

Article

Bacterial Co-Infection as a Potential Threat to Farmed Flathead Grey Mullet (*Mugil cephalus*): Phenotypic and Molecular Diagnosis, Histopathology, Immunity Response, and In Vitro Antibacterial Evaluation

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Abstract: *Vibrio alginolyticus*, *Vibrio cholerae*, and *Enterococcus cloacae* were the main bacterial pathogens. investigated during recurrent episodes of mortalities among farmed flathead grey mullet (*Mugil cephalus*) in the Bahar El-Baqar area, Sharqia governorate, Egypt, during the summer season in 2021. Nevertheless, the contamination of water with heavy metals was evaluated. In this study, a total number of sixty moribund and freshly dead *Mugil cephalus* were examined clinically and upon post-mortem. Then, the bacterial isolates were subjected to bacteriological phenotypic examination, 16S rRNA gene sequencing, and antibiotic sensitivity testing. Histopathological screening and the immunity response of naturally infected *M. cephalus* were also implemented. In areas where bacterial strains were recovered in a high percentage, water contamination with Cu, Fe, and Ni was higher than the permissible limit. The results showed a high similarity (99–100%) to *Vibrio* and *E. cloacae* strains using phylogenetic analysis of 16S rRNA. Most isolates exhibited sensitivity to florfenicol, ciprofloxacin, and norfloxacin, while they were resistant to erythromycin and amoxicillin. Hemorrhage, oedema, hemosiderosis, and melanomacrophage activation in most affected tissues of infected *M. cephalus* were detected by light microscopy. Nevertheless, higher levels of AST and ALT, as well as serum inflammatory markers, CRP, and lysozyme activity, were shown in infected fish. There were higher MDA levels, along with a reduction of GSH, SOD, and catalase, in the gills and brain of infected *M. cephalus*, compared to the control fish. Our obtained data confirmed the high prevalence of *Vibrio* serovars and *E. cloacae* in infected grey mullets, describing their histopathological alterations, higher inflammatory response, and oxidative stress impact. These results could be useful indicators for understanding the pathogenesis of these pathogens in correlation with water parameter deterioration. Thus, the emergence of *Vibrio* and *E. cloacae* species in this study threatens the brackish water aquaculture industry in Egypt and poses a public health concern.

Keywords: *Vibrio alginolyticus*; *Enterococcus cloacae*; *Mugil cephalus*; heavy metals; 16S RNA sequencing; histopathology; immune response

Key Contribution: Pathogenic *Vibrio* and *E. cloacae* strains were identified at molecular levels in the infected *M. cephalus* that were associated with higher levels of heavy metals in the affected fish farms.



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1. Introduction

In the last few decades, flathead grey mullet (*Mugil cephalus*) has been one of the most significant candidate fish species in Egyptian aquaculture with a total global production of 250,000 tons in 2018 [1]. Mulletts are the next most farmed fish species after Nile tilapia in brackish water, in which tilapia, carp, and mullets are raised together in polyculture earthen pond environments [2,3]. Moreover, grey mullet is the preferred choice of consumers due to its taste, texture, fewer spines, and good nutritive value [3,4].

In Egypt, aquatic ecology is frequently vulnerable to accidental contamination by agrochemical and sewage disposal in addition to the deterioration of water parameters. All of these stress factors have emphasized the outbreaks of bacterial infection among fish species [5,6], particularly flathead grey mullet [7]. Thus, the knowledge base of hazardous bacterial co-infections together with environmental stressors that alter fish health is crucial for investigating effective preventive strategies [8]. In general, concurrent mixed microbial infections have caused serious impacts on the affected fish [9].

Vibriosis is the most threatening bacterial disease facing coastal and estuarine environments [10]. *Vibrio* species are halophilic Gram-negative, facultative anaerobes, and motile rod bacteria. These pathogens are plentiful in aquatic environments, where they are free-living in water or in association with aquatic fauna, considering opportunistic pathogens in many aquatic animals, such as marine fishes, crustaceans, and bivalves [11,12]. In addition, many *Vibrio* species have been implicated as causes of human foodborne illnesses as *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and *V. alginolyticus* are associated with gastroenteritis, severe dehydration, and primary septicemia due to the consumption of contaminated drinking water and undercooked or raw sea food [13,14]. Moreover, the relationship between *Vibrio* spp. abundance and impairment of environmental factors has been documented in numerous studies and which is strongly correlated with high salinity (30–35 ppt), high temperature (>28 °C), and increased ammonia levels [6,15,16]. These factors suppress the immunity and promote the susceptibility of fish to vibriosis [17,18]. Given these obstacles, *Vibrio* infection represents a dreadful economic loss in aquaculture and so awakes public concern.

Vibrio alginolyticus has caused mass mortalities among many marine fish farms throughout the Mediterranean area with severe economic losses in aquaculture worldwide [11,19–21]. *V. alginolyticus* has been described as pathogenic bacteria for turbot (*Scophthalmus maximus* L.) [22], sea bream (*Sparus aurata* L.) [23,24], grouper (*Epinephelus malabaricus*) [25], white leg shrimp (*Litopenaeus vannamei*) [26], grey mullet (*Mugil capito*) [27], and *M. cephalus* [18].

Naturally, fish have a mutualistic relationship with *V. cholera* as this bacterium secretes proteases and chitinases, with which they digest food (e.g., chitin and protein) in the fish gut [13,14,28]. Previous studies have reported the isolation of *V. cholerae* from the internal organs of diseased ayu (*Plecoglossus altivelis*) and guppy fish (*Poecilia reticulata*) in Japan and Iran, respectively [29–31], as well as from Nile tilapia (*Oreochromis niloticus*) that were cultured in floating cages in Thailand [32]. In addition, *V. cholera* was identified from the intestine of *Sarotherodon galilaeus* and *M. cephalus* [33]. In addition, an intraperitoneal injection of *V. cholerae* into common carp and rainbow trout caused severe mortalities [34].

Enterobacter cloacae (*E. cloacae*) is an enteric Gram-negative bacterium belonging to the family Enterobacteriaceae and widely distributed in nature. This opportunistic bacterium is found in the fecal matter of humans and animals, water, soil, plants, insects, and dairy products. *Enterobacter* species have a resistance to many antibiotics, such as colistin, by expressing the mobile colistin resistance-1 (*mcr-1*) gene to modify the lipid A component of lipopolysaccharide; subsequently, many human bacterial diseases are becoming incurable. Moreover, foodborne diseases are widely caused by Enterobacteriaceae, which is highly spread among various aquatic systems to be colonized in humans, causing urinary tract infections. In addition, the abuse of antibiotics in the aquatic environment has led to the emergence of new bacterial *Enterobacter* strains [35–40].

Enterobacter strains are not the normal flora in the intestinal tract of fish and, hence, their presence in the infected fish may be related to polluted waters and the feeding behavior of fish [41,42]. Previous studies have assayed the isolation of various *Enterobacter* strains from brown bull head catfish (*Ictalurus nebulosus*, LeSueur) [43], dolphin fish (*Coryphaena hippurus* L.) [44], grey mullet *Mugil cephalus* [41,45], ornamental fish, *Etroplus maculatus* [42], and Nile tilapia (*Oreochromis niloticus*) [46]. Moreover, recent studies documented that *E. cloacae* is a new-borne bacterial pathogen causing severe deaths to farmed crayfish [47].

