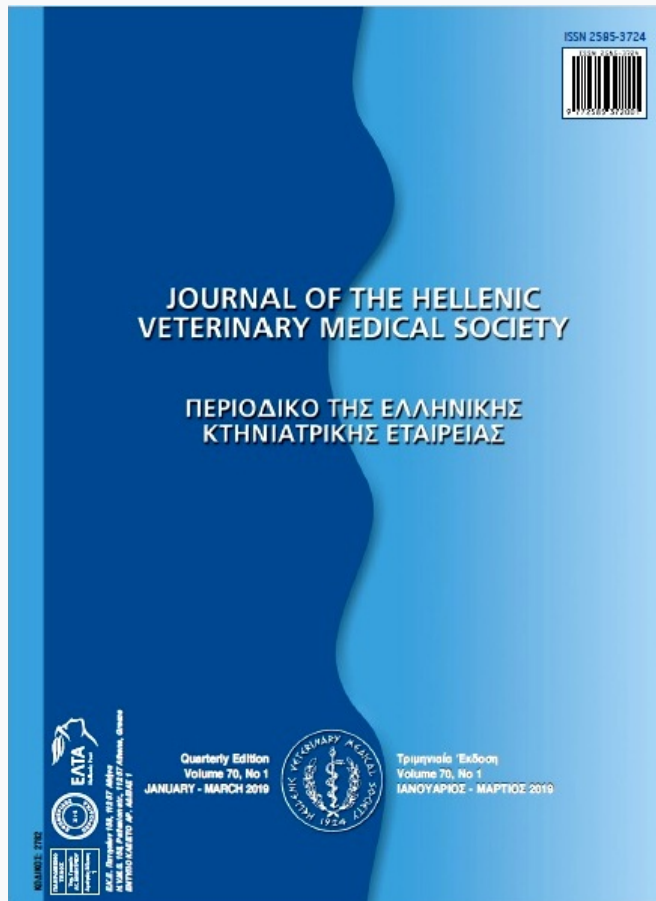


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## Advanced diagnosis of vibriosis among some marine fishes in lake Tamsah, Egypt

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**ABSTRACT.** Vibriosis is a serious disease affecting marine water fishes. The present study was applied on 320 marine fishes of four equal different species (*Sparus auratus*, *Mugil cephalus*, *Mugil seheli* and *Tilapia zillii*) each 80. Fishes were collected seasonally and randomly from Lake Tamsah in Ismailia governorate from September 2015 to August 2016. The signs and lesions of severe septicemia, hemorrhages and ulcerations were observed among the examined fishes. Isolated bacteria were observed as yellow and green pigmented colonies on TCBS media, and as creamy colored colonies on TSA media with 2.5% NaCl concentration. The causative agent was identified as vibrio sp. Concerning gene expression, most isolates were molecularly identified using the pvsA gene primers giving a product size of 338-bp size and 348-bp for *V. alginolyticus* and *V. parahemolyticus* respectively. The highest prevalence of vibriosis was recorded in *T. zillii* (63.75%), *M.seheli* (37.5%) then *M.cephalus* and *Sparus auratus* (28.75%) while the total prevalence was (39.69%). The highest seasonal prevalence was recorded in summer (81.25%) followed by spring (35%) then autumn (23.75%) and winter (18.75%). The highest prevalence of organ specificity was in liver, kidneys then spleen and gills. The histopathological studies showed activation of melanomacrophage centers, degeneration, necrosis and congestion in liver, kidney, spleen, and gills.

**Keywords:** Marine fishes, Vibriosis, pvsA gene, Histopathological exam.

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## INTRODUCTION

Egypt depends on fish as one of the essential sources of the national profit, stimulating local market economies and essential source of foreign exchange. Furthermore, marine waters are the main sources for water needed for mariculture and luckily, Egypt has more than one marine resources such as the Red and Mediterranean Sea (Sadek, 2000).

The universal trend of commercial aquaculture is towards condensation of culture practice in the target to increase productivity per unit area (Elgendy et al., 2015 and Kolkovski, et al. 2011).

