Characterization of *Vibrio Alginolyticus* Infection in Gilthead Seabream (*Sparus Auratus,* L) Cultured in Egypt

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Abstract

The present study investigated Vibriosis in cultured Gilthead Seabream at the Suez Canal area, Egypt. Two hundred moribund fish samples subjected to clinical, microbiological and histopathological examinations. Retrieved isolates were biochemically identified using API 20 E system then confirmed by conventional PCR. Antibiogram profiling of the retrieved isolates was also done. Naturally infected fish showed severe ascites, hemorrhages and erosions in the skin and fins. A sum of 29 *V. alginolyticus* isolates were retrieved from the examined samples. Targeting *16Sr*RNA and *gro*El genes produce an expected product with a length of 336, 301bp size bands; respectively, were found from all tested strains of *V. alginolyticus*. The antibiogram profiling of *V. alginolyticus* isolates showed resistance to Ampicillin, Gentamycin followed by Ciprofloxacin with high sensitivity to Sulphamethoxazole/Trimethoprim and oxytetracycline. Histopathological alterations in the affected tissues showed, inflammatory reactions together with degenerative and /or necrotic changes in branchial, liver and kidney.

Ultimately, the current study emphasizes the critical fish health threats posed by *V. alginolyticus* with subsequent influences on human. Thus, competent biosecurity strategies should be adopted to control Vibrio infection in marine fishes and to minimize the antimicrobial resistance hazards in mariculture.

Keywords: *Vibrio alginolyticus*, Gilthead Seabream, *16s rRNA* gene, *gro*El gene, antibiogram and histopathology.

Introduction

The Mediterranean mariculture is an expanding industry with steadily growing production capable of fulfilling the national needs as well as international export ***(Eissa et al., 2017).*** Gilthead Seabream (*Sparus auratus*), Seabass (*Dicentrachus librax*), Mullets (*Mugil cephalus; Liza ramada*), Meagre *(Argyrosomus regius*) are the most commonly reared marine species

across the Mediterranean basin ***(FAO, 2018).*** Based on the magnitude of production, the Mediterranean mariculture is currently lead by Turkey, Greece, Spain, Italy, and Egypt, which altogether supply 96% of the total regional production ***(FAO, 2018).***

Gilthead Seabream, *(Sparus aurata,* L*)* is a common species through the Mediterranean and considered as one of the most popular porgies for food. The overall breams production is estimated as 191 500 tones and almost 18% of them are Gilthead seabream ***(FAO, 2018).*** With the development of national mariculture mega projects, Egypt became one of the top producer of Gilthead Seabream ***(Mehanna, 2007, FAO, 2018).*** Triangle of Deeba (a triangle area between Damietta and Port Said Province) represents the north region for its production while the northeast regions are indicated by Suez Canal (Ismailia).

In warm-water, considering the potential of impediments to aquaculture sustainability, bacterial diseases came on the top list of infectious threats endangering their entire existence ***(Pridgeon and Klesius, 2012).*** Globally, Vibriosis is considered one of the most important bacterial diseases affecting marine fish species under culture condition ***(Toranzo et al., 2005, Austin and Austin, 2012)*** and also a affecting farmed Mediterranean fish species ***(Gudding and Goodrich, 2014).*** 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, *Vibrio alginolyticus* which is a Gram-negative facultative anaerobic, halophilic bacterium, was formerly regarded as an opportunistic pathogen causing Vibriosis in marine fish and shellfish ***(Austin and Austin, 2007).*** Also, ***Abdel-Aziz et al. (2013)*** stated *V. alginolyticu* as the main causative agent of marine Vibriosis and was frequently isolated from many epizootic outbreaks among the Gilthead Seabream and European Seabass populations ***(Zorrilla et al., 2003b).*** Vibriosis outbreaks were often related to the immunosuppression because of the stress. For this particular disease, sudden water temperature fluctuation was among the main triggering factors. So historically, this problem was related to a spring syndrome (a fall syndrome) ***(Winfield, 2018).***

The random use of antibiotics to control such bacterial infections is a

potential initiator of vast spectrum of antibiotic resistance among aquatic species as well as their human consumers ***(Aly, 2013, Li et al., 2015).***

Thus, the current research proposed to diagnose *V. alginolyticus* infection among cultured Gilthead Seabream through clinical, postmortem, bacteriological and molecular examinations, together with histopathological investigations during episodes of mass mortalities in some private marine aquaculture in Egypt. This in addition to, antibiogram profile of the retrieved isolated *V. alginolyticus* was also done.

