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Investigation of Summer Mortalities in the Farmed Nile Tilapia, *Oreochromis niloticus*, From Three Provinces in Egypt

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ABSTRACT

The Nile tilapia represents one of the most widely cultured fish species in Egypt. In recent years, the Egyptian tilapia farms have been suffering from unknown high mortalities during the summer season resulting in high economic losses. Investigation of mortalities by different research groups revealed a significant association with various microbial agents, however, the main causes of summer mortality in tilapia are still unknown. The current study aimed to identify the possible causes of summer mortalities in tilapia farms from three main tilapia-producing governorates in Egypt. Clinical, microbial, histopathological and environmental examinations have been carried out over a period of a year in different tilapia farms and results were recorded. Clinical examination of moribund and recently dead fish showed extensive cutaneous haemorrhages with scales losses and erosion, abdominal distension, and ulcers. Screening of fish samples by viral and bacterial examination and molecular assays, including PCR and sequencing, showed negative results for tilapia lake virus and positive detection of *Aeromonas hydrophila* subsp. *dhakensis*, *A. sobria*, *A. veronii*, *A. enteropelogenes*, and *Streptococcus iniae*. The current study may contribute to our knowledge of potential causes of summer mortality in tilapia and would help in the development of control strategies for the devastating tilapia losses in the Egyptian fish farms.

INTRODUCTION

Aquaculture is considered one of the most important food-producing sectors supplying the needs for animal protein and seafood sources globally **(Troell *et al.*, 2017)**. Egypt ranked the first Nile tilapia (*Oreochromis niloticus*) producer in Africa and the third globally after China and Indonesia, with an estimated annual production of ~1.2 million tons **(FAO, 2020)**. The Nile tilapia are characterized by rapid growth rate and significant adaptation to different culture systems, affordability and high content of protein and nutrients **(Leaño, 2018)**. In addition, tilapia have the ability to survive different water quality parameters, such as low dissolved oxygen, high ammonia, high and low salinities, and are physiologically more resistant to diseases compared to other cultured species **(Fitzsimmons, 2000)**.

While tilapia farming has witnessed significant growth over the past decades, the intensification of production resulted in the emergence of infectious diseases globally **(Leal *et al.*, 2019)**. Cultured tilapia are susceptible to various microbial diseases during the production cycle, including viral, bacterial and parasitic diseases **(Surachetpong *et al.*, 2020)**. Many viruses have been detected in farmed tilapia such as viral nervous necrosis (VNN), tilapia larvae encephalitis virus (TLEV), infectious spleen and kidney necrosis virus (ISKNV), herpes-like virus, and recently tilapia lake virus (TiLV) **(Crane & Hyatt, 2011; Eyngor *et al.*, 2014)**.

Bacterial diseases are the most commonly reported pathogens in farmed tilapia. There are about 30 species of bacterial pathogens isolated from cultured tilapia, such as *Aeromonas* spp., *Streptococcus* spp., *Flexibacter columnaris, Yersinia ruckeri*, *Pseudomonas* spp., *Edwardsiella tarda, E. ictulari*, and *Francisella orientalis* **(Klesius *et al.*, 2008; Austin *et al.*, 2012)**.

In Egypt, recurrent high mortalities have been recorded in various tilapia farms during the summer season over the past 10 years. Investigation of these mortality events from the different outbreaks identified numerous pathogens including *Aeromonas hydrophila*, *Vibrio mimicus, V. cholerae Flavobacterium columnaris, Edwardsiella tarda, Yersinia ruckeri, Streptococcus agalactiae, S. iniae* **(Aly, 2013; Abdel-Latif *et al.*, 2020)**. In addition, some outbreaks showed co-infection between bacteria and parasites such as *F. columnare* and *Myxobolus tilapiae* **(Eissa *et al.*, 2010)**, *V. alginolyticus, V. harveyii, Enterococcus faecalis, A. hydrophila, Ichthyophthirius multifiliis, Trichodina* sp. and *Gyrodactylus* sp. **(Nofal & Abdel-Latif, 2017)**, and *A. hydrophila* and *Gyrodactylus cichlidarum* **(Abdel-Latif & Khafaga, 2020)**. Identification of bacteria associated with mortalities in cultured tilapia in most low- and middle-income countries is commonly performed using basic assays, including bacterial isolation and biochemical characterization. However, the previously mentioned assays are time-consuming, labor-intensive, and may lead to inconclusive results **(Abbott, Cheung & Janda, 2003)**. In 2019, unknown mortalities of farmed tilapia from three provinces in Egypt were reported causing high economic losses. In the current study, we investigated the potential causes of mortality using microbiological and molecular approaches. In addition, we studied histopathological associated lesions in the diseased tilapia. This study may contribute to our understanding of the newly emerging diseases associated with summer mortality events in cultured tilapia in Egypt and will facilitate setting up control measures for these diseases.

