including the water salinity, the number of fishes, species of the fish, season, water quality and area of collection (Lee et al., 2006). In large-scale aquaculture, negative conditions, for example, overcrowding, high temperature, quick development, occasional water restoration rate and despicable evacuation of injured and dead fishes from the cultivating range make great conditions for the rise of bacterial ailments (Almeida et al., 2009). The data obtained herein showed that, the prevalence rate of different *Vibrio* species isolated from the examined fishes was varied, with *V. harveyi* is the predominant isolate (29.48%, 23/78), followed by *V. anguillarum* (28.20%, 22/78), *V. vulnificus* (16.67%, 13/78), *V. alginolyticus* (15.38%, 12/78), and *V. fluvialis* (10.25%, 8/78). This result is not the same as that got

previously (Moustafa et al., 2015; Wang et al., 2001), who showed that, *V. alginolyticus* is the predominant *Vibrio* serovar recovered from fishes and infection with this serovar usually associated with severe economic losses. *V. harveyi* is mainly a pathogen of marine aquaculture has been isolated from several diseased marine animals (Hashem and El-barary, 2013; Ransangan et al., 2012). The Nile tilapia *Oreochromis niloticus* is a fish of both fresh and salt water, and the main pathogenic *Vibrio* serovars involved in salt water infections are *V. anguillarum*, *V. parahaemolyticus* and *V. vulnificus*. While in freshwater cultures *V. mimicus* and *V. cholerae* are the main pathogenic serovars involved (Plumb, 1999; Sakata and Hattori, 1988). Here in, isolation of *V. harveyi* from fresh water fishes and cultivate farms in Egypt warrants more investigations to clarify its public health impact. Moreover, additional studies are required to elucidate the pathogenesis of *V. harveyi*-induced infection in both marine and fresh water fishes.

The virulence of *Vibrio* pathogen is primarily administered via a variety of virulence encoding genes. Herein, *Vibrio alginolyticus* and *Vibrio harveyi* were further tested for the presence of collagenase and *hhv* virulence encoding genes respectively using specific primers set. The data showed that, the isolated serotypes exhibited a specific band of 737 bp and 235 bp respectively. This result is consistent with that obtained by others (Di Pinto et al., 2005; Khamesipour et al., 2014; Maiti et al., 2009; Moustafa et al., 2015). PCR has been introduced as an easy, accurate, less expensive, rapid (only takes few hours) tool used for affirmation of pathogenic *Vibrio* serovar (Bramhachari and Dubey, 2006). The use of PCR as a simple, quick and inexpensive method in the diagnosis of *Vibrio*-related diseases in marine and other large-scale aquaculture systems will help specialists develop accurate preventive and treatment measures to reduce the economic losses associated with these diseases (Shruti and Haldar, 2012).

The histopathological examination of the livers of naturally infected fishes showed congested portal vein, bile pigment, coagulative necrosis of hepatocytes, another showed bile ductin filtration and mononuclear cells infiltrations. Gills showed necrosis, congestion of blood vessels, mononuclear cells infiltrations and thickening of the secondary lamellae. Spleens showed severe fibrosis hemosiderin phages and hemorrhage. While kidneys showed mononuclear cells infiltration around renal tubules, severe hemorrhage, congested glomerular capillaries, degeneration, and necrosis of renal tubular epithelium. The obtained data are consistent with the findings obtained by others (Diggles et al., 2000; El-Bassiony, 2001; Korun and Timur, 2008). The presence of histopathological lesions in the internal organs clearly explained the septicemic nature of *Vibrio* infection. Altogether, the full confirmation of *Vibrio* infection ought to incorporate, examination of fishes for the visible clinical

signs, postmortem examination, isolation of the causative agent using specific media and biochemical tests, and histopathological examination of the internal organs as a proof of septicemic nature of the *Vibrio* infection.

The contamination of water with heavy metals has been increased markedly during the last decades. Accumulation of heavy metals in marine and freshwater fishes will adversely affect the fishing industry and the consumer's health. Therefore, regular monitoring of water for heavy metals contamination is a priority for the food safety regulations (Bosch et al., 2016). In this study, water samples were collected from the areas where fishes collected were examined for the level of heavy metals in particular Copper and Iron. The data presented showed that there is a positive relationship between the incidence of infection with *Vibrio* and the level of water contamination with Iron and Copper. Contamination of water with heavy metals most likely altered the biochemical and physiological status of the fishes render them more susceptible to infection with bacterial diseases (Vinodhini and Narayanan, 2009). To the best of our knowledge, there are no studies about the precise role of Copper and Iron as heavy metals in the pathogenesis of vibriosis among freshwater fishes. Therefore, the data of this study provides a new finding required further investigation in future studies. This will be a crucial step on the road to reduce stressors on freshwater fishes and thus will legitimately diminish the seriousness of bacterial disease specifically infection with *Vibrio* spp.

CONCLUSION

Under the condition of this study, the obtained data revealed that *Vibrio* is an important bacterial disease affecting freshwater and cultivate farms fishes with wide range host. *V. hareveyi* is the predominantly isolated serovar from freshwater and cultivate farms fishes. Further investigations are required to elucidate its public health impact. Monitoring of the water for the contamination with heavy metals is a crucial step to minimise the severity of bacterial infections, in particular, infection with *Vibrio* serovars. These findings expand the previous information of *Vibrio*-induced infection in freshwater ecosystems and present new findings for future investigations.

FUNDING SOURCES

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

ACKNOWLEDGEMENTS

The authors would like to thank all the staff members of the Department of Pathology, Faculty of veterinary Medi-

cine, Sadat City University, Egypt and the Department of Fish Diseases, Animal Health Research Institute, Dokki, Giza, Egypt for their valuable support.

CONFLICT OF INTEREST

The authors would like to declare there is no conflict of interest

AUTHORS CONTRIBUTION

El-Sharaby S, Khalil R, Ali M, El Ballal S, design of the experiment, bacteriological and histopathological work, Abd-Elgaber M, and Tarabees R, bacteriological histopathological and molecular work. Tarabees R and Abd-Elgaber M write the manuscript; all the authors approved the final version of the manuscript.

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