Materials and methods:

Clinical and postmortem examination:

A total number of 200 moribund Gilthead Seabream (*Sparus auratus*, L) 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. The clinical signs and the full external postmortem examination was done according to the method described by ***Austin and Austin (2012).***

The internal postmortem examination was performed on moribund fish according the method described by ***Whitman (2004)*** to inspected any internal lesions.

Isolation and identification of V. alginolyticus:

The isolation and biochemical identification of *V. alginolyticus* was carried according to ***Buller (2004).*** The biochemical identity of the suspect *V. alginolyticus* isolates were confirmed using API 20E test (Biomérieux, France).

Molecular identification of V. alginolyticus:

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 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 primer (V.16S-700F, V.16S-1325R and groEl F, groEl R), 4 μL of previously extracted

DNA and add ddh2o 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 Vibrio *16sr*RNA gene (universal primers):

16SrRNA primers with target 663 bp band size, used for gene amplification for universal Vibrio *16sr*RNA gene. The primers sequences were defined V.16S-700F (5 CGGTGAAATGCGTAG AGAT-3) and antisense V.16S-1325R (5-TTACTAGCGATTCCGAGTTC-3) ***(Tarr et al., 2007)***. The reaction was preformed according to ***Carvalho et al. (2016).***

Detection of (*gro*El gene):

Application of PCR for identification of *gro*El as species-specific gene for demonstration of *V. alginolyticus.* Primers targeted 301 bp band size were used for *gro*El gene amplification as species specific gene which appeared highly conserved in *V. alginolyticus* isolates that defined groEl (F) **(5-**GATTCGGTGAAGAAGAGATG ATCTC**-3)** and antisense groEl (R) **(5-**TCTTCGTTGTCACCCGTTAGGT GA **-3)** ***(Raju et al., 2016).***

The PCR reaction was performed using the following parameters: initial denaturation at 95℃ for 30 sec followed by 35 amplification cycles, each cycle consisting of denaturation of 5 sec at 95℃, primer annealing for 15 sec at 66℃ and primer extension for 30sec at 72℃. After the last cycle, the PCR mixtures were incubated for 5 min at 72℃ ***(Raju et al., 2016).***

Antibiogram profile of V. alginolyticus:

Antibiotic susceptibility profile of retrieved Vibrio 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^{TM} (Thermo-scientific, UK). Susceptibility testing was conducted according to the recommendations of the Clinical and Laboratory Standards Institute **(Clsi, 2017).**

Histopathological examination of V. alginolyticus:

Specimens from the gills, liver and kidney of moribund Gilthead Seabream were fixed in neutral buffered formalin 10%. This technique done as described by ***Buller (2004).***

Results:

Clinical and postmortem examinations:

A total number of 200 moribund Gilthead Seabream (*Sparus auratus,* L) examined clinically which exhibited erosion in the skin of the

dorsal muscles with remarkably hemorrhages in pectoral fins and tail. Exophthalmia with severe bilateral destination of abdomen besides vent prolapse were seen. The necropsy findings of the examined fish revealed decaying odor with profuse serosanguinous fluid during dissecting the abdomen. Marked adhesion of the internal organs were observed **(**Error! Reference source not found.**).**

Bacteriological assay:

Morphologically, out of 99 isolated bacterial strains there were 29 isolate detected as *V. alginolyticus*, which exhibited 2-3 mm round, yellow colonies on TCBS. The biochemical identity of the retrieved isolates coincided with the standard biochemical criteria of *V. alginolyticus* **(Table 1).**

The highest prevalence of *V. alginolyticus* were recorded during the spring, summer while the highest intensities of infection were in kidney followed by spleen **(Table 2-4)**

Molecular assay:

The molecular analysis of all *V. alginolyticus* retrieved isolates depending on *16sr*RNA gene and the *gro*El gene, generated product sized 663-301-bp respectively. (Error! Reference source not found.**-3**).