Analysis of 16S rRNA is the most precise and prompt diagnostic technique for different bacterial pathogens [48,49], such as *Vibrio* species [50] and *Enterobacter* species [41]. In addition, histopathological and immunological alterations, as well as oxidative stress impact, are adopted to be useful indicators for understanding *Vibrio* species pathogenesis [10].

In this study, we investigated the main pathogenic bacterial agents accompanied by poor water quality as a cause of summer mortalities in flathead grey mullets that were farmed in the Bahr El-Baqar area, Egypt. We initially performed a molecular characterization of bacterial isolates using 16S rRNA as a target marker followed by sequencing and phylogenetic analyses. Moreover, the immune response as well as histopathological changes in *M. cephalus* were evaluated in response to natural co-infection by *Vibrio* spp. and *E. cloacae*.

2. Materials and Methods

2.1. Study Area

Cases of mortality (approximately 40–50 fish/day) were reported in flathead grey mullet, *M. cephalus* (350 and 500 gm) during the late summer season of 2021 on 3 fish farms in the Bahr El-Baqar area, Sharqia governorate, Egypt (Figure 1). Mullet fish were cultured in 3 different polyculture earthen ponds (70, 15, and 25 acres) at an approximate density of 500–1000 per acre in an integrated system with *Mugil capito* (*M. capito*) (3000/acre) and Nile tilapia (15,000–20,000/acre). The aquatic ecosystem was contaminated by agrochemical and sewage drainage. No paddlewheel aerators were used in the farm. Between 40–50 estimated fish deaths were recorded daily per each pond for 15 successive days, with mortality rates peaking on these days and then a gradual decrease in mortalities was noticed after treatment trials.

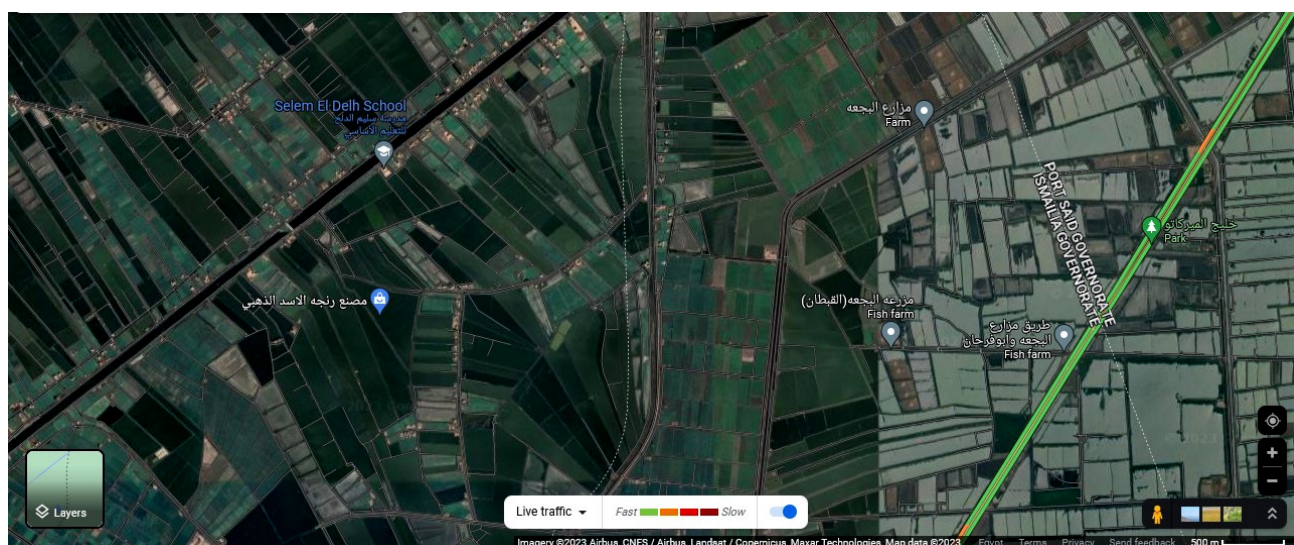


Figure 1. Satellite map from Google Earth showing different fish farms located at El-Baqar area, Sharqia governorate, Egypt. (<https://www.google.com/maps/@30.9269852,32.1553382,4806m/data=!3m1!1e3!5m1!1e1?entry=ttnu>, accessed on 22 June 2023).

2.2. Fish Sampling and Clinical Examination

At the peak time of mortalities, a total number of 60 moribund *M. cephalus* were collected by repeated dip-netting from 3 earthen ponds (20 fish/farm) for further diagnostic procedures and classified as a diseased group. Recently moribund fish were kept in tanks with aerators and then transferred to the laboratory of fish disease and management in the Faculty of Veterinary Medicine, Mansoura University. The clinical signs and post-mortem (PM) lesions were examined according to previously described methods [51]. The procedures for the hygienic disposal of dead fish were performed. Ten apparently healthy *M. cephalus* specimens were collected from the other earthen ponds of the same farms (ten fish per farm) and examined, to be named as the control group. In addition, water samples were collected from the control and diseased ponds.

Fish were euthanized with clove oil at 60 mgL^{-1} [52]. Blood samples were then collected from both groups from the caudal vessel using a 5 mL heparinized syringe and a 24 GA needle; serum was separated by centrifugation at 3000 rpm for 10 min at 4°C , removed and stored in 1.5 mL microcentrifuge tubes at -80°C for subsequent analysis of some biochemical and immunity parameters. Then, fish were carefully dissected and internal organs were used for bacterial isolation. Gills and brain were excised and stored in cold phosphate buffer saline PBS (pH 7.5) at -20°C for the determination of oxidative enzymes. Finally, gills, buccal cavity, and internal organs were stored in formalin for histopathological examination.

2.3. Water Quality Measures

The physicochemical characteristics of the pond water were investigated through the collection of water samples from the control and diseased ponds at a depth of 50 cm by a column sampler in sanitary plastic bottles then stored according to typical methods described by [53]. A water thermometer (Yellow Springs Comp., Ohio-model 33) was used to evaluate the water temperatures. Levels of dissolved oxygen (DO) were measured using an oximeter model DO200A EcoSense® (YSI, Brannum Lane, Yellow Springs, OH, USA, EcoSense® products) which was used to measure the percentage of water salinity. A portable Digital PH meter was used to measure the pH value (EcoSense® pH100A Meter). The kits (Palintest, Water Analysis Technology, Golden, CO, USA) were used for the evaluation of the levels of un-ionized ammonia (NH_3) and nitrate (NO_3). In addition, the analysis of the heavy metals in the water (mgL^{-1}) was carried out using the flame atomic absorption spectrophotometer (AAS) technique [54].

2.4. Bacterial Strains Isolation and Identification

Bacteriological samples were collected aseptically from the kidney, liver, and spleen of diseased *M. cephalus*, then cultured onto tryptic soya agar plates (TSA; Oxoid, Basingstoke, Hampshire, UK) and thiosulphate citrate bile salts sucrose (TCBS, Difco TM, Becton and Dickinson, ND, USA). Cultures were incubated for $30 \pm 2^\circ\text{C}$ for 24 h. Then, a single distinct colony was carefully chosen from each plate, sub-cultured on the TSA and TCBS plates, and re-incubated under the same conditions. Subsequently, purified bacterial cultures were preliminarily recognized using Gram's stain, motility, oxidase, and catalase tests using standard biochemical classification techniques API®20-NE, according to the manufacturer's instructions (BioMérieux, Marcy-l'Étoile, France) [49,55].

