Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 24(1): 553 – 571 (2020)
www.ejabf.journals.ekb.eg

Characterization of *Vibrio parahaemolyticus* Infection in Gilthead Seabream (*Sparus auratus*) Cultured in Egypt

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ARTICLE INFO

Article History:
Received:Dec. 3, 2019
Accepted:Feb. 26, 2020
Online: Feb. 29, 2020

Keywords:
Vibrio parahaemolyticus,
Gilthead seabream,
Sparus auratus
*tox*R gene,
kangawa phenomena,
antibiogram,
histopathology.

ABSTRACT

This study aimed to diagnose *V. parahaemolyticus* infection among cultured Gilthead Seabream from the Suez Canal area of Egypt. A total of 200 Gilthead Seabream fish samples were clinically examined for the presence of any abnormal external or internal clinical findings. Samples from internal organs of moribund fish were subjected to histopathological and microbiological examinations. Retrieved isolates were biochemically identified using API 20 E system then further confirmed by regular PCR. Antibiogram profiling of the retrieved isolates was also done. A sum of 64 *V. parahaemolyticus* isolates was retrieved from the examined moribund Gilthead seabream samples. A total of 18 (32.8%) of the retrieved isolates were positive for the Kanagawa phenomenon (KP+ve). An expected product with a length of 368 bp size bands was found from tested strains of *tox*R targeted gene. Further, the PCR amplification of *pR72*H target gene generated an expected product with a length of 387-bp. Most of the isolates showed resistance to Ampicillin (AMP 10µg) and Gentamycin (CN 10µg) with high sensitivity to Sulphamethoxazole/Trimethoprim (SXT 25µg). Pathologically, severe inflammatory reactions together with melanomacrophage center alterations were recorded within the examined splenic, hepatic, renal and branchial tissues. Ultimately, the current study emphasizes the critical fish health threats posed by *V. parahaemolyticus* with consequent impacts on human consumers. Thus, competent biosecurity strategies should be adopted to control Vibrio infection in marine fishes and to minimize the antimicrobial resistance hazards in mariculture.

INTRODUCTION

Gilthead seabream is one of three top marine fish species that are progressively cultured by Egyptian Private as well as Governmental sectors. Production mostly comes from two main geographic regions within the north and northeast regions of Egypt. Triangle of Deeba (a triangle area between Damietta and Port Said Province) represents the north while the northeast regions are indicated by Suez Canal (Ismailia). The deteriorated aquatic environment is a main trigger of bacterial infections eruptions among the cultured marine fish populations in these regions. In the past few years, Vibrio infection were incriminated as main cause of mass mortalities among Gilthead seabream cultured populations in both North and Northeast regions **(Megahed and Aly, 2009).**

As a part of the natural microbiota of estuarine, costal and marine ecosystems, Vibrio species represent a large portion of heterotrophic bacteria that distributed widely in these environments **(Hongping *et al.*, 2011; Urakawa and Rivera, 2006)**. Vibrios represent a wide spectrum of pathogens colonizing poor quality aquatic marine environments. Most of the Vibrios are opportunistic pathogens that commensally exist in marine environments without posing a health threat to immunologically competent fish. Environmental stress will upregulate virulence genes of these organisms converting them into potentially pathogenic disease agents. Parasitism, high organic matter and fluctuation of water temperature are all potential stress factors associated with pathogenic vibrio invasion.

A member of *Vibrio* species from the Vibrionaceae family, *V. parahaemolyticus* which is a Gram-negative halophilic and emerging pathogenic bacterium that is widely disseminated in estuarine, marine and coastal areas **(Zhang and Orth, 2013; Letchumanan *et al.*, 2014).**

Regarding aquatic animals, *V. parahaemolyticus* is one of the most bacterial pathogen responsible for mass mortalities among fish stocks in many marine fish farms throughout the Mediterranean area with severe economic losses worldwide **(Zorrilla *et al.*, 2003b).** Outbreaks tend to be concentrated along coastal regions during the summer and early fall when higher water temperatures engorge higher levels of bacteria.

Concerning human, *V. parahaemolyticus* was first isolated from Shirasu, semi dried juvenile sardine, which implicated in an outbreak of food poisoning during 1950 in Osaka, Japan where 272 patients were ill and 20 deaths were recorded **(Miwatani and Takeda, 1976).**

Commonly, not all strains of *V. parahaemolyticus* are considered pathogenic **(Dileep *et al.*, 2003),** the pathogenic ones that responsible for onset of disease symptoms and outbreaks are characterized by the production of thermostable toxins and/or TDH-related haemolysin encoded by *tdh* and *trh* genes; respectively, these enzymes lyse red blood cells on Wagatsuma blood agar **(World Health Organization and Food Agriculture Organization, 2011).** This hemolytic activity on high salt blood agar is identified as Kanagawa phenomenon (KP) **(Alipour *et al.*, 2014)**.This has been used as a

significance pathogenic indicator for *V. parahaemolyticus* along few decades. Only 1–2% of the environmental samples is reported to be KP-positive and the rest are categorized as KP-negative strains **(Alipour *et al.*, 2014).** While a haemolysin produced by the bacterium is thought to be an important virulence factor **(Joseph *et al.*, 1982),** however the overall virulence mechanisms of pathogenesis by *V. parahaemolyticus* remain unclear. Iron rich aquatic marine environment is a rich media for Vibrio colonization. Vibrios are considered highly selective siderophores who swiftly / competently utilize sequestered iron to enhance their pathogenic mechanisms **(Eissa *et al.*, 2015).**

Thus, the current research proposed to diagnose *V. parahaemolyticus* infection among Gilthead seabream populations through clinical, postmortem, bacteriological and histopathological examinations together with utilizing the virulence genes based molecular tests during episodes of mass mortalities in some private marine aquaculture in Egypt. Antibiogram profile of the isolated *V. parahaemolyticus* was also done.