Antibiogram:

Most of the isolates showed resistance to Ampicillin, Gentamycin and Ciprofloxacin. On the other hand, isolates showed highly sensitivity to Sulphamethoxazole and Trimethoprim and novobiocin followed by Oxytetracycline as illustrated in **table (4).**

Histopathological results:

The histopathological alterations in the tissues of the naturally infected Gilthead Seabream revealed inflammatory reactions together with degenerative and /or necrotic changes in branchial and visceral tissues as fully described in **figure (4-6).**

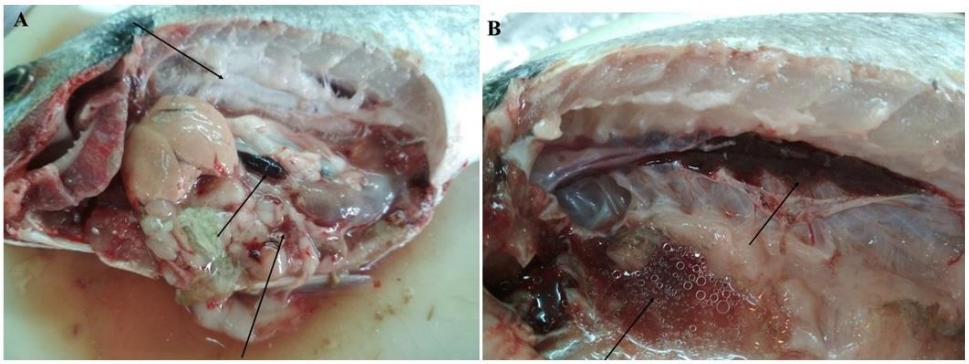


Figure 1: Postmortem findings in moribund Gilthead Seabream showing; Sever visceral adhesion, pale liver, congested spleen and hemorrhages in surface of the abdominal viscera.

Profuse ascetic fluid in the dissected abdomen and congested kidney.

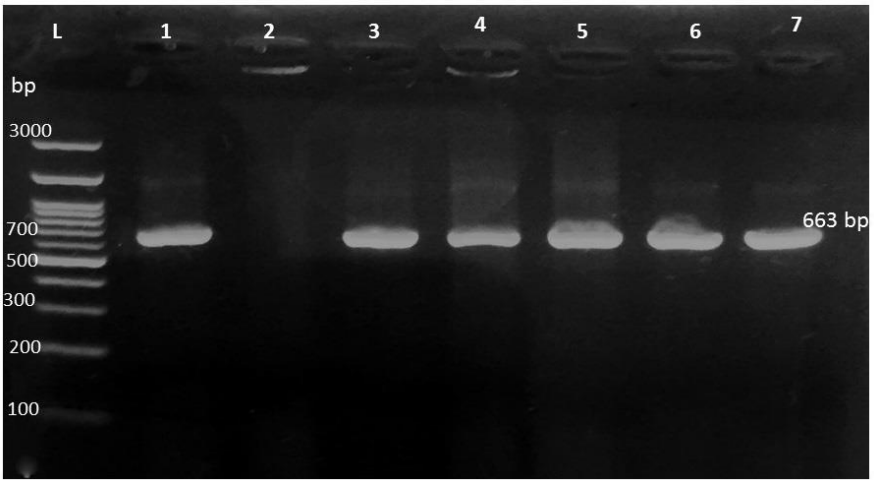


Figure 2: Agarose gel electrophoresis of PCR products corresponding to amplification of 16S rRNA target (V.16S-700F and V.16S1325R primers) at various vibrio bacteria isolated from naturally infected sea bream. L: Molecular weight marker (Gene Direx) used as a reference for fragment size; 3, 4, 5, 6 and 7: Vibrio strains and 1: control (+ve) and 2: control (-ve) non-Vibrio bacterial strain. The 663 bp fragments correspond to the known type of 16S rRNA PCR products.