2.5. Molecular Identification of Bacterial Isolates

2.5.1. Bacterial DNA Extraction

The QIAamp DNA Mini kit (Qiagen, Hilden, Germany, GmbH, Catalog no. 51304) was used for DNA extraction from the biochemically recognized *V. alginolyticus*, *V. cholera*, and *E. cloacae* isolates, according to the manufacturer's protocol. Briefly, 200 μL of the sample suspension was incubated with 10 μL of proteinase K and 200 μL of lysis buffer at 56°C for 10 min. After incubation, 200 μL of 100% ethanol was added to the lysate, which

was then washed and centrifuged. The nucleic acid was then eluted with 100 µL of elution buffer and stored at -20°C for further use in the PCR steps [6].

2.5.2. 16S rRNA PCR, Sequencing, and Phylogenetic Analysis

One universal 16S rRNA primer (5'AGAGTTTGATCMTGGCTCA-3') and (3'TACGGYTACCTTGTACGACTT-5') was used for the molecular identification of *V. alginolyticus*, *V. cholera*, and *E. cloacae*, which amplified a 1485 bp fragment [56]. PCR amplification was performed in a 25 µL reaction mixture containing 12.5 µL of Emerald Amp Max PCR Master Mix (Takara, Kusatsu, Japan), 1 µL of each primer (20 pmol; Metabion, Planegg, Germany), 4.5 µL of distilled water, and 6 µL of DNA template. The reaction was performed in an Applied Biosystems 2720 thermal cycler (Applied Biosystems, Foster City, CA, USA). Amplification started with an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 56°C for 45 s, and 72°C for 45 s, and a final extension step was adjusted at 72°C for 10 min. Next, 15 µL of the amplified PCR products was analyzed by electrophoresis on 1.5% agarose gel (AppliChem, Darmstadt, Germany, GmbH) using gradients of 5 V cm^{-1} , and a 100 bp DNA Ladder (Fermentas, Thermo Scientific, Bremen, Germany) was used to determine the fragment sizes. The bands were visualized by ultraviolet (UV) light and photographed using the agel documentation system (Alpha Innotech, San Leandro, CA, USA; Biometra, Gottingen, Germany).

The QIAquick PCR Product extraction kits (Qiagen, Valencia, Spain) were used to purify PCR products. Then, Bigdye Terminator V3.1 cycle sequencing kits (Perkin Elmer Applied Biosystems, Foster City, CA, USA) were utilized for the sequencing reaction. The genome DNA sequences were obtained using the Applied Biosystems 3130 × Genetic Analyzer (HITACHI, Chiyoda City, Japan). The 16S rRNA sequences were evaluated for their homology using the BLASTn program (<http://www.ncbi.nlm.nih.gov/Blast>, accessed on 29 May 2023). Multiple alignment through Muscle tool and maximum likelihood phylogenetic analysis were conducted based on the 16S rRNA sequence cluster that positioned our bacterial isolates in a very close proximity to the reference strains of *V. alginolyticus*, *V. cholera*, and *E. cloacae* using the Mega software version 7.0 [57].

2.6. Antibigram Testing

The antibiotic susceptibility of the pathogenic bacterial strains was carried out using the disc diffusion method of Muller–Hinton Agar (Oxoid, Hampshire, UK) [58,59] supplemented with 1.5% (*w/v*) sodium chloride. After 24 h of incubation at $30 \pm 2^{\circ}\text{C}$, the organisms were classified as sensitive, intermediate, or resistant in accordance with the instructions of the Clinical and Laboratory Standards Institute guidelines [60] through measuring the diameters of the inhibition halos surrounding the discs. The selected antimicrobial discs were [ciprofloxacin (CIP, 5 µg), amoxicillin (AML, 25 µg), norfloxacin (NOR, 10 µg), doxycycline (DO, 30 µg), erythromycin (E, 15 µg), and florfenicol (F, 30 µg)], which were obtained from Bioanalyse[®] (Ankara, Turkey).

2.7. Serum Biochemical and Immune Markers

The serum activities of alanine aminotransferase (ALT, Catalog No.; MBS038444), (MyBioSource Co., San Diego, CA, USA), and aspartate aminotransferase (AST, Catalog No.; EK12276) (Biotrend Co., MD, USA), as well as levels of total protein (Catalog No.; MBS9917835) (MyBioSource Co., San Diego, CA, USA) and albumin (Catalog No.; SB-028-500) (Stanbio Laboratory, Boerne, TX, USA), were estimated spectrophotometrically according to the standard protocol of their specific pamphlets using a spectrophotometer (Lambda EZ201; Perkin Elmer, Waltham, MA, USA) [61,62].

The serum levels of immunoglobulin M (IgM, Catalog No.; CSB-E12045Fh, complement (C3, Catalog No.; MBS281020)) and C-reactive protein (CRP, Catalog No.; MBS016586) were assayed using the kits obtained from Cusabio Co. (Houston, TX,

USA) and MyBioSource Co. (San Diego, CA, USA), respectively, based on the manufacturer's instructions [63–65].

Serum lysozyme activity was measured according to the previously described method [66] based on the lysis of *Micrococcus lysodeikticus* (Sigma Co., Ronkonkoma, NY, USA), with some modifications. The serum and *M. lysodeikticus* suspension (0.2 mg/mL in 0.05 M PBS, pH 6.2) mixture was reacted at 25 °C for 5 min. The optical density was then measured at 1 min intervals for 5 min at 540 nm (5010, Photometer, BM Co., Herne, Germany). The concentration of serum lysozyme was calculated using a calibration curve constructed using different dilutions of lyophilized chicken egg-white lysozyme (Sigma Co., Ronkonkoma, NY, USA).

2.8. Oxidative Stress/Antioxidant Parameters

Parts of gills and brain tissues from apparently healthy and diseased striped mullet were washed three times using cold NaCl solution (0.9%) and homogenized in cold PBS (pH 7.5). Later, the homogenates were cold centrifuged for about 15 min at 3000 rpm and the supernatants were carefully collected in a clean tube to be used in the evaluation of antioxidant and oxidative stress parameters [67]. The homogenate protein level was determined using the protocols described [68].

The gills and brain levels of malondialdehyde (MDA, catalog No.; MD 25 29), glutathione (GSH, catalog No.; No. GR 25 11), catalase (catalog No.; CA 25 17), and superoxide dismutase (SOD, catalog No.; SD 25 21) were estimated spectrophotometrically using commercial test kits obtained from Biodiagnostics company (Cairo, Egypt).

2.9. Histopathological Examination

The brain, liver, spleen, and gills were dissected from the control and diseased *M. cephalus* and fixed in 10% neutral buffered formalin for 24 h. The dissected organs were placed in tissue cassettes, processed, and embedded in paraffin wax. Embedded samples were sliced into 5 µm sections by a microtome. The sections were stained using haematoxylin and eosin [69]. The stained slides were examined under a light microscope (Olympus CX 31, Tokyo, Japan).