One of the common genera in aquatic habitat, particularly in marine, is genus vibrio, several species of it are pathogenic for freshwater especially where organic loads are high (Alicia et al., 2005).

One of the most frequent diseases occurring in marine aquaculture, is vibriosis caused by many vibrio species (Alcaide, 2003). It is a serious epizootic disease affecting both wild and farmed marine fish species worldwide, and has become a limiting factor for the development of intensive aquaculture industry. Several factors have been proposed to influence the survival, persistence and ability of vibrios to cause infection (Lipp et al., 2002).

The present study was conducted to investigate the prevalence of different vibrio spp. among some marine fishes in relation to clinical picture, conventional and advanced diagnosis using *pvsA* gene along with histopathological examination.

## MATERIALS AND METHODS

### 1- Fishes:

A total number of 320 marine fishes of four equal various species, average body weights  $100 \pm 5$  for *Sparus auratus* and *Mugil cephalus* and  $50 \pm 5$  for *Mugil seheli* and *Tilapia zillii*, each were 80 were represented as (*Sparus auratus*, *Mugil cephalus*, *Mugil seheli* and *Tilapia zillii*). They were collected seasonally and randomly. They were examined freshly from Lake Tamsah in Ismailia governorate, Egypt from September 2015 to August 2016 and subjected to full clinical, postmortem and bacteriological examinations.

### 2- Bacterial examination:

#### *a-Isolation:*

The bacterial isolates were taken from ulcers, gills, liver, kidneys and spleen of naturally alive and freshly dead infected *Sparus auratus*, *Mugil cephalus*, *M. seheli* and *Tilapia zillii*. They were streaked on TCBS agar and incubated at  $27^\circ\text{C} \pm 1^\circ\text{C}$  for 24-48 hr. Suspected colonies were subcultured on TSA (2.5% NaCl) and subjected to microscopic and biochemical analysis according to Farmer et al., (1992).

#### b-Phenotypic Identification:

##### *b.1) Morphological and biochemical examination:*

The pure colonies were identified morphologically according to Cruickshank et al., (1982) and biochemically according to Thompson et al., (2004). They were identified using the traditional biochemical tests only on Gram negative, catalase positive and Oxidase positive. These tests were accomplished by API20E strips (BioMerieux, France).

##### *b.2) Molecular characterization of Vibrio alginolyticus & Vibrio parahaemolyticus using pvsA gene:*

###### *b.2.1) Genomic DNA Extraction:*

Genomic DNA was extracted from *V. alginolyticus* and *V. parahaemolyticus* strains using boiling technique according to (Devi et al. 2009).

###### *b.2.2) Oligonucleotide primers:*

Four primer sequences, two sense (F) and two antisense (R), were used for identification of both strains. The primers were designed from the published *pvsA* gene sequences (accession no. DQ201184.2 and AB082123.1) for *V. alginolyticus* and *V. parahaemolyticus* strains respectively using the web-based software Primer3Plus (Untergasser et al., 2007). Primers sequences and characteristics are shown in Table 1.

###### *b.2.3) PCR and agarose gel electrophoresis:*

The PCR reaction mixture was contained 5.5µl genomic DNA, 12.5µl PCR master mix, 1µl of forward primer, 1µl of reverse primer in a total volume of 25µl. Reaction cycles were performed using TC-25/H thermal cycler, the cycle conditions as a follow: pre- denaturation at  $94^\circ\text{C}$  for 3 min then followed by 35 cycles of denaturation at  $94^\circ\text{C}$  for 30

**Table 1:** *V. alginolyticus* and *V. parahemolyticus* pvsA gene Primers sequence and characteristics.

Primer name	Reference gene for location	Location (np)	Sequence	Tm(°C)	Gc%	Product size
VA 1F	DQ201184.2	10391-10410	CAG TAA CCG CCT TAC CGT GT	60.1	55	338 bp
VA 1R		10709-10728	CAC TCC AGC GTG TCG ACT TA	60	55	
VP 1F	AB082123.1	721-740	TTC CCT GTC GTA GCA AAA CC	60.1	50	348 bp
VP 1R		1049-1068	AAA TTC GCT GTG GCA AAC TC	60.3	45	

sec, annealing at 60°C for 30 sec; and extension at 72°C for 3 min. in order to confirm the amplification of the target sequences the PCR products were electrophoresed on 1% agarose gel stained with ethidium bromide, the resulting fragments were visualized by UV transillumination (Slime line™ series).