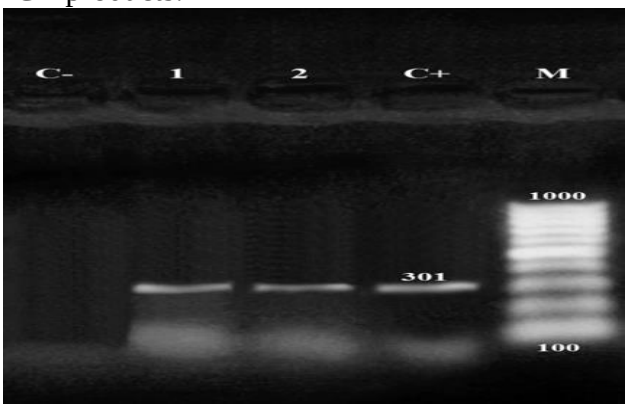


Figure 3: Agarose gel electrophoresis for PCR analysis of *gro*El gene (groEl F and groEl R primers) for molecular characterization of *V. alginolyticus* strains.

Lane M: 100 bp ladder as molecular size DNA marker. Lane C+: Control positive strain for *gro*El gene. Lane C-: Control negative. Lanes 1 & 2: Positive *V. alginolyticus* strains for *gro*El gene. The 301bp fragments correspond to the known type of *gro*El segment PCR products.

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

Criteria	***V. alginolyticus***
API 20 E id	4346125
Culture characters On TCBS	Yellow colony 3 mm
Blood agar	β-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. alginolyticus bacteria from moribund Gilthead Seabream.*

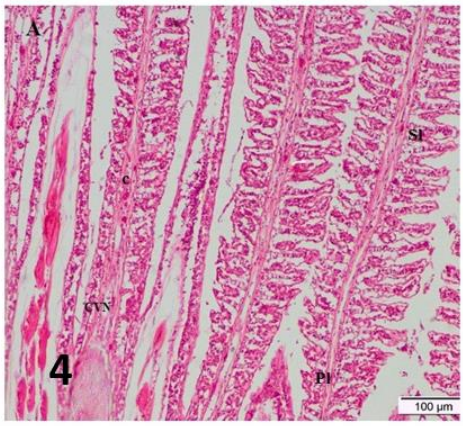
Identified retrieved Isolates	**Prevalence**	**Summer 2017**	**Autumn 2018**	**Winter 2018**	**Spring 2018**	**Total**
V. alginolyticus	**No**	6	8	6	9	29
	%	20.69	27.58	20.69	31.03	100

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

Identified retrieved isolates	Number and percentages of retrieved isolates.			
	Liver	**Kidney**	**Spleen**	**Total**
V. alginolyticus	9 (31.03%)	10 (34.48%)	10 (34.48%)	29 (100%)

Table 4: *Antibiogram profiling of the recovered V. alginolyticus 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. alginolyticus*** isolates.						
Sensitive	25 86.2%	-	6 20.7%	9 31.0%	29 100%	29 100%
Mild sensitive	4 3.8%	12 41.4%	3 10.3%	14 48.3%	-	-
Resistant	0 0%	17 58.6%	20 69.0%	6 20.7%	-	-



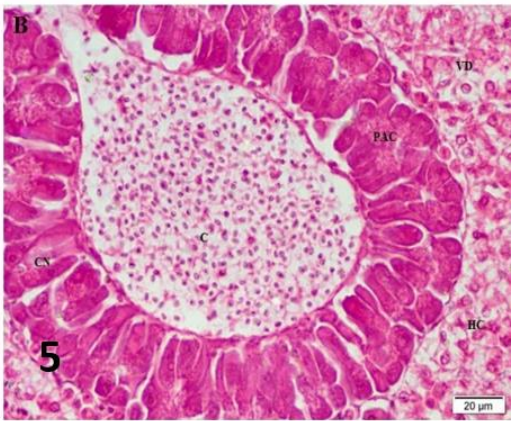


Figure 4: Histopathological alterations in the gills of moribund Gilthead Seabream.
Micrograph (A) showing the sloughing in the two types of gill lamella with congestion and mononuclear cell infiltration. CVS (Central venous sinus), PL (primary lamella), SL (secondary lamella) and C (Congestion). H&E stain.