2.10. Statistical Analysis

Data were expressed as mean ± standard error (SE). The differences among all parameters were applied via the SPSS software program (version 20, (IBM Corporation, SPSS Statistics, Chicago, IL, USA) using independent samples *t*-test to determine the differences between groups.

3. Results

3.1. Clinical Signs and Necropsy Findings

The diseased *M. cephalus* samples were anorexic and exhibited external generalized erythematic signs, such as severe hemorrhages on the opercula, buccal cavity, and on the base of fins with eroded fins, as well as an inflamed swollen vent was noticed (Figure 2). On necropsy, the PM lesions revealed congested gills. In addition, the abdominal cavity was loaded with copious amounts of bloody ascetic fluids with a distended gall bladder. Congested kidney tissue with surface hemorrhages over the brain, liver, and intestinal mucosa were noticed (Figure 3).

3.2. Water Physicochemical Properties

The quality parameters of the pond water collected from the Bahr El-Baqar fish farms during the mass mortalities of *M. cephalus* are summarized in Table 1. The results showed a decrease in dissolved oxygen (4 mgL^{−1}) and elevated levels of un-ionized ammonia (0.90 mgL^{−1}) and nitrite (0.07 mgL^{−1}) were detected. In addition, the measured levels of heavy metals were 0.64, 1.9, 0.8, 0.09, and 0.40 mgL^{−1} for copper (Cu), iron (Fe), nickel

(Ni), cadmium (Cd), and lead (pb), respectively. These levels are over the recommended permissible levels [70].

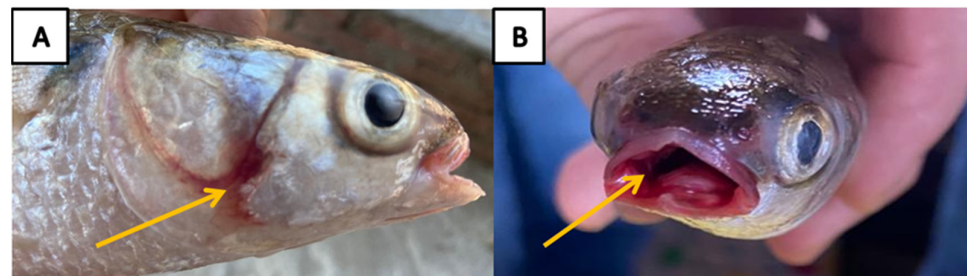


Figure 2. (A) Naturally diseased cultured flathead grey mullet (*Mugil cephalus*) showed opercular hemorrhage (yellow arrow). (B) Buccal cavity hemorrhage (yellow arrow) was also observed.

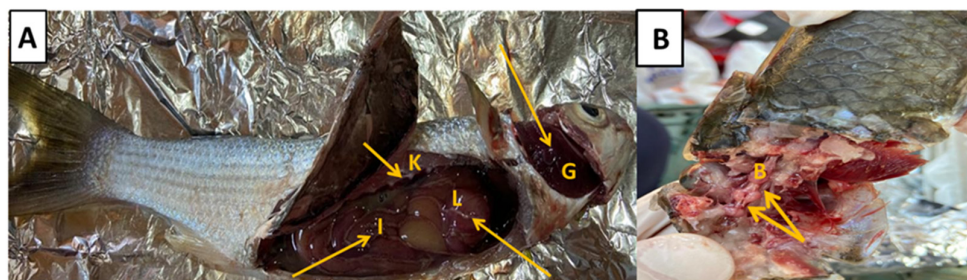


Figure 3. (A) Naturally infected grey mullet (*Mugil cephalus*) showed congested gills (G), hemorrhage of intestinal mucosa (I), congested kidney (K), and surface hemorrhages over liver (L) (yellow arrows). (B) Severe hemorrhages over the brain (B) were revealed (yellow arrow).

Table 1. The physicochemical properties of pond water in Bahr El-Baqar fish farms during mortalities.

Water Parameters	Values
Dissolved oxygen (DO) (mgL ⁻¹)	4
Temperature (°C)	33
Nitrite (NO ₂) (mgL ⁻¹)	0.07
Un-ionized ammonia (NH ₃) (mgL ⁻¹)	0.90
Copper (Cu) (mgL ⁻¹)	0.64
Iron (Fe) (mgL ⁻¹)	1.9
Nickel (Ni) (mgL ⁻¹)	0.80
Cadmium (Cd) (mgL ⁻¹)	0.09
Lead (mgL ⁻¹)	0.40

3.3. Phenotypic Identification of Bacterial Pathogens

The strains of *V. alginolyticus*, *V. cholera*, and *E. cloacae* were isolated and identified from the diseased fish following phenotypic characterization, as illustrated in Table 2. *V. alginolyticus* was isolated from 50% (30/60) of the sampled *M. cephalus*. Meanwhile, *V. cholerae* was isolated from 25% (15/60) of the sampled fish and *E. cloacae* was successfully isolated from 10% (6/60). In addition, 15% of the fish harbored a mixed infection of two or three isolates which were identified.

3.4. Molecular Identification of Bacterial Pathogens

PCR successfully amplified the 1485 bp band of the universal 16S rRNA primers of Gram-negative bacteria (Figure 4). One sequence of *V. alginolyticus* was deposited in the GenBank (Accession No. OM459825) and two sequences of *V. cholerae* were deposited in the GenBank (Accession No. OM185314 and OM841535). One sequence of *E. cloacae* was also deposited in the GenBank (Accession No. OM914983). BLAST analysis of the nucleotide sequence of the 16S rRNA genes from *V. alginolyticus* in this study shared 94.16% to 100%

identity with other published sequences of *V. alginolyticus* obtained from the GenBank database (Figure 5). Similarly, the 16S rRNA genes of *V. cholerae* sequenced in this study shared 99.44% to 99.87% identity with other published sequences of *V. cholerae* obtained from the GenBank database (Figure 4). In addition, the sequence of *E. cloacae* in our study shared 99.16% to 100% identity with other published sequences of *E. cloacae* obtained from the GenBank database (Figure 6). The phylogenetic analysis of the amplified 16S rRNA sequences from *V. alginolyticus*, *V. cholerae*, and *E. cloacae* were grouped together with their relevant sequences in the GenBank database, respectively (Figures 5 and 6).

Table 2. The physicochemical properties of pond water in Bahr El-Baqar fish farms during mortalities.

Item	<i>V. alginolyticus</i>	<i>V. cholerae</i>	<i>E. cloacae</i>
Gram staining	-ve	—	—
Cell morphology	Short rod with curve shaped	Short rod with curve shaped	Bacilli
Motility	+	+	—
Catalase	+	+	+
Growth on TCBS	Green	Green	—
Sorbitol (SOR) fermentation	—	—	+
Rhamnose (RHA) fermentation	—	—	+
Sucrose (SUC) fermentation	+	+	+
Melibiose (MEL) fermentation	—	—	+
Amygdalin (AMY) fermentation	+	+	+
Arabinose (ARA) fermentation	—	—	+
Cytochrome oxidase	+	+	—
Arginine dihydrolase	+	+	—
Lysine decarboxylase (LDC)	+	+	+
Ornithine decarboxylase (ODC)	+	+	—
Citrate utilization	+	+	+
H ₂ S production	—	—	—
Urease production	—	—	—
Tryptophane deaminase (TDA)	+	+	—
Indole (IND) production	+	+	—
Voges–Proskauer Test (VP)	—	V	+
Gelatine (GEL) hydrolysis	+	+	—
Glucose (GLU) fermentation	+	+	+
Mannitol (MAN) fermentation	+	+	+
Inositol (INO) fermentation	—	—	+

Note: + = positive, — = negative, V = Variable.