MATERIALS AND METHODS

1. Sampling and clinical examination

The recently died or moribund Nile tilapia (*O. niloticus*) were collected from May to October 2019 (summer season) off 24 tilapia farms from three main fish-producing provinces in Egypt, including, El-Behera, Kafr-Elsheikh, and Port-Said governorates. Twenty-four fish farms from the 3 provinces showed high mortality rates which reached 15- 30%. Stocking density in the screened farms ranged between 15,000- 20,000 fish/ acre, while the weight of the examined

fish was between 10 and 450g. Seventy-two fish were euthanized using an overdose of MS-222 (Syndel, USA) and transported to the aquatic animal medicine laboratory at the faculty of veterinary medicine, Damanhour University, for clinical examination. Upon the arrival of the fish, a thorough examination, necropsy and recording of clinical signs were performed.

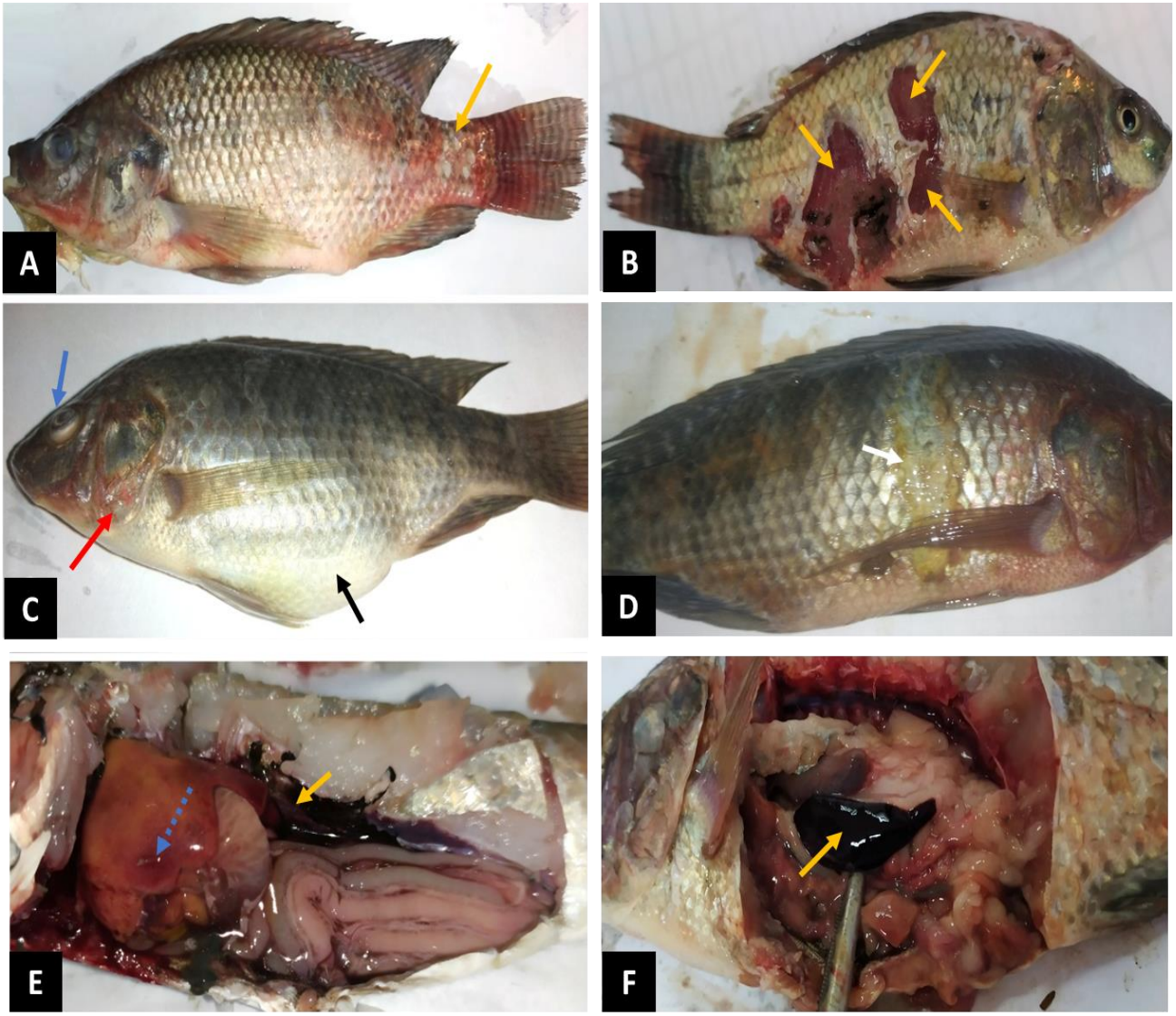

Fig. 1. Clinical signs and necropsy findings of moribund tilapia collected during the summer mortality event from Egypt showing: **(A)** Extensive cutaneous haemorrhages at caudal peduncle and tail fin with scales loss (yellow arrows), **(B)** Deep cutaneous lesions and haemorrhages at muscles (yellow arrows), **(C)** Abdominal distension (black arrow) and exophthalmia (blue arrow) with pustules at the operculum (red arrow), **(D)** Scale loss and discoloration of underlying muscles (white arrow), and **(E, F)** Congested and enlarged spleen (yellow arrows), as well as an enlarged hepatopancreas with haemorrhagic patches on the surface (blue dotted arrow)

2. Water sample

Twenty-four water samples (one sample/ farm) were taken from different sites and analyzed to determine the water-quality parameters, including total ammonia nitrogen (TAN), hardness, and pH **(Alabaster & Lloyd, 2013)**. The analysis was performed using a commercial kit (Tetra, USA) following the manufacturer's instructions.