MATERIALS AND METHODS

Samples collection and preparation:

A total number of 200 moribund gilthead Seabream (*Sparus auratus*) were collected randomly from different marine fish farms at Ismailia and Port-said Governorates during outbreaks, throughout the period from August 2017 until July 2018. The fish samples were transferred immediately to Microbiology Lab at the Fish Farming and Technology Institute to perform postmortem and bacteriological examinations. Fish were cut opened using three line technique **(Conroy and Herman (1970).** Fish were examined externally and internally for any possible lesions before being sampled for bacteriological examination.

Isolation and identification of V. parahaemolyticus:

The isolation and biochemical identification of *V. parahaemolyticus* was carried out as recommended in the FDA's Bacteriological Analytical Manual (BAM) **(Kaysner and Depaola, 2004)**.

Loopfuls from different internal organs were inoculated onto TCBS agar plates (Oxoid CM0333B). Inoculated plates were incubated at 28 ^{o} C for 24 hrs. *V. parahaemolyticus* was presumptively identified by its green or blue green colonies on TCBS agar plates. The biochemical identity of the suspect *V. parahaemolyticus* isolates were confirmed using API 20E test (Biomérieux, France).

Kanagawa phenomenon:

The Kanagawa reaction was carried out according to **(Miyamoto *et al.*, 1969).** Kanagawa phenomenon demonstrates the presence of specific virulent Factor (Thermostable direct haemolysin; TDH) in *V. parahaemolyticus*. Young pure colonies were streaked onto high salt blood agar (NaCl 7%) containing fresh human red blood cells. The plates incubated at 28^{o}C for 18-24 hrs. A zone of β- hemolysis was detected.

Molecular identification of V. parahaemolyticus by PCR:

Extraction of genomic DNA:

The extraction of genomic DNA of all retrieved vibrio isolates were performed according to the technique described by **Santos *et al.* (2001)**. From enrichment broths, DNA preparation was carried out by the thermal shock method from all the harvested colonies. The colonies were grown in 2mL of TSB supplemented with 2%NaCl that harvested after 24 h at 28◦C. One mL of the medium was transferred to sterile Eppendorf tubes and centrifuged for 3 min at 3000 rpm. The supernatant was discarded.

The pellets were then washed by mixing in 1 ml NaCl 0.85% followed by centrifugation then re-suspended in 400 μL of pure sterile water. After homogenization, the supernatant was boiled for 10 min and centrifuged at 8000 rpm for 5min then collected. The obtained DNA extracts were stored at −30 °C until PCR analyses were performed.

All the gene amplifications for various suspect vibrio isolates were performed in a thermal cycler DNA (Eppendorf- Vapo.protect, Germany).The PCR amplification was conducted in a final volume of 25μL reaction mixture using One PCR Gene Direx master mix (USA), ready to use solution containing Taq DNA polymerase, PCR buffer, dNTP, gel loading dyes and fluorescence dye. The reaction mixture contained 12.5 μL of 2xGene Direx master mix, 0.4 μL for each primers (*tox*R 1, ToxR 2, VP32 and VP33), 4 μL of previously extracted DNA and add ddH_2O to 25μL. In all PCR reactions, DNA from pure cultures of reference vibrio strain was used as a positive control, whereas molecular grade water was used as a negative control.

Detection of *tox*R gene:

primers for *tox*R gene, with target 368 bp band size, used for gene amplification for species specific gene (*tox*R) which appeared highly conserved in *V. parahaemolyticus* isolates were defined ToxR1(5-GTCTTCTGACGCAATCGTTG-3) and antisense ToxR2 (5-ATACGAGTGGTTGCTGTCATG-3) **(Kim *et al.*, 1999)**. The reaction was preformed **(Rosec *et al.*, 2009, Abd-Elghany and Sallam, 2013)** with slight modifications. The conditions for *toxR* gene were an initial denaturation at 94◦C for 2min followed by 30 cycles of 94◦C for 1 min, 63◦C for 1min, and 72◦C for 1min and a final elongation step at 72◦C for 5min, for all reactions.

Detection of pR72H-cloned segment:

Primers, 387 bp in size, used for gene amplification for gene *pR72H* chromosome *V. parahaemolyticus* were defined; VP32 (5-AATCCTTGAACATACGCAGC-3) and antisense. VP33 (5- TGCGAATTCGATAGGGTGTTAACC-3) **(Lee *et al.*, 1995).** The PCR reaction was performed using the following parameters: initial denaturation at 94 ℃ for 5 min followed by 35 amplification cycles, each cycle consisting of denaturation of 1 min at 94 ℃, primer annealing for 1 min at 60 ℃ and primer extension for 1 min at 72 ℃. After the last cycle, the PCR mixtures were incubated for 5 min at 72 ℃ **(Lee *et al.*, 1995).**

Antibiogram profile of V. parahaemolyticus:

Antibiotic susceptibility profile of bacterial isolates to various commercial antibiotic disks was determined by Kirby-Bauer Disk Diffusion Susceptibility method **(Bauer *et al.*, 1966).** The isolates were tested against the following antibiotics; oxytetracycline (OTC/30IUµg disk−1), trimethoprim sulphamethoxazole (SXT/1.25ug–23.75ug disk−1), ampicillin (AMP/10IU disk−1), gentamycin (CN/10µg disk−1); ciprofloxacin (Cip/5µg disk−1) and novobiocin (E/, 30µg disk−1). Discs were supplied by Oxoid™ (Thermo-scientific, UK). At the end of incubation period, antibiotic inhibition zones were measured in mm using a measuring caliber. Susceptibility testing was conducted according to the recommendations of the Clinical and Laboratory Standards Institute **(ClSI, 2017).**

Histopathological examination of V. parahaemolyticus:

Specimens from the liver, spleen, kidney and gills of moribund Seabream were fixed in neutral buffered formalin 10%. Paraffin blocks were prepared after routine processing of the specimens. Then, sectioned (5 *um*) and stained with haematoxylin and eosin **(Drury *et al.*, 1980).** Slides were examined under low power (X 10) and medium power (X40).

RESULTS

Postmortem examinations:

Moribund Seabream exhibited remarkably dark operculum, pectoral fins and tail. Thinning of the head, obvious marked elevation in the lateral line with severe bilateral destination of abdomen and vent prolapse were seen. Hemorrhage was detected with erosion in the skin and the dorsal muscles **(Fig. 1).**

The necropsy findings of the examined fish revealed decaying odor with profuse serosanguinous fluid during dissecting the abdomen. Marked adhesion of the internal organs and the intestine were observed. Pale, friable liver as well as adhered rubbery, congested and enlarged spleen were seen. Stomach was enlarged and filled with milky fluid. The kidney was congested with black spots on its surface. Severe hemorrhage was noticed on the surface of liver, gonads, abdominal fat as well as hemorrhages in the swim bladder and thickening on its wall **(Fig. 1).**

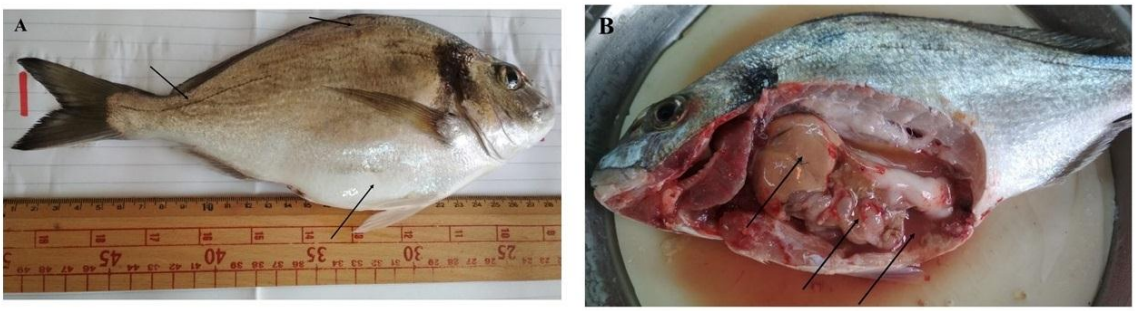

Fig. 1. Postmortem findings in moribund Gilthead Seabream showing;

(a) Severe bilateral destination of abdomen, elevated lateral line and detached scales.

(b) Visceral adhesion, pale liver, hemorrhages in abdominal viscera and ascetic fluid.

1. Bacteriological assay:

Morphologically, the retrieved colonies exhibited round, green and 2-3 mm colonies with dark blue center on TCBS **(Fig. 2).** Such colonies were able to grow on TSA with different salt concentrations (2%, 6% & 8%). Morpho-chemically, the bacterial isolates were Gram-negative, straight or curved rods, facultative anaerobic, motile, cytochrome oxidase positive, and catalase positive with high sensitivity to vibriostatic agent (novobiocin, 30µg). The biochemical identity of the retrieved isolates coincided with the standard biochemical criteria of *V. parahaemolyticus* **(Table 1)**.

The highest prevalence of *V. parahaemolyticus* were recorded during the spring and summer while, the lowest were at autumn and winter. In respect to intensity of infection, the highest intensities were in kidney (46.87%) followed by spleen (39%) and the lowest level recorded in liver (14%) **(Table 2 Table 3)**.

Table 1. Phenotypic characters & API20E profile of *V. parahaemolyticus* isolated from Gilthead Seabream.

Criteria	*V. Parahaemolyticus*
API 20 E ID	4146107 & 4146105
Culture characters on TCBS	Round green (2-3 mm) with dark blue center
Blood agar	Beta- hemolysis.
Gram stain character	-Ve
Salt tolerance	
0% NACL	-
6% NACL	+
8% NaCl	+
Temperature tolerance	
28 ^{o}c	+
37 ^{O}C	+
40 ^{O}C	+
OPNG	-
ADH	-
LDC	+
ODC	+
CIT	-
H_2S	-
URE	-
TDA	-
IND	+
VP	-

GEL	+
GLU	+
MAN	+
INO	-
SOR	-
RHA	-
SAC	-
MEL	-
AMY	+
ARA	+/-
OXIDASE	+

Table 2. Seasonal prevalence of recovered *V. parahaemolyticus* bacteria from moribund Gilthead Seabream.

Identified retrieved Isolates	Prevalence	Summer 2017	Autumn 2018	Winter 2018	Spring 2018	Total
V. Parahaemolyticus	No	18	16	9	21	64
	%	28.13	25	14.1	32.8	100

Table 3. Retrieved *V. parahaemolyticus* isolates from different organs from moribund Gilthead Seabream.