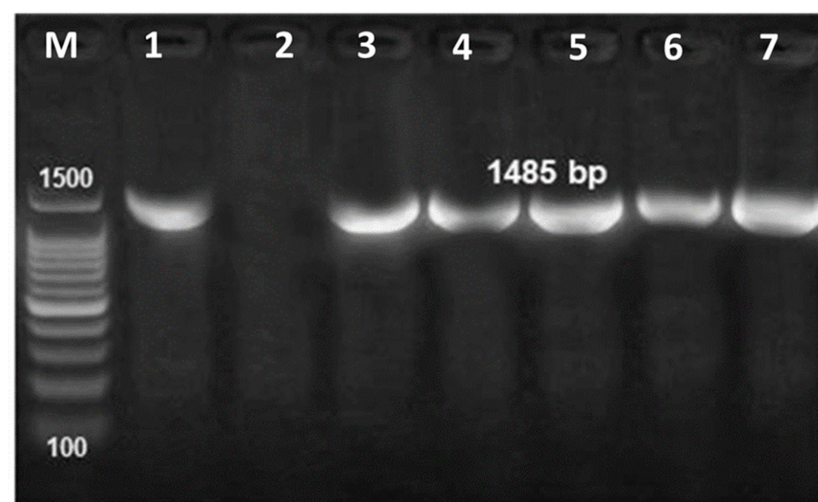


Figure 4. PCR amplification of the universal 16S rRNA primers of Gram-negative bacteria at 1485 bp. M: 1000–1500 bp DNA size marker, Lane 1: control positive and Lane 2: control negative, Lane 3–7: bacterial strains obtained from naturally infected flathead grey mullet.

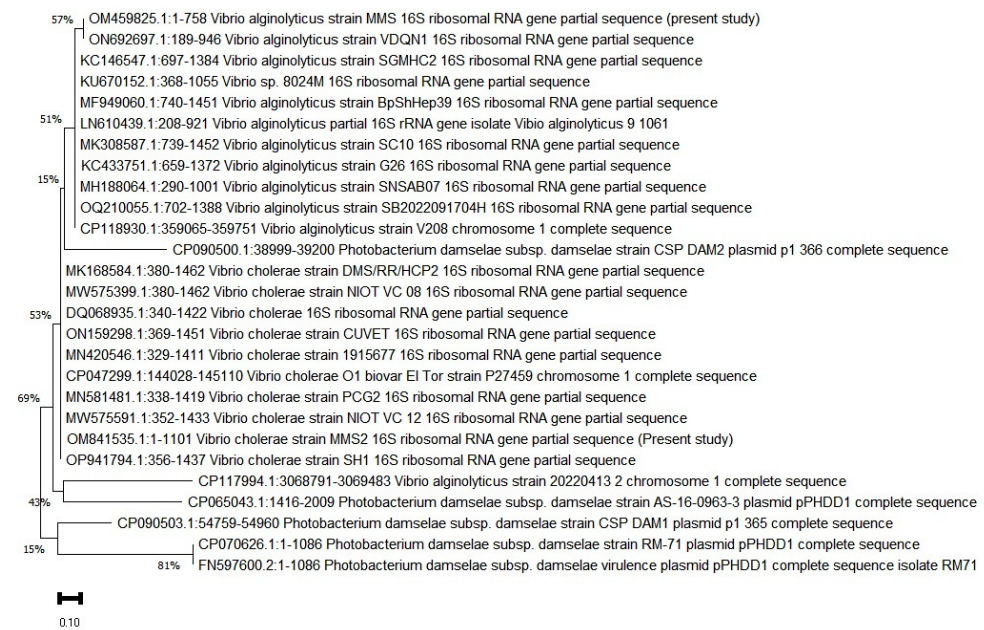


Figure 5. Maximum likelihood phylogenetic tree showing the relationships between the *Vibrio* strains (three strains) isolated from diseased grey mullet, *Mugil cephalus*, and other reference strains of *Vibrio* spp. based on the 16S rRNA sequences of *Vibrio* spp. The numbers above the branches are the percentages determined in a bootstrap analysis (1000 replicates).



Figure 6. Maximum likelihood phylogenetic tree showing the relationships between the *E. cloacae* strain isolated from diseased grey mullet, *Mugil cephalus*, and other reference strains of *E. cloacae* spp. based on the 16S rRNA sequences. The numbers above the branches are the percentages determined in a bootstrap analysis (1000 replicates).

3.5. Susceptibility of Pathogenic Strains to Antibiotics

The *Vibrio* strains were sensitive to norfloxacin, florfenicol, and ciprofloxacin, whereas they were resistant to amoxicillin. They also exhibited a moderate sensitivity to erythromycin and doxycycline. The *E. cloacae* strain was sensitive to doxycycline, while was exhibited an absolute resistance to erythromycin, as well as a moderate sensitivity being exhibited to ciprofloxacin and amoxicillin (Table 3).

Table 3. Antibigram profile of pathogenic *Vibrio* spp. and *E. cloacae* strains.

Antibiotic	Concentration (μg)	<i>V. alginolyticus</i>	<i>V. cholera</i>	<i>E. cloacae</i>
Amoxicillin (AML)	25	09.7 ± 0.6 (R)	10.5 ± 1.0 (R)	22.7 ± 1.5 (S)
Erythromycin (E)	15	23.1 ± 1.7 (S)	21.7 ± 1.5 (S)	07.3 ± 0.6 (R)
Doxycycline (DO)	30	23.3 ± 1.1 (S)	22.3 ± 1.1 (S)	26.0 ± 1.0 (S)
Florfenicol (FFC)	30	20.3 ± 1.1 (S)	21.3 ± 1.1 (S)	24.0 ± 1.0 (S)
Ciprofloxacin (CIP)	5	20.7 ± 1.2 (S)	22.7 ± 1.3 (S)	25.0 ± 1.0 (S)
Norfloxacin (NOR)	10	10.3 ± 0.6 (R)	10.3 ± 0.6 (R)	26.0 ± 1.0 (S)

The sensitivity criterion used was based on the standardization of surveillance of antimicrobial usage and antimicrobial resistance in aquaculture [60]. S: Highly sensitive; R: Resistant.

3.6. Serum Biochemical and Inflammatory Findings

As illustrated in Table 4, the obtained data demonstrated significantly higher serum activities of ALT ($p < 0.01$) and AST ($p < 0.001$) in the diseased striped mullet than that of the controls. There were no significantly valuable differences in the serum levels of total protein, albumin, globulin, and C3 ($p \geq 0.05$) between the groups.

Table 4. Serum biochemical and immune markers in control and diseased *M. cephalus*.