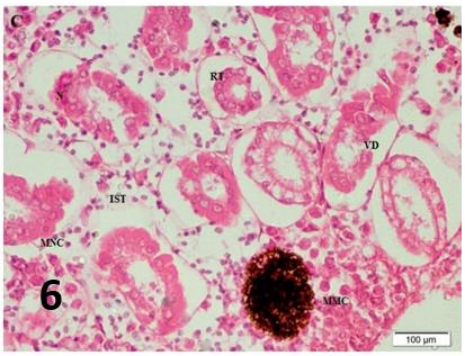


Figure 5: Histopathological alterations in the hepatopancreas of moribund Gilthead Seabream.
Micrograph (B) showing vacuolar degeneration (VD) and coagulative necrosis (CN) in hepatic cells (HC) and pancreatic acinar cells (PAC). Marked congestion in blood vessels of pancreatic tissue. H&E stain.

Figure 6: Histopathological alterations in the renal tissue of moribund Gilthead Seabream.
Micrograph (C) showing focal depletion in the hematopoietic tissue with marked activation of melanomacrophage centers (MMC) and tubular nephrosis (RT, renal tubules) in the form of vacuolar degeneration (VD) and coagulative necrosis (N). H&E stain

Discussion:

Vibrio species gain the global interest of the microbiology community and zoonotic diseases experts for being pathogen of public health concern ***(Austin and Austin, 2016).***

The clinical signs of naturally infected Gilthead Seabream were matched with those obtained by ***Gomathi et al. (2013)*** and ***Winfield (2018).***

The characteristic internal postmortem lesions were similar to those previously reported in naturally infected Gilthead seabream ***(Labella et al., 2011, Abdel-Aziz et al., 2013, Winfield, 2018)*** and in moribund common pandora fish **(Eissa *et al.*, 2017).**

These findings attributed to the production of toxic proteases associated with virulence ***(Kahla-Nakbi et al., 2009*)** and lethal to the fish**,** that released during the growth of Vibrio bacteria in iron-limited conditions and in fish serum**.** In addition to hemolysins, one of the most powerful ECPs, that were exotoxins causing lysis of erythrocytes and hence the releases of the intracellular hem, which identified as important virulence factors of *V. alginolyticus* owing to their contribution to the hemorrhagic septicemia.

The morphological characteristics and biochemical activities of *V. alginolyticus* coincided with the standard criteria that also reported by ***Austin and Austin (2016) and Eissa et al. (2017) .***

The highest prevalence of all retrieved *V. alginolyticus* was at the spring (31.03%) and summer (20.69%) which corresponded to the findings of ***Gomathi et al. (2013) and Winfield (2018)***. This may attributed to the immune-suppression because of the stress thus facilitate Vibrios invasion and outbreaks; High water temperature and sudden water temperature fluctuation were among the main triggering factors that establishing a strict connection with the appearance of outbreaks in spring and summer ***(Khalil et al., 2014) and (Winfield, 2018).*** The most predictable sites for such pathogens were in the kidney (34.48%), spleen (34.48%) followed by liver (31.03%). This findings harmonized with earlier literatures ***(Botella et al., 2002, Zorrilla et al., 2003a, Abdel-Aziz et al., 2013)*** which reported the kidney as the main target organs for isolation of *V. alginolyticus* in Gilthead Seabream. On the pathophysiological level, this tissue preference could be related to some of the virulence determinants owned by these pathogens, which enhance their septicemic nature with final predisposition into the main immune warrior (kidney), hematopoietic site (spleen) and toxin neutralizing site (liver).

The molecular characterization of Vibrios retrieved isolates to the species level were identified using PCR and targeting the universal Vibrio *16S r*RNA gene that produce a specific genomic band with 663bp. These results were consistent with earlier literatures ***(Tarr et al., 2007, Pascual et al., 2010, Carvalho et al., 2016, Kiani et al., 2016)*.**

The molecular characterization of retrieved *V. alginolyticus* isolates using *groE*l primers corresponded to the results of ***Raju et al. (2016)*** who found that the primer set based on *groE*L gene was specific for *V. alginolyticus* detection that produce band at 301bp size.