### 3-Experimental infection:

A total of 60 apparently healthy *Tilapia zillii*, weighting  $30 \pm 5$ g were selected for detection of the pathogenicity of the most common bacterial isolates. Fish were classified into duplicate three groups each contained 10. The inocula processed for isolated bacteria as I/P and I/M injections were prepared according to Austin & Austin, (2007). Fish were noticed daily for 14 days. one group was consistently inoculated I/P and the other group was inoculated I/M with the bacterial suspension of *Vibrio alginolyticus* in a dose of 0.2 ml of ( $3 \times 10^7$  CFU). The control groups were injected I/P and I/M with 0.2 ml of sterile tryptic soya broth.

### 4-Histopathological examination:

Specimens were collected freshly from liver, kidney, spleen and gills of naturally infected fishes. Fixed in 10% phosphate buffered formalin and processed by traditional method, sectioned at 5micron thickness. They were stained with H&E stain then examined microscopically according to Roberts, (2001).

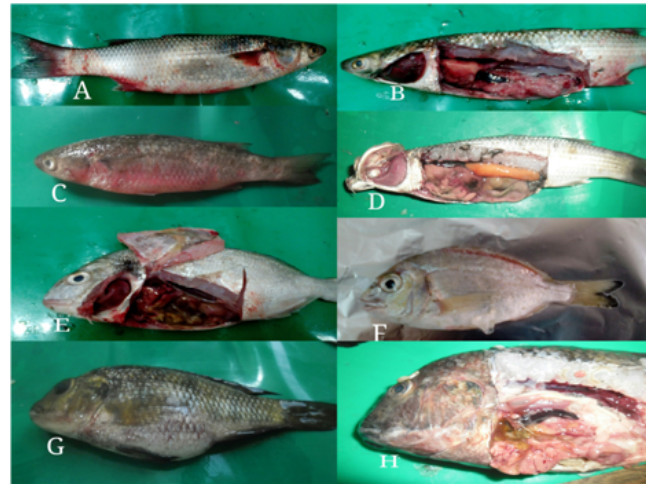
## RESULTS

### A-Clinical examination of naturally infected fishes:

The clinical examination of naturally infected fishes was recorded in Plate (1).

### B-Results of bacteriological examinations:

The colonies were creamy color on tryptic soya

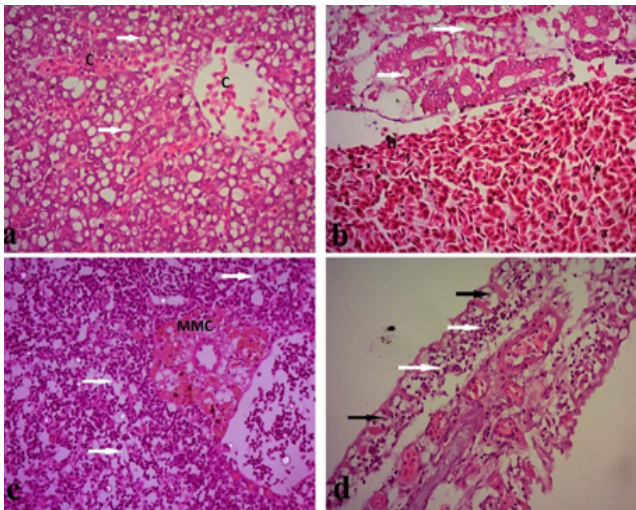


**Plate 1:** A. Naturally infected *M.cephalus* showing severe hemorrhage on the abdomen, pectoral and tail fin. B. Severe hemorrhagic gills & kidneys and enlarged spleen. C. Naturally infected *M.seheli* showing severe external hemorrhage on external body and detached scales. D. pale gills and liver in advanced case. E. Naturally infected *S.auratus* showing hemorrhagic gills, liver and kidneys. F. Hemorrhage under the dorsal fin. G. Naturally infected *T.zillii* showing severe abdominal distention and turbid eye. H. Hemorrhagic kidneys and enlarged spleen.