Parameters	Groups		Sig
	Healthy <i>M. cephalus</i>	Infected <i>M. cephalus</i>	
ALT (U L^{-1})	13.25 ± 1.09	29.04 ± 1.71	**
AST (U L^{-1})	68.53 ± 2.57	99.1 ± 2.32	***
T. protein (g dL^{-1})	5.84 ± 0.27	6.02 ± 0.33	NS
Albumin (g dL^{-1})	3.19 ± 0.12	2.73 ± 0.18	NS
Globulin (g dL^{-1})	2.65 ± 0.32	3.30 ± 0.29	NS
IgM (mg dL^{-1})	9.31 ± 0.64	4.98 ± 0.76	***
CRP (mg L^{-1})	2.63 ± 0.41	7.12 ± 0.67	**
C3 (mg mL^{-1})	0.36 ± 0.04	0.54 ± 0.08	NS
Lysozyme ($\mu\text{g mL}^{-1}$)	7.82 ± 1.32	14.71 ± 2.19	**

Data were represented as Mean $\pm$ SE, NS; Non-significant, **; $p < 0.01$, ***; $p < 0.001$. ALT, alanine aminotransferase; AST, Aspartate aminotransferase; IgM, Immunoglobulin M; CRP, C-reactive protein; C3, complement 3.

To assess the mechanisms involved in the progression and damage induced by bacterial infection, some serum inflammatory markers were estimated in our study (Table 4). The serum CRP level and lysozyme activity ($p < 0.01$) were found to be highly elevated with a lower IgM level ($p < 0.001$) in the naturally infected flathead grey mullet compared to the control one.

3.7. Oxidative Stress/Antioxidant Status

The progression of infection caused the generation of an accentuated ROS and impairment of antioxidant molecules confirmed in our results by the higher MDA levels, along with a reduction of GSH, SOD, and catalase in both the gills and brain of the diseased flathead grey mullet compared to the control one (Table 5).

Table 5. Gills and brain oxidative stress/antioxidant status of healthy and infected *M. cephalus*.

Parameters	Tissues	Groups		Sig
		Healthy <i>M. cephalus</i>	Infected <i>M. cephalus</i>	
MDA (nmol mg ⁻¹ protein)	Gills	8.55 ± 0.94	12.48 ± 0.54	***
	Brain	5.24 ± 0.49	9.20 ± 0.56	**
SOD (U mg ⁻¹ protein)	Gills	116.81 ± 7.77	95.24 ± 4.55	**
	Brain	50.49 ± 3.64	32.15 ± 1.06	**
Catalase (U mg ⁻¹ protein)	Gills	69.11 ± 6.29	50.46 ± 2.27	*
	Brain	31.01 ± 3.76	16.29 ± 1.21	**
GSH (mg mg ⁻¹ protein)	Gills	2.95 ± 0.51	1.42 ± 0.22	**
	Brain	5.89 ± 0.28	3.16 ± 0.14	***

Data were represented as Mean ± SE. NS; Non-significant, *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$. MDA, malondialdehyde; GSH, reduced glutathione; SOD, superoxide dismutase.

3.8. Histopathological Findings

The diseased and control *M. cephalus* were examined for histopathological changes during the peak of mortalities. The liver had diffuse necrosis with a cellular detachment that was accompanied by an eosinophilic cytoplasm and pyknotic to a complete loss of the nucleus. Furthermore, there was diffuse edema and hemorrhage admixed with hemosiderin, widely separated damaged hepatocytes, and congested blood vessels (Figure 7A–C). In the spleen, there was a multifocal splenic granuloma and necrosis infiltrated with numerous melanomacrophages that were surrounded by numerous lymphocytic aggregations and leukocytic infiltrations (Figure 7D–F). The gills had marked diffuse lamellar sloughing, necrosis with lamellar telangiectasis, and interlamellar necrotic debris with severe interlamellar hemorrhage (Figure 7A–C). The most characteristic findings of the fish brain are congested and dilated blood vessels, multifocal granuloma with few inflammatory cells, and numerous degenerated cells with focally leukocytic infiltrations (Figure 8D–F).

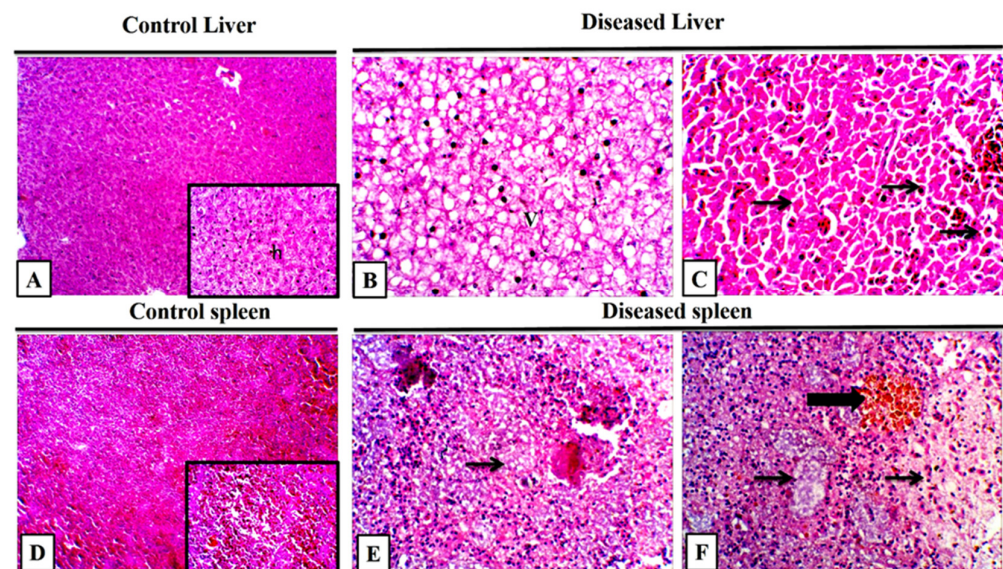


Figure 7. Representative photomicrograph of grey mullet (*Mugil cephalus*) liver and spleen. (A) Control liver showed normal hepatic architecture (h) in low and high power, H and E, 100×, and 400×. (B) Diseased mullet liver showed a diffuse hepatic vacuolation (v), H and E, 400×. (C) Diseased liver showed a diffuse hepatic individualization with pyknotic nuclei (arrows), H and E, 400×. (D) Control spleen showed normal splenic architecture. (E,F) Diseased spleen showed a loss of normal architecture with lytic necrosis (thin arrows) and melanomacrophage aggregations (thick arrow), H and E, 400×.