Identified retrieved isolates	Number and percentages of retrieved isolates.			
	Liver	**Kidney**	**Spleen**	**Total**
V. Parahaemolyticus	9 (14.06%)	30 (46.87%)	25 (39.06%)	64 (100%)

1. ***Kanagawa phenomenon (KP):***

KP-positive reaction in *V. parahaemolyticus* isolates from examined moribund Gilthead Seabream samples were presented in **Fig. 2**. The result revealed (21) 32.8% of the total *V. parahaemolyticus* isolates were KP+ve.

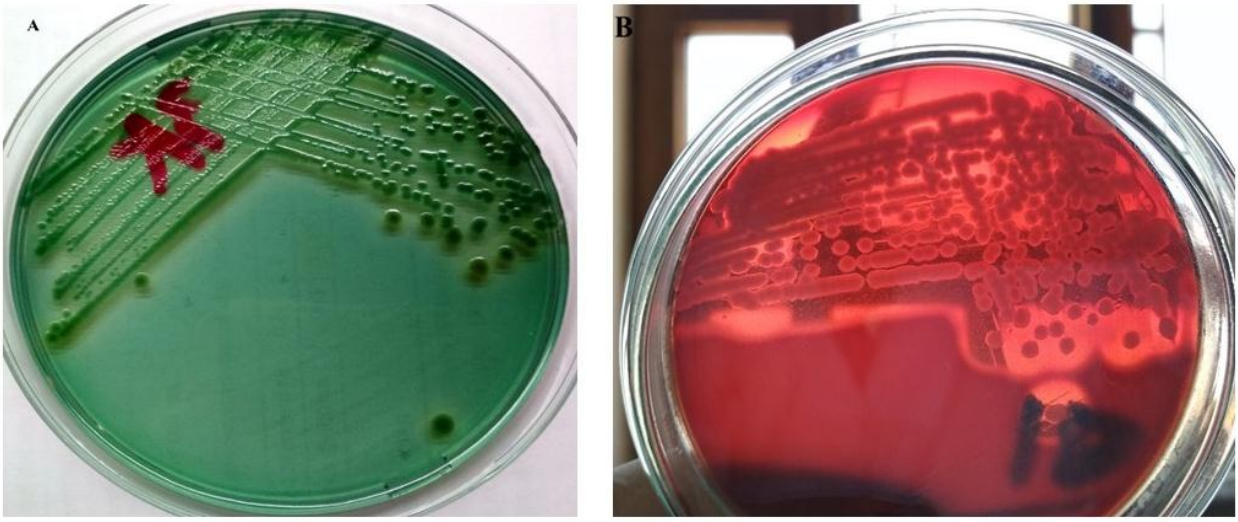

Fig. 2. Showing Culture characteristics of the retrieved *V. parahaemolyticus* on TCBS (A) and Blood agar (B).

2. Molecular assay:

Upon targeting the *tox*R gene primers which are species specific for the *V. parahaemolyticus* strain, an expected product with a length of 368 bp size bands were found from the tested strains **(Fig. 3).** Moreover, when using DNA-specific marker *pR72*H, the PCR amplification generated an expected product with a length of 387-bp that was detected in the examined five strains of *V. parahaemolyticus*, while non-specific products were not found **(Fig. 4).**

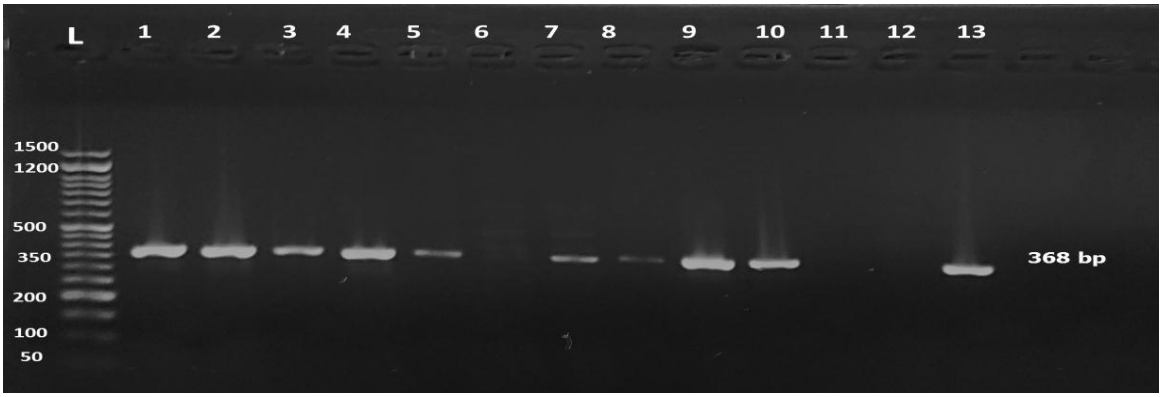

Fig. 3. Agarose gel electrophoresis for PCR analysis of *tox*R target (ToxR 1 and ToxR 2 primers) at *V. parahaemolyticus* isolated from moribund Seabream. Chromosomal DNA from all the presumptive identified *V. parahaemolyticus* was used as a template for PCR amplification using specific oligonucleotide primer for *tox*R gene. L: DNA marker *(Gene Direx);* used as a reference for fragment size; Lanes with the key numbers from 1 to5 and 7 to 10 and 13 are the selected positive strains for *tox*R gene.

While, lanes with the key numbers 6,11 &12 were non *V. parahaemolyticus* strains. The 368 bp fragments correspond to the known type of *tox*R gene PCR products.