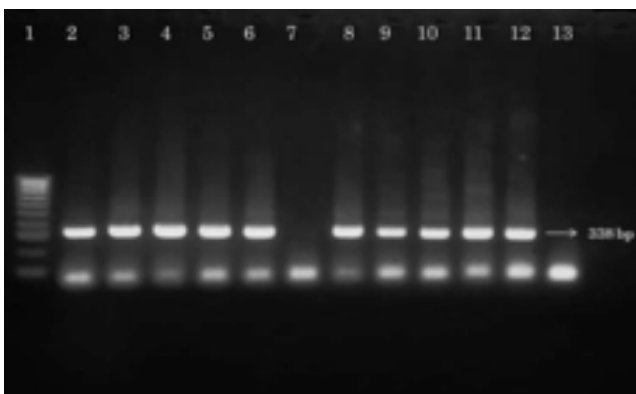
medium with a range of (2-3 mm in diameter) while on TCBS medium, they were yellow and green colored colonies with yellow pigmentation. Also, sensitive to vibriostate O/129(150 µg). Isolates were Gram- negative, comma or rod shaped scattered in arrangement and motile. The result of the conventional and commercial systems using API20E for biochemical tests declared the results in (Table 2).

### C-Molecular characterization of *V.alginolyticus* & *V.parahemolyticus* using pvsA gene:

Fig 1 shows the amplification of 338 bp PCR amplicon representing pvsA gene in *V.alginolyticus* strain using VA1F and VA1R primer pair. While, Fig



**Plate 2:** Histopathological changes induced by *Vibrio* spp. in *Tilapia zilli* fish., H&E. X 400 (a) Liver showing congestion of central vein & hepatic sinusoids and presence of distinct fat globules (arrow). (b) kidney showing massive hemorrhage and degenerative changes of renal tubules epithelium (arrow). (C) Spleen showing hyperplasia of melano-macrophage centers, congestion of blood vessels & sinusoids and depletion of white pulps (arrows). (d) Gills showing focal congestion, leukocytic infiltration (white arrows), atrophy of secondary lamellae and vacuolar degeneration of lamellar epithelium (black arrows).

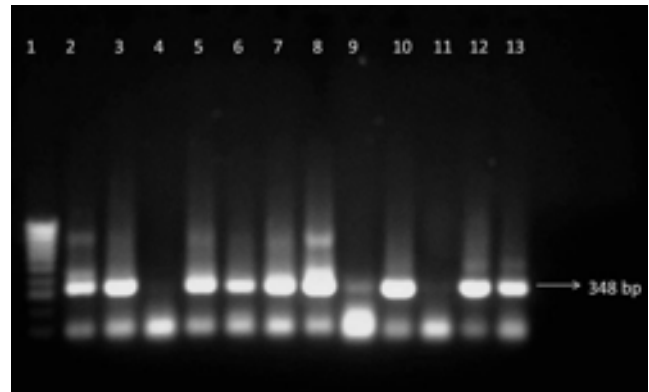


**Figure 1.** Showing 338- bp PCR amplicon representing pvsA gene in *V.alginolyticus*, Lane 1: 100 bpDNA ladder.

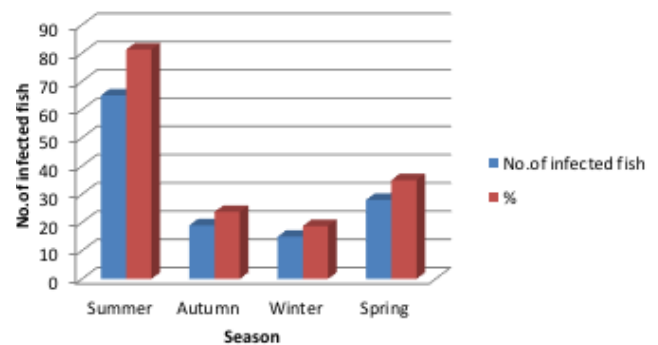
2 shows the amplification of 348 bpPCR amplicon representing pvsA gene in *V.parahemolyticus* strain using VP1F and VP1R primer pair.