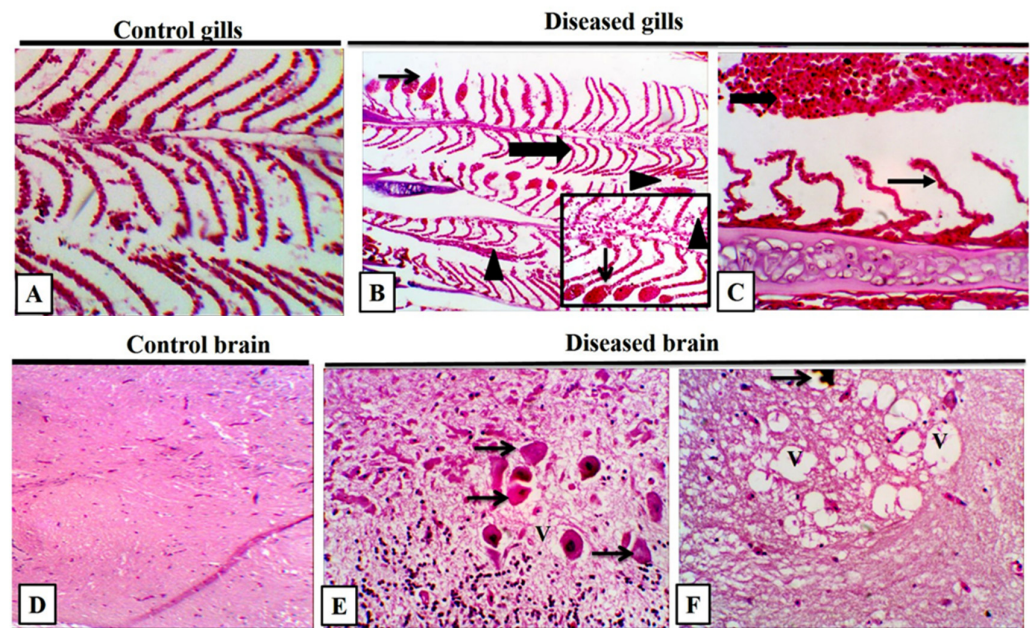


Figure 8. Representative photomicrograph of grey mullet (*Mugil cephalus*) gills and brain. (A) Control gills showed a normal lamellar architecture with minimal sloughed epithelium. (B) Diseased gills showed clubbing (thick arrow) and telangiectasis of the gill lamellae (thin arrow) with detachment of gill epithelium (arrow heads), high power focus on telangiectasis (thin arrow), and interlamellar sloughed epithelium admixed with RBCs (arrow head). (C) Lamellar lifting (thin arrow) with extensive interlamellar hemorrhage (thick arrow) was revealed. H and E, 400 $\times$. (D) Control brain showed a normal histological appearance. (E) Diseased brain showed nerve cell bodies exhibiting pyknotic or complete loss of nuclei with shrunken cellular outline and hypereosinophilic cytoplasm (neuronal necrosis) (thin arrows) with moderate neuropil vacuolation H and E, 400 $\times$. (F) Neuronal parenchyma with clustered, focal variably sized, clear vacuoles (V), few vacuoles had brown pigment (thin arrow) H and E, 400 $\times$.

4. Discussion

The opportunistic bacteria from the genus *Vibrio* are ranked number one among all the pathogenic agents which have caused mortalities in estuarine and marine fish farms [10,48]. Environmental stressors have been correlated with *Vibrio* spp. abundance that can be used as a predictive strategy to determine when these pathogens may be present [6,11,71]. Moreover, *Vibriosis* outbreaks mainly occur during the summer season, as a result of the deterioration of water parameters such as the temperature, pH, DO, and salinity [72]. This pathogen load was also enhanced, particularly in developing countries that have disputed sanitary circumstances [73]. Given these constraints, the prevalence of bacterial infections has become a crucial problem faced via an *M. cephalus* culturing strategy [7].

In the current work, the most common generalized septicemic clinical signs and PM lesions were assigned in the infected striped mullet *M. cephalus* infected with *Vibrio* spp., reporting a sudden onset of mortalities during the summer season in many fish farms native to the Bahr El-Baqar region, Sharqia governorate. These findings were similarly reported in *M. capito* infected with *V. alginoticus* in the Wadi-mariut region, Borg El-Arab city, Alexandria governorate, Egypt [27]. In concordance with our results, many studies have reported the same typical signs of *Vibrio* infection in *M. capito* and *M. cephalus* [11,16,48,74]. A recent study showed septicemic lesions in diseased *M. cephalus* with *V. parahaemolyticus* in Lake Al-Manzala [6]. These results could be attributed to the detrimental effect of several virulence factors produced by *Vibrio* spp., such as siderophore anguibactin, iron-binding capacity, the adherence capacities to epithelial cells of *V. alginoticus*, and extracellular products (e.g., hemolysin, cytotoxin, lipase, protease, and phospholipase) [75,76]. These factors are responsible for invasive and proliferative pathogenicity in fish. Of particular

concern, circulatory component disturbances and immune suppression are mediated by these extracellular products [10]. It has been documented that the *Enterococcus* species caused septicemic-like infections in Nile tilapia in Egypt, Thailand, and Bangladesh [77,78].

Our data represented decreased DO levels accompanied by elevated levels of NH_3 and NO_2 along with a high temperature and increased heavy metal levels (more particularly, Cu, Fe, Ni, Cd, and Pb) in water. The above-mentioned substandard water quality results are positively associated with increased fish susceptibility to bacterial diseases which are consequent to the immune suppression of fish. It was noted that the continuous unhygienic disposal of agricultural and sewage pollutants in the water supply of the Bahr EL-Baqar drain usually led to a deterioration of the water quality parameters [79,80]. A potential correlation was observed between the *Vibrio* spp. infections in diseased *M. cephalus* which suffered from mortalities in numerous Egyptian fish ponds during the summer season and poor water characteristics including high levels of heavy metals [6,81]. In similar studies, a sharp increase in NH_3 , water salinity, and temperature with decreased dissolved oxygen and ability to scavenge iron from iron-binding proteins are the most likely triggering factors for the spread of *V. alginoticus* infections in coastal areas in China [82], and also in many Egyptian coastal sites [83], including Lake Al-Manzala's fish farms [8,84]. Moreover, several reports assayed that abnormal water parameters are the pre-disposing factors for Enterococcosis in Egyptian fish farms [46,77]. Higher organic matter and un-ionized ammonia detected in the earthen ponds in the Kafr El Sheikh governorate and supplied with agriculture drainage water increased the risk of infectious *Enterococcus faecalis* in Nile tilapia [46]. Nevertheless, a higher prevalence of *Vibrio* species in *Mugil cephalus* were isolated from Lake Qarun, and this could be related to the deterioration of water quality via the sewage and agriculture discharges of Lake Qarun [18]. Changes in the water quality parameters in highly polluted Abbassa farms with subsequent heavy metal bioaccumulation enhanced the bacterial infections in many farmed fish species [85].

Additionally, heavy metals pollution, such as Ni [85,86] and Cu [87], exhibited an immunosuppressive effect in many teleost fish. Iron, an excellent oxygen transporter, has been shown to stimulate the growth of *Vibrio* spp. by iron acquisition through chelation to host iron-binding proteins (e.g., transferrin and lactoferrin) [88,89]. Copper toxicity has caused respiratory stress from the coagulation of the mucus layer of the gills, prohibiting oxygen transport, which was accompanied by reduced phagocytic activity [90]. Elevated levels of Fe, Cu, and Ni were positively related with vibriosis in Nile tilapia and silver carp from fish farms in Lake Al-Manzala during the summer season [8]. In addition, a high percentage of *Vibrio* was isolated from farmed *M. cephalus* in many Egyptian provinces where water was contaminated with Cu and Fe [91]. Consequently, it is plausible that the levels of metals detected in our study could have contributed to the outbreaks of disease with the resulting immunosuppression in striped mullet.

The insight analysis of the morpho-chemical characteristics for the majority of retrieved isolates coincided with the *Vibrio* sp. profiles as previously reported in diseased farmed *M. cephalus* [6,11,18,92]. Similarly, the phenotypic characteristics of the *E. cloacae* isolate in farmed *M. cephalus* corresponded with the description of *E. cloacae* in infected *M. cephalus* in the lagoon of Muttukadu, Chennai, India [41], and also with the *E. cloacae* strain from diseased crayfish (*Procambarus clarkii*) in China [47]. Biochemically identified isolates were further confirmed by the 16S rRNA PCR gene; however, this gene has a low distinguishing power to differentiate closely related *Vibrio* species that have nearly identical sequences [18,93].