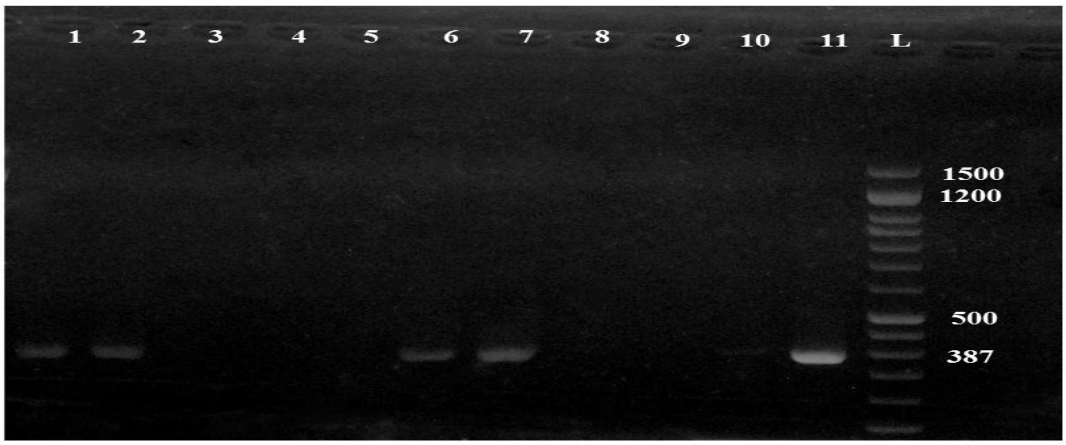

Fig. 4. Agarose gel electrophoresis for PCR analysis of pR72H target (VP33 and VP32 primers) of *V. parahaemolyticus.* Lane L: molecular weight marker (Gene Direx); used as a reference for fragment size; lanes with the key numbers 1,2,6,7 and 11 are the positive for pR72H of tested *V. parahaemolyticus* while, lanes 3 to5 and 8 to10, tested strains of non *V. parahaemolyticus* isolates. The 387 bp fragments correspond to the known type of pR72H segment PCR products.

1. Antibiogram:

Most of the isolates showed resistance to Ampicillin (AMP 10µg) and Gentamycin (CN 10µg) with intermediate susceptibility to Ciprofloxacin (CIP 5µg). On the other hand, isolates showed highly sensitivity to Sulphamethoxazole and Trimethoprim (SXT 25µg) followed by Oxytetracycline (OT 30µg) as illustrated in **Table 4**.

Table 4. Antibiogram profiling of the recovered *V. parahaemolyticus* isolates.

Standard inhibition zones of the antibiotics used in the antibiogram						
Antibiotic	Ot-30	CN-10	Aml-10	CIP-5	SXT-25	E-30
Resistant	11	12	13	20	10	10
Mildly sensitive	12-14	13-14	14-16	21-30	11-15	11-16
Sensitive	15	15	17	31	11-16	17
Antibiotic sensitivity for the retrieved *V. parahaemolyticus* isolates.						
Sensitive	41 64.1%	-	6 9.4%	12 18.8%	64 100%	64 100%
Mild sensitive	12 18.8%	17 26.6%	0 0%	33 51.6%	-	-
Resistant	11 17.2%	47 73.4%	58 90.6%	19 29.7%	-	-

2. Histopathology:

Examined moribund seabreams showed sloughing in some primary and secondary gill lamellae. Leukocytic mononuclear cells were infiltrated the secondary lamella. The gill arch and lamella exhibited marked edema and focal congestion **(Fig. 5).**

The hepato-pancreatic tissue of moribund gilthead Seabream, displayed congestion and wide spread vacuolar degeneration and coagulative necrosis in the hepatic and pancreatic cells **(Fig. 6).** A tubular nephrosis in the majority of the renal epithelium was evident in the form of vacuolar degeneration and coagulative necrosis. Moreover, the interstitial tissue revealed congestion of renal blood vessels with some mononuclear cells infiltration. Focal depletion in the lymphoid and haematopoietic tissue with marked activation of melanomacrophage centers were evident in both splenic and renal tissue **(Fig. 7).**