Using antibiotics as a therapeutic mean for control of bacterial diseases as Vibriosis is now discouraged because, antibiotic overuse/misuse leads to resistant pathogens, leaving antibiotics

ineffective as a treatment ***(Pridgeon and Klesius, 2012, Tuševljak et al., 2013, Haenen et al., 2014, Austin and Austin, 2016)***. Increasing antibiotic resistance poses important risks to human health and can affect the course of infectious diseases ***(Aly, 2013).*** Antibiogram for retrieved *V. alginolyticus* recorded resistance against ampicillin (69%), gentamycin (58.6%) and ciprofloxacin (20.7%) with highly susceptible to trimethoprim/sulphamethoxazole (100%) followed by oxytetracycline (86.2%). Similarly, previous studies documented that most *V. alginolyticus* 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 that some *V. alginolyticus* isolates was resistant to sulfamethoxzole/trimethoprim and oxytetracycline. The results were also consistent with those of ***Enany et al. (2011)*** and ***Abdellrazeq and Khaliel (2014).***

The histopathological alterations at tissues of naturally infected Gilthead Seabream revealed degenerative changes, circulatory disturbances and inflammatory reactions. These lesions of vibriosis have been attributed to endo- and/or exotoxins. ***Letchumanan et al. (2014)*** explained that during tissue cell infection, T3SS1 initiates a series of events that involves autophagy, membrane blebbing, cell rounding, and lastly cell lysis. Similar findings documented by ***Aly et al. (2000)*** for vibriosis in catfish and in experimentally infected rainbow trout by ***Avci et al. (2012)*** who stated 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 MMCs of the remaining splenic and renal hematopoietic tissue ***(Hendrikson and Zenoble, 1983, Roberts, 2012).***

Conclusion:

It could be concluded that; Vibriosis is a highly pathogenic disease not only to Gilthead Seabream but also to other marine fish and human thus the prevention measures and strict veterinary hygienic regulations should be implemented to control such infections and improve mariculture production in Egypt that is to minimize the antimicrobial use in fish farms which construct a high risk to human health, aquaculture and all environment.

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الملخص العربى

توصيف الإصابة بميكروب الفيبريو الجينوليتكس في أسماك الدنيس المستزرعة في مصر

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عنيت الدراسة الحالية بتقصى مدى الإصابة بمرض الفيبريوزس في أسماك الدنيس المستزرعة في منطقة قناة السويس ، مصر. خضعت مائتي عينة من الأسماك المجمعه التى ظهرت عليها علامات مرضية لفحوصات إكلينيكية وميكروبيولوجية ونسيجية. كما تم تعريف العزلات البكتيرية كيميائيا باستخدام نظام API 20 E ، ثم تم التأكيد عليها بواسطة PCR التقليدي. إلى جانب اجراء اختبار الحساسية للمضادات الحيوية للمعزولات. أظهرالفحص الاكلينيكى للأسماك المصابة طبيعياً استسقاء شديد ونزيف وتآكل في الجلد والزعانف. تم عزل و تعريف عدد ٢٩ عزلة ميكروب الفيبريو ألجينيوليتيكوس من العينات التي تم فحصها. و باستهداف الجينات التالية (*16Sr*RNA و *gro*El) ظهرقواعد بيبتيدية اثبتت تواجدهم بكل العترات المختبرة. وأظهرت الحساسية للمضادات الحيوية لعزلات ميكروب الفيبريو ألجينيوليتيكوس مقاومة للأمبيسيلين ، وجنتاميسين يليه سيبروفلوكساسين مع حساسية عالية لسولفاميثوكسازول / تريميثوبريم وأوكسيتيتراسيكلين. كما أظهرت التغيرات النسيجية في الأنسجة المصابة ، ردود الفعل الالتهابية جنبا إلى جنب مع التغيرات التنكسية و / أو نخرية في الأنسجة الحشوية والخيشومية.

وقد خلصت الدراسة إلى تاكيد المخاطر التى يسببها ميكروب الفيبريو ألجينيوليتيكوس و التي تهدد صحة الأسماك مع الآثار المترتبة على المستهلكين. وبالتالي ، ينبغي اعتماد استراتيجيات مختصة للأمن الحيوي للسيطرة على إصابة الفيبريوزس في الأسماك البحرية وتقليل مخاطر مقاومة الميكروبات للمضادات الحيوية في تربية الأحياء البحرية.