The results of the phylogenetic analysis based on the 16S rRNA gene sequences of the bacterial isolates retrieved during the current study proposed that this gene is useful in allocating species to various branches of the Vibrionaceae family [94] and to the Enterobacteriaceae family [7]. The bacterial isolates identified in the present study shared nearly identical sequences in the 16S rRNA gene (99.5% to 100% identity). Subsequently, 16S rRNA gene sequencing analyses are highly discriminative as phylogenetic identifiers in ascertaining *Vibrio* and *E. cloacae* species.

The isolated *Vibrio* strains were highly sensitive to norfloxacin, florfenicol, and ciprofloxacin, whereas they were resistant to amoxicillin. These findings are in line with previous studies [83,95]. In addition, the *E. cloacae* strain was sensitive to doxycycline, while it exhibited absolute resistance to erythromycin, as well as an intermediate sensitivity being exhibited to ciprofloxacin and amoxicillin. Similar results were reported in virulent *E. cloacae* isolated from diseased crayfish, *Procambarus clarkii* [47]. Therefore, these selected antibiotics could be recommended for prevention during the peak season of disease manifestation.

Many fish species have shown changes in blood chemistry parameters during acute bacterial infections. The biomarkers ALT and AST are important indicators of liver dysfunction and injury [96], and their detection in blood is due to the release of these enzymes into the bloodstream by damaged hepatocytes [97]. Our data revealed significantly higher serum activities of ALT ($p < 0.01$) and AST ($p < 0.001$) with insignificant changes in the serum levels of the total protein, albumin, globulin, and C3 ($p \geq 0.05$) of the diseased stripped mullet than that of the controls. Similarly, the ALT and AST activities were higher in the *Aeromonas hydrophila* and *Pseudomonas fluorescens*-infected Nile tilapia [98]. Furthermore, *Rhamdia quelen* infected with *Aeromonas hydrophila* had higher ALT levels than the control group [99]. Additionally, another previous study found that *Vibrio vulnificus* or *Streptococcus iniae* infection caused dramatically higher ALT and AST, with lower total protein and albumin levels in infected fish [100]. Unlike our findings, complement pathway activation has been recorded in *M. amblycephala* after infection with *A. hydrophila*, mandarin fish (*Siniperca chuatsi*) infected with *Flavobacterium columnare*, and soiny mullet (*Liza haematocheila*) in response to *Streptococcus dysgalactiae* infection [101–103].

IgM and lysozyme are involved in a fish's innate and adaptive immune responses to bacterial infection [104]. C-reactive protein (CRP) is a cyclic serum protein that is released from the serum of many fish species during inflammation, making it a well-acknowledged biomarker of fish health status [105]. In our study, the serum CRP level and lysozyme activity were significantly elevated with a lower IgM level ($p < 0.001$) in the naturally infected flathead grey mullet compared to the control one. A previous report documented that the common carp was injected with *Aeromonas hydrophila* and *Escherichia coli* lipopolysaccharide [106]. In addition, CRP levels in rainbow trout serum have been shown to be high interacting with *Aeromonas salmonicida*-derived LPS, suggesting that a number of virulence factors are involved in causing a pro-inflammatory response to bacterial infection in fish [107]. Likewise in our results, lysozyme activity was considerably higher in the *A. hydrophila* and *Photobacterium damsela*-infected Nile tilapia [65]. On day 1, 3, 5, 14, and 21 after *A. hydrophila* infection, a similar reaction was observed in *P. damsela* subsp. *piscicida* (Phdp)-infected *Solea senegalensis* [108,109], as well as in blunt snout bream (*Megalobrama amblycephala*) [110]. Furthermore, in the liver of *S. senegalensis* infected with Phdp, the lysozyme gene was dramatically increased [111]. The high level of serum lysozyme in infected fish implies a natural protective mechanism in fish against infection [112].

Oxidative stress is defined as an imbalance in antioxidant–oxidant status, which can impact the redox status of cell compartments in a reversible manner, causing harm to biological cell components such as lipid peroxidation and protein carbonylation [113]. Overproduction of reactive oxygen species (ROS) and/or a weakening of the defense antioxidant system during bacterial infection can cause oxidative stress in fish [114]. *Pseudomonas aeruginosa* infection caused an elevation in the brain lipid peroxide (MDA) of silver catfish, which led to the emergence of clinical symptoms and disease pathogenesis [115]. Moreover, *Providencia rettgeri* infection induces oxidative damage in infected *O. niloticus* via increasing hepatic and renal MDA levels and reducing SOD and catalase activities [116]. Similarly, the SOD and GSH levels were diminished with higher ROS levels but were not affected in the liver and kidney of silver catfish (*Rhamdia quelen*) experimentally infected with *Aeromonas caviae*, and in the liver of sea trout (*Salmo trutta* L.) experimentally infected with *A. hydrophila* [114,117]. Our data demonstrated that *Vibrio*

and *Enterococcus* infection caused the generation of ROS (higher MDA levels) with an impairment of antioxidant molecules (reduction of GSH, SOD, and catalase) in both the gills and brain of the diseased flathead grey mullet compared to the control one. This finding could indicate that the antioxidant systems failed to protect the gills and brain tissues from the harmful effects of ROS during *Vibrio* and *Enterococcus* infection, thereby contributing to disease pathogenesis.

The pathological lesions in naturally exposed fish to *Vibrio* spp. and *E. cloacae* were presented in this study. Here, we clearly monitored that the main pathological changes in the affected tissues of diseased *M. cephalus* were septicemic lesions, such as hemorrhage, oedema, hemosiderosis, and proliferative and necrotic alterations. These lesions are considered the main changes in the natural infection in the acute phase of septicemic diseases, including vibriosis [49,118]. Similar findings were stated in the internal organs and gills of seabass [118,119], thin lip grey mullet, *M. capito* [27], and *M. cephalus* [91] upon infection with *V. alginolyticus*. In addition, extensive inflammatory and degenerative histopathological changes in fish infected with *Enterococcus* species were observed in recent reports [46,47]. A recent study observed similar lesions in *M. cephalus* infected with *Streptococcus agalactiae* and *Enterococcus faecalis* [7]. Altogether, the obtained data of the full diagnostic techniques accompanied by immunological and histopathological alterations are considered as proof of the septicemic nature of the bacterial co-infection in *M. cephalus*.

5. Conclusions

The deterioration in water quality via sewage and agriculture discharges was strongly related to a high prevalence of vibriosis and Enterococcosis in flathead grey mullet farmed in the Bahar El-Baqar area, Egypt. *V. alginolyticus*, *V. cholera*, and *E. cloacae* are the most causative bacterial species that were identified from diseased *M. cephalus* via phenotypic and molecular characterization. In addition, they are associated with septicemic clinical signs, pathological, and immunological alterations. Under the conditions of this study, the obtained data revealed that the monitoring of the water for heavy metals pollution is a crucial step to diminish the severity of *Vibrio* serovars infections. Subsequently, further investigations are required to assess the food safety regulations and the consumer's health in the growing aquaculture sector in Egypt.

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