**D-Prevalence of vibriosis among naturally infected examined marine fishes:**

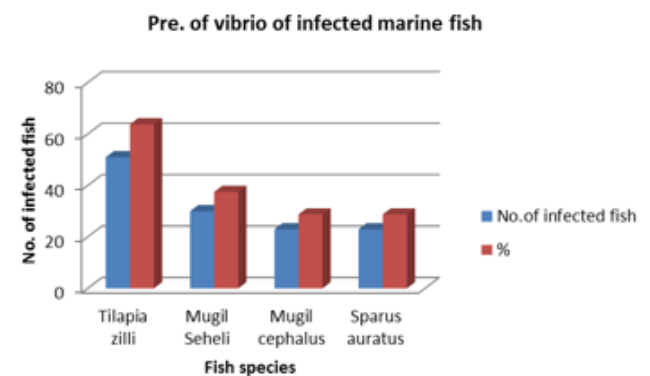
1) Total and Seasonal prevalence of vibriosis



**Figure 2.** Showing 348- bp PCR amplicon representing pvsA gene in *V.parahemolyticus*, Lane 1: 100 bpDNA ladder.



**Figure 3.** Showing the total prevalence of Vibriosis among the examined marine fishes.

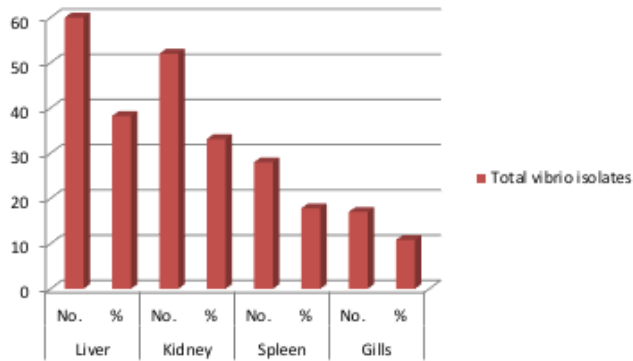


**Figure 4.** Showing the prevalence of Vibriosis in infected marine fishes.

among naturally infected marine fishes: results are summarized in figures 3 and 4

2) Prevalence of vibrio spp. isolates in different organs of examined fishes:

Results are shown in figure 5.