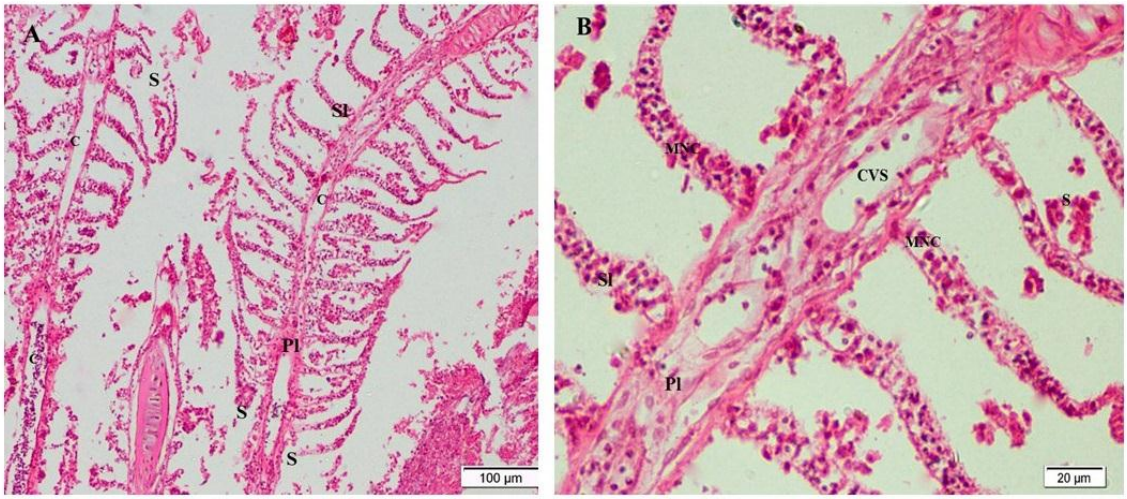

Fig. 5. Histopathological alterations in the gills of moribund gilthead Seabream. Micrograph (a&b) showing the sloughing in the two types of gill lamella with congestion and mononuclear cell infiltration. CVS (Central venous sinus), PL (primary lamella), SL (secondary lamella) MNC (mononuclear cells), S (Sloughing). H&E stain.

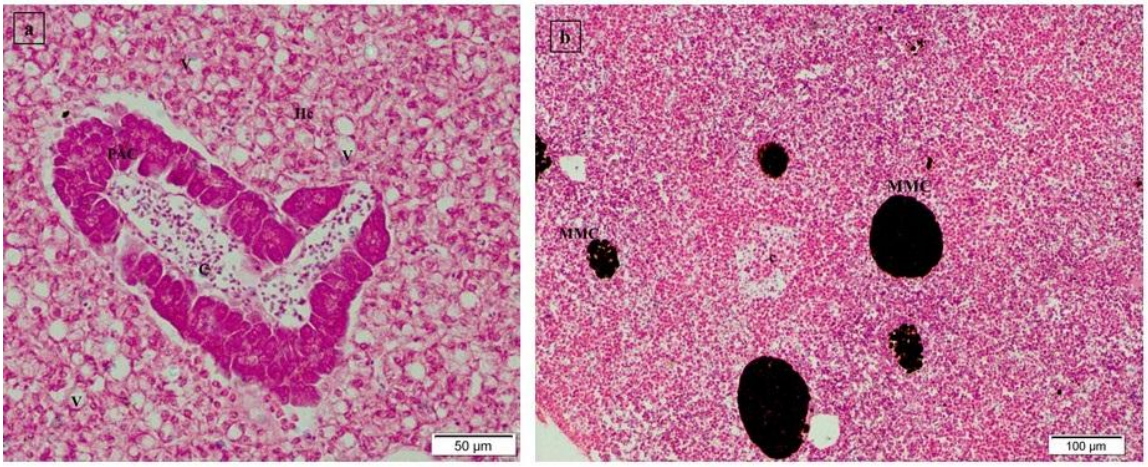

Fig. 6. Histopathological alterations in the hepatopancreas and the spleen of moribund Gilthead Seabream.

Micrograph (a) showing vacuolar degeneration (VD) and coagulative necrosis (CN) in hepatic cells (HC) and pancreatic acinar cells (PAC). Marked congestion in blood vessels of pancreatic tissue.

Micrograph (b) showing activated melanomacrophage centers and focal depilation of lymphoid tissue. H&E stain.

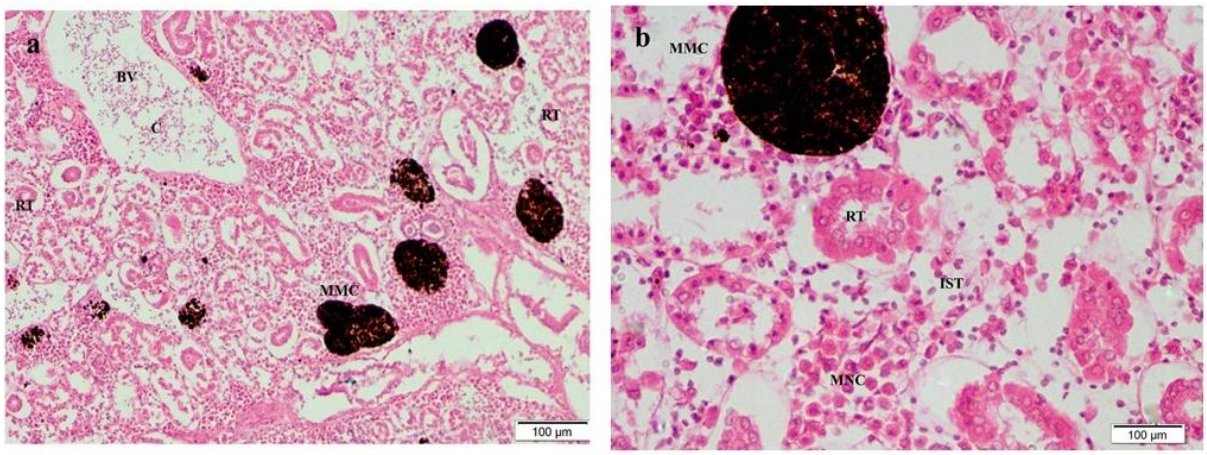

Fig. 7. Histopathological alterations in the renal tissue of moribund Gilthead Seabream.

Micrograph (a) showing focal depletion in the hematopoietic tissue with marked activation of melanomacrophage centers (MMC) and congested renal vessels (BV, blood vessel). Micrograph (a, b) showing tubular nephrosis (RT, renal tubules) in the form of vacuolar degeneration (VD) and coagulative necrosis (N). H&E stain.