3. Screening of fish for tilapia lake virus (TiLV) using real-time polymerase chain reaction

3.1 RNA extraction, complementary DNA (cDNA) synthesis

Hepatopancreas and brain were collected under aseptic conditions after the euthanization of seventy- two fish from 24 farms (3 fish/ farm) and fixed in RNAlater (Sigma Aldrich, NC, USA) at 4°C for 24hr before being stored at −80°C till analyzed **(Fathi *et al.*, 2017)**. The RNA was extracted from the hepatic and brain tissues using Viral Gene-spin™ viral DNA and RNA extraction kit (iNtRON Biotechnology, Korea) following the manufacturer's instructions. The total RNA concentration was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, USA), adjusted to 100ng/ µL using molecular grade water (Thermofisher Scientific, GA, USA) and stored at -80°C for further analysis. The cDNA was synthesized using a Qiagen Quantitec cDNA kit® (Qiagen, Valencia, California) following the manufacturer's instructions.

3.2 Real-time quantitative polymerase chain reaction (qPCR)

The qPCR assay was performed in a QuantStudio 5 Real-time PCR system (ThermoFisher, USA) in a 12µL reaction containing 5µL of template cDNA, 7µL of SYBR Green PCR Master Mix (Applied Biosystems, NY, USA) and 1µL of the primers TiLV-112F 5'-CTGAGCTAAAGAGGCAATATGGATT-3′ and TiLV- 112R 5'-CGTGCGTACTCGTTCAGTATAAGTTCT-3′ **(Tattiyapong *et al.*, 2017)**, and RNAse free water (ThermoScientific, GA, USA) up to volume. The cycling conditions were as follows: 1 cycle at 95°C for 10min, 45 cycles of amplification at 95°C for 15s, and annealing at 60°C for 1min. The data were collected during each elongation step. Negative (DEPC-treated water, ThermoScientific, CA, USA) and non-reverse transcriptase controls were included in each run. Each sample was run in triplicate.

2.4 Screening of fish for fungal infections

Samples of skin were collected under aseptic conditions from freshly euthanized fish (3 fish/ farm) under aseptic conditions and cultured on Sabaroud dextrose agar (SDA) (Oxoid LTD Laboratories, Hampshire, UK) and incubated at 28°C for 7 days **(Buller, 2014)**.

2.5 Screening for bacterial infections

Swabs of the posterior kidney, hepatopancreas and spleen of 3 fish per farm were plated on tryptic soy agar (TSA) (Oxoid LTD Laboratories, Hampshire, UK) and incubated at 28°C for 24- 48hr. Dominant colonies were subcultured on TSA plates and incubated at the same condition described above. Morphological characterization, including colony morphology and

Gram staining and biochemical tests, including cytochrome oxidase, catalase and triple sugar iron test, were performed following the methods described by **Abbott *et al.* (2003)** and **Austin *et al.* (2012)**. Pure bacterial colonies were transferred to brain heart infusion broth (BHI, Oxoid LTD laboratories, UK), incubated at 28°C for 24hrs with shaking at 150rpm, followed by storing in 20% glycerol at −80°C **(Rodger, 2010)**.

2.6 Molecular identification of bacterial pathogens

2.6.1 DNA extraction

The DNA was extracted from pure single colonies by PureLink™ Genomic DNA Mini Kit (Invitrogen™, MA, USA), according to the manufacturer's instructions. The final DNA was eluted in 100µL of elution buffer and stored at 4°C till analyzed.

2.6.2 PCR amplification and sequencing

Colonies that were tentatively identified as *Aeromonas* sp. and *Streptococcus* sp. by morphological and biochemical tests were further screened by conventional PCR using primers listed in Table (1). The PCR assays for both pathogens were run in a 50µL reaction containing 25µL of 2x DreamTaq Green PCR Master Mix (Thermo Scientific™, UK), 1µL of each primer, 20µL of PCR RNase-free water, and 3µL of DNA template.

For *Aeromonas* sp., PCR conditions were as follows: initial denaturation for 3min at 95°C, 35 cycles of denaturation for 30s at 95°C, annealing for 35s at 60°C and an extension at 72°C for 1min, and a final extension at 72°C for 5min. For *Streptococcus* sp., PCR conditions were as follows: initial denaturation for 3min at 95°C, 35 cycles of denaturation for 30s at 95°C, annealing for 35s at 50°C, and an extension at 72°C for 1min, and a final extension at 72°C for 5min.

The amplified PCR