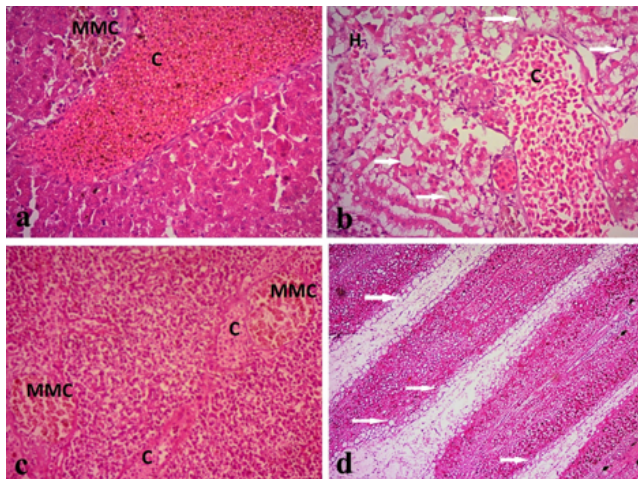


**Figure 5.** Showing numbers and percentages of vibrio isolates from (liver, kidney, spleen & gills) of examined fish.

### E-Experimental infection:

#### Results of histopathological examination

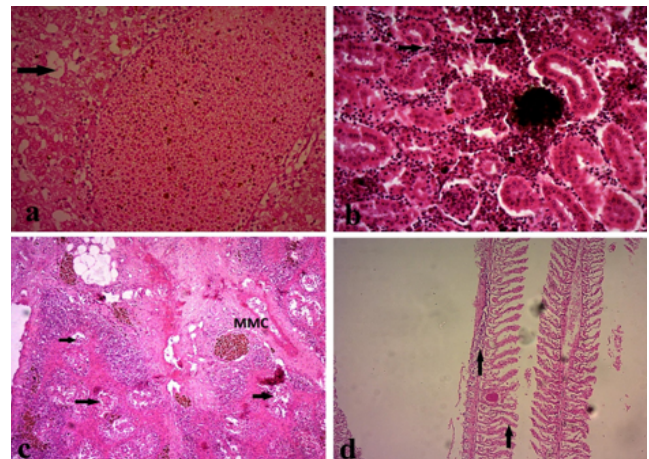
Results are expressed in (plates 2,3,4,5) for *Tilapia zillii*, *Mugil cephalus*, *M.seheli* and *Sparus auratus* respectively.



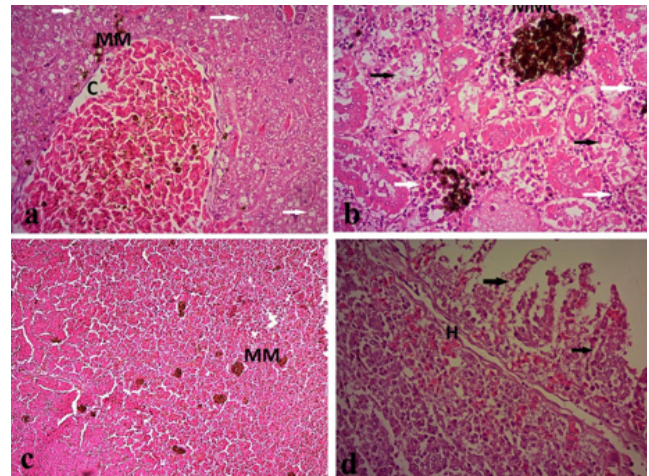
**Plate 3.** Histopathological changes induced by *Vibrio* spp. in *M. cephalus* fish .H&E X400. (a) liver showing severe congestion of blood vessels and activation of melano-macrophage centers. (b) kidney showing severe congestion and extensive degeneration (arrows) & necrosis of renal tubules along with focal hemorrhage. (c) Spleen of showing congestion of blood vessels, activation of melano-macrophage centers and mild depletion of white pulp. (d) Gills showing congestion of blood vessel, hyperplasia of primary & secondary lamellae and severe mucinous degeneration (arrows).

### Discussion:

Regarding to clinical signs, it was revealed that darkness of external body surfaces & hemorrhages at abdominal and anal regions, swollen abdomen, turbid eyes, ulcer and detached scales in *T. zillii*. Also, there was redness all over the external body surface and



**Plate 4.** Histopathological changes induced by *Vibrio* spp. in *M.seheli* fish. H&E X400. (a) liver showing severe congestion, vacuolar degeneration of hepatocytes and mild focal necrosis also observed (arrows). (b) kidney showing focal inter-tubular hemorrhage admixed with leukocytic infiltration (white arrows) and mild degeneration of renal tubules with focal hyperplasia of melano-macrophage centers. (c) Spleen showing depletion and necrosis of lymphoid follicles (arrows) and hyperplasia of melano-macrophage centers (MMC). (d) Gills showing severe congestion, loss of secondary lamellae and mild leukocytic infiltration (arrows).