DISCUSSION

Being pathogen of public health concern, the Vibrio species gain global interest of the microbiology community and zoonotic diseases experts **(Austin and Austin, 2016).** *V. parahaemolyticus*, is a pathogenic Vibrio that present in plenty loads in the common reservoirs such as shellfishes, mussels and mollusks **(Austin *et al.*, 2012, Abd-Elghany and Sallam, 2013, Eissa *et al.*, 2015)**. It is associated with sewage pollution which is one of the leading cause of bacterial food poisoning pathogens linked to raw or inefficiently cooked seafood feeding **(Austin *et al.*, 2012).**

The current study revealed that, most of the moribund Gilthead Seabream with *V. parahaemolyticus* strains showed lesions similar to those previously reported among moribund Gilthead Seabream **(Labella *et al.*, 2011, Abdel-Aziz *et al.*, 2013, Winfield, 2018)** and in common pandora fish **(Eissa *et al.*, 2017).**

Concerning, the insight analysis of the phenotypic characteristics for the majority of retrieved isolates, our findings were in close agreement with those reported for the *V. parahaemolyticus* profiles **(Buller, 2004, Yiagnisis and Athanassopoulou, 2011, Abdel-Aziz *et al.*, 2013, Eissa *et al.*, 2017).**

Winfield (2018), reported that historically, this diseases was related to a spring syndrome and a fall syndrome (sudden temperature fluctuations), establishing a strict connection with appearance of outbreaks in this two seasons when temperature fluctuations are more frequent. Our findings revealed the highest prevalence of *V. parahaemolyticus* was recorded at spring and summer.

In respect to intensity of infection, our results showed the highest intensities were in kidney 46.87% followed by spleen 39% and the lowest level recorded in liver 14%. This finding was similarly in agreement with previous authors **(Botella *et al.*, 2002, Zorrilla *et al.*, 2003a, Abdel-Aziz *et al.*, 2013)** who reported the liver and kidney as the main target organs for isolation of *V. parahaemolyticus* in Gilthead Seabream. On the pathophysiological level, this tissue preference could be related to some of the virulence determinants owned by this pathogen, which augment their septicemic nature with final predisposition into the main immune warrior (kidney) and toxin neutralizing site (liver).

It is worth to mention that, in our results only 32.8% of examined isolates were positive in KP reaction. This finding confirms the association between *tdh* and non-*tdh* producing strains which coincided with previous results reported by **Alipour *et al.* (2014)** who illustrated that *V. parahaemolyticus* strains that produce *tdh* gene shows hemolysis in the form of a halo on Wagatsuma agar.

Liu (2003) mentioned that, most of clinical isolated *V. parahaemolyticus* could produce a well-defined, β-type hemolytic zone on a Wagatsuma blood agar. However, only 0.3–3% of non-pathogenic environmental isolates are KP positive **(Nordstrom *et al.*, 2007).** In this context, we should take a strict control on the isolation of pathogenic *V. parahaemolyticus*. Thus, in some countries, for example, China, the KP test has been

widely used in food-quarantine practice for screening those pathogenic *V. parahaemolyticus* strains on a routine basis **(Hongping *et al.*, 2011).**

Vibrio, a diverse genus of aquatic bacteria, currently includes 72 species, 12 of which occur in human clinical samples. The PCR based assay, targeting of *tox*R gene (the toxin operon gene), which considered well conserved among *V. parahaemolyticus,* were become a popular molecular technique for detection and identification of *V. parahaemolyticus* in seafood samples **(Kim *et al.*, 1999, Vimala *et al.*, 2010, Paydar *et al.*, 2013, Suffredini *et al.*, 2014).** The biochemically identified isolates were further genetically verified by PCR for detection of *V. parahaemolyticus tox*R gene. The use of two specific primer sets for the *tox*R gene, target bands of 368 bp were obtained. These findings further support the idea of **Kim *et al.* (1999)** who clarify that, the *V. parahaemolyticus tox*R sequence is perfectly conserved among *V. parahaemolyticus* strains and targeting this virulence gene for clinical isolates always be appropriate diagnostic test. The *tox*R gene stimulates the expression of *tdh* gene and it is present in either pathogenic or non-pathogenic *V. parahaemolyticus* isolates **(Sujeewa *et al.*, 2009).** Besides, the *tdh* and TDH-related hemolysin (*trh*) are the two virulence factors associated with *V. parahaemolyticus* hemolysis and cytotoxicity activity in the host cell **(Broberg *et al., 2011,* Zheng *et al.*, 2014).** Moreover, these results match those observed in earlier studies **(Kim *et al.*, 1999, Vimala *et al.*, 2010, Paydar *et al.*, 2013, Suffredini *et al.*, 2014).** Conversely, **Venkateswaran *et al.* (1998)** proposed the use of the *gyr*B gene, encoding the B subunit of DNA gyrase, to detect *V. parahaemolyticus* and closely related *V. alginolyticus* strains. **Pascual *et al.* (2010)** demonstrated that, of six protein-encoding genes, only two – *tox*R and *rpo*D – served the purpose of reliable species identification based on sequence similarity. Of the other genes, *rec*A, *pyr*H and *gyr*B gave poor resolution.

Upon the species level, *V. parahaemolyticus* and *V. alginolyticus* isolates are very closely related. *V. alginolyticus* was prior designated as a biotype 2 of *V. parahaemolyticus*, but now universally known as *V. alginolyticus*. DNA-DNA re-association studies revealed that *V. alginolyticus* shares only 60-70% homology with *V. parahaemolyticus* **(Robert-Pillot *et al.*, 2002).** In order to distinguish between these two strains, with the use of Primer pair (VP33-VP32) to amplify target pR72H DNA fragment. Our PCR amplification generated bands of 387-bp. specific for *V. parahaemolyticus* tested strains while no PCR amplicons were found for *V. alginolyticus* tested strains. This finding verifies the results of **(Lee *et al.*, 1995)** who developed a pair of oligonucleotide sequences (Vp32 and VP33) that bind to opposite ends of a 320 - 387bp DNA fragment termed pR72H, from the chromosome of *V. parahaemolyticus* that was shown to be found only in this species and conserved in *V. parahaemolyticus*. He added that the expected PCR products were obtained from all *V. parahaemolyticus* strains tested, while no PCR amplicons were found in other vibrios or related genera. On contrast, **Gugliandolo *et al.* (2011)** compare the two sets of targeted genes in the

diagnosis of *V. parahaemolyticus* and suggested the IGS primer set was higher sensitivity than that targeting the *pR72*H conservative fragment. Consequently, the *gyr*B PCR and the *pR72*H fragment PCR were the least reliable and most difficult to assess.

Antibiotic resistance poses important risks to human health and can affect the course of infectious diseases **(Aly, 2013)**. Regarding this issue, the susceptibility screening of all retrieved *V. parahaemolyticus* isolates to various antibiotics presented. All isolates revealed resistance to ampicillin and gentamycin with intermittent resistance to ciprofloxacin. Nevertheless, *V. parahaemolyticus* showed high sensitivity to sulphamethoxazole/ trimethoprim and oxytetracycline. These results were nearly similar to those obtained by **Abdel-Aziz *et al.* (2013)** who found most *V. parahaemolyticus* isolates were resistant to ampicillin, amoxicillin and gentamycin, intermediate sensitive to erythromycin while, sensitive to ciprofloxacin, oxytetracycline, tetracycline, trimethoprim– sulfamethoxazole and novobiocin. Similarly, noticed previous studies documented that most isolates of *V. parahaemolyticus* isolates were resistant to ampicillin **(Eissa *et al.*, 2017, Abdellrazeq and Khaliel, 2014, Laganà *et al.*, 2011, Zulkifli *et al.*, 2009)**. Controversially, **(Eissa *et al.*, 2017)** reported resistant to sulfamethoxzole-trimethoprim and oxytetracycline. The findings also were consistent with those of **Abdellrazeq and Khaliel (2014)** who noticed resistance to amoxicillin, penicillin, oxytetracycline and variable susceptibility to erythromycin, streptomycin as well. However, sensitivity to chloramphenicol, ampicillin and gentamycin were also reported.

However, some sort of resistance to ampicillin, oxytetracycline, sulfamethoxzole-trimethoprim has been observed. This could be attributed to the repetitive usage of these antibiotics in controlling previous outbreaks. The outbreaks of Vibriosis were controlled by using a feed additive of oxytetracycline and the officially licensed antibiotics **(Aly and Albutti, 2014, Laganà *et al.*, 2011, Manfrin *et al.*, 2009)**. Surprisingly, the mild resistance to ciprofloxacin that recorded in this study might explained by the consistent presence of such antibiotic in coastal waters receiving agricultural/municipal wastes, that could contain poultry, large animals and human antibiotic wastes of the same type. This result confirmed by **Carvalho *et al.* (2016)** who found 72 Vibrio isolates showed resistance to ciprofloxacin and gentamicin with higher rates in those isolated from crabs samples. Similarly, **Laganà *et al.* (2011)** indicated that, all vibrios isolated from fish, shellfish and shrimps that were tested in his study showed different levels of resistance to the antibiotics used in human medicine as well as to those commonly used in aquaculture which is a consequence of the selective pressure exerted by the extensive use of these agents in fish farms. This result explains the increasing incidence of bacterial resistance to antimicrobial compounds **(Smith, 2008).**

The histopathology of infected fish revealed degenerative changes, circulatory disturbances and inflammatory reactions in the gills and internal organs with focal proliferation of melanomacrophage centers and depletion in the hematopoietic tissue.

These lesions have been attributed to endo- and/or exotoxins. **Letchumanan *et al.* (2014)** indicated that T3SS1 initiates a series of events that involves autophagy, membrane blebbing, cell rounding, and lastly cell lysis during the course of tissue infection. Similar findings documented by **Aly *et al*. (2000)** in vibrio infected catfish and in experimentally infected rainbow trout by **Avci *et al.* (2012)**. This author reported that the histopathological alterations were cellular degeneration and tissue necrosis mixed with bacteria clumps as well as the increase in eosinophilic granular cells (EGCs) and degranulation in gills. Moreover, severe hemolytic anemia induced by the lytic toxin of Vibrio results in heavy deposition of hemosiderin in the melanomacrophage centers of the remaining splenic and renal hematopoietic tissue **(Hendrikson and Zenoble, 1983, Roberts, 2012).**

CONCLUSION

Vibriosis is a worldwide aquatic animal disease posing an actual threat for both aquatic species and human consumers. Epidemiologically, *V. parahaemolyticus* comes on the top list of Vibrios responsible for fatal diseases in both fish and human. The use of specific diagnostic panel of biochemical tests augmented with determinative molecular assays based *tox*R gene and *pR72*H cloned fragment have accurately unveiled the full identity of retrieved *V. parahaemolyticus* isolates. Ultimately, the current research can be considered as an alarm for the growing inevitable antibiotic resistance in aquaculture. Thus, strict veterinary hygienic regulations should be implemented to control such infections and minimize the antimicrobial use in fish farms.

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