**Plate 5.** Histopathological changes induced by *Vibrio* spp. in *Sparus auratus* fish. H&E X 400(a) Liver showing severe congestion of blood vessels and mild to moderate vacuolation of hepatocytes (arrows). (b) kidney showing severe vacuolar degeneration of renal tubules (black arrows), focal leukocytic infiltration (white arrows) and hyperplasia of melano-macrophage centers. (c) Spleen showing hyperplasia of melano-macrophage centers, congestion of sinusoids and mild degeneration of white pulp (d) Gills showing focal hemorrhage, leukocytic infiltration, degeneration and necrosis of secondary lamellae (arrows).

pectoral fin, hemorrhage with detached scales in some areas in *M.seheli*. Other cases showing external hemorrhages and gill hemorrhages in *M.cephalus*. Besides, there was hemorrhage under the dorsal fin, turbid eye, hemorrhagic gills and pale gills in severe cases. These results agree with the findings of Alicia et al., (2005) and Eissa et al., (2013). These signs may be attributed to the adhesion ability which is an important factor in bacterial pathogenicity since it precedes penetration of the microorganisms in the host tissues promoted by the production of toxins according to Lee, (1995).

Regarding the postmortem lesions in cases suffering from vibriosis, we noticed that in naturally infected *S. auratus*, there was slight congestion in gills and liver while in severe cases; there was pale liver and gills. Some cases showing congestion in the body cavity and kidneys with yellow serous mucus in the intestines. In naturally infected *T.zillii* there was pale liver & gills, and in other cases revealed congested and hemorrhagic kidneys and inflamed intestines filled with mucus and enlarged gall bladder. Otherwise, in naturally infected *M.seheli* there was pale liver with hemorrhagic spots and pale gills. Some cases in *M.cephalus* showed hemorrhagic gills and enlarged spleen. These results agreed nearly with Golomazou et al., (2006), Robert et al., (2012) and Eissa et al., (2013). This may be attributed to the manner of infection in fish consist of three main steps: (i) the bacterium penetrates the host tissues by chemotactic motility; (ii) bacterium deploys iron-sequestering systems within the host tissues the, e.g., siderophores, to “steal” iron from the host; and (iii) the bacterium damages the fish by means of extracellular products, e.g., hemolysins and proteases according to Larsen and Boesen ,(2001).

The master factor that permits vibrios to survive and can cause infection within their host is their iron-sequestering systems (Tolmasky et al., 1988). These systems center upon the production of iron-scavenging compounds which known as siderophores and the consequent transport of the ferric-siderophore complex back into the cell cytosol (Crosa, 1997 and Actis et al., 1999).

So, according to researchers Anzaldi and Skaar, (2010) and Kustusch et al., (2011) who found that

there are iron-scavenging compounds siderophores known as vibrioferrin in *vibrio* species, these siderophores are the base of iron-sequestering systems in these bacteria helping them to uptake of iron from tissues of the hosts to their cytosol. So, it could be investigated that bacteria used iron into infected fishes and that lead to decrease their concentration in the different organs.

In this study, the isolated bacteria were gram –ve motile, fermentative catalase and oxidase positive, VP, H<sub>2</sub>S, URE –ve and there were yellow and green colonies and code number on API20E strips was 0146125 & 4047125 for *V. alginolyticus* and 401604 for *V. parahemolyticus* while Buller, (2004) described *V. anguillarum* isolated from fish as Gram-negative, motile and fermentative in O/F test. Isolates were positive for oxidase, catalase and Voges proskauer. On the other hand, it was negative in respect to H<sub>2</sub>S and urease tests. It grows well on TCBS producing yellow colonies.

According to Tomotaka et al., (2003), several genes are expected to be involved in the biosynthesis and transport of vibrioferrin have been identified in the surrounding genomic regions of the *pvuA* gene. One of these genes called *pvsA* gene which constitute an operon that is expressed under iron-limiting conditions. In this study concerning gene expression most isolates were molecularly identified using the *pvsA* gene primers giving a product size of 338-bp size and 348-bp for *V. alginolyticus* and *V. parahemolyticus* respectively.

In the present study, the seasonal prevalence of vibriosis was found that the highest was recorded in summer (81.25%) followed by spring (35%) then autumn (23.75%) and winter (18.75%). The seasonal prevalence among examined fishes revealed that; in *T. zillii* was (95%) in summer followed by spring (75%) then autumn (50%) and winter (40%), In *M. seheli* was (80%) in summer followed by spring (30%) then (25%) autumn and winter (15%), In *S. auratus* was (70%) in summer followed by spring (20%) then (10%) autumn and winter (15%), in *M.cephalus* was (80%) in summer followed by 20, 10 and 5% for spring ,autumn and winter respectively. These results nearly agreed with Eissa et al., (2013) who found that the highest seasonal prevalence

was recorded in summer followed by spring then autumn and winter. Also, it was found that seasonal prevalence of *Vibrio alginolyticus* in *S. auratus* and *T. zillii* high in summer followed by in spring then autumn and at the last the winter. Also, these result in agreement with Lee et al., (2006) and Ming et al., (2011) who all confirmed that the temperature is the main predisposing factor of vibriosis and help in its development.

Owing to prevalence of vibriosis in the examined fishes, the highest prevalence was recorded in *T.zillii* (63.75%) followed by *M.seheli* (37.5%) then *S.auratus* and *M.cephalus* (28.75%) .Which nearly similar to Enany et al., (2011) who reported that *V.alginolyticus* was isolated from *M.capito* with percentage 37.5%. These results agree with Wafeek et al., (2007) who isolated *V.alginolyticus* from Grey mullet fish (*M.cephalus*) collected from Sharm El-Sheikh with high percent (39%). This may be attributed to the high immune response in *S.auratus* and *M.cephalus* rather than *T.zilli*.

Regarding to total prevalence, it was found in examined species (39.69%) which is slightly higher than which recorded by Eissa et al., (2013) who revealed that the total prevalence of vibriosis among naturally infected marine fishes (36%).The result is lower than that obtained by Adebayo-Tayo et al., (2011) who recorded (44.2%) of examined seafood samples obtained from Oron creek infected with vibrio spp. Also, it was lower than which obtained by Balebona et al., (1998) who reported (67.8%) in three fish farms with intensive culture of gilt-head seabream, *S.aurata* L. in southwestern Spain infected with vibrios. That attributed to number of fish, age, site of collection and water temperature.

Regarding to prevalence of vibriosis in different organs of examined fishes, It was shown that the highest prevalence of vibriosis in liver (38.22%) followed by kidneys (33.12%) then spleen (17.83%) and gills (10.8%) which are similar to Eissa et al., (2013) who recorded the same arrangement in *V.alginolyticus*. These results can be explained that, the liver and kidneys are the common target organs

of infection. Similarly to El-Bassiony, (2001) who reported that liver was the highest organ (4 isolates) followed with kidney and spleen about (3 isolates) And not agreed with Wafeek et al., (2007) who approved that the distribution of *V.alginolyticus* in different organs was higher in mouth, gills, liver, kidneys and spleen.

Concerning to challenge test, the mortality rate increased in *T. zillii* that mostly high in the first three days post inoculation in the two inoculation methods (I/P ,I/M) then decreased continuously reached the total mortality rate 80% and 70% respectively The re-isolation and identification of inoculated bacteria from the challenged *Tilapia* proved that, it was corresponded to the same tested strain for challenge the infected fish revealed the same clinical and postmortem changes nearly as Vibriosis. Infected fish revealed ulcers, hemorrhages all over the body and dorsal fin. Besides, pale liver and gills, enlarged gall bladder and congested kidneys which agreed with Sakata and Hattori (1988) who found that experimental moribund tilapia revealed signs of a hemorrhagic septicemia as the same as the natural disease. This proved that the injected *V. alginolyticus* strain was mostly virulent.

Concerning the histopathological results induced by vibriosis in examined fishes, severe congestion, hemorrhages and degenerative changes were observed in most types of fish and this could be attributed to the severity of isolated strains. Our results come in agreement with Roberts, (2001a), El-Bassiony, (2001) and Stephens et al., (2006).

## CONCLUSION

1-Seasonal prevalence of vibrio spp. was highest in summer followed by spring, autumn and winter.

2-PCR yielded amplification for *V.alginolyticus* and *V.parahemolyticus* strains isolated from some marine fish representing *pvsA* gene and is considered as an important tool for diagnosis.

## CONFLICT OF INTEREST

The authors declare no conflict of interest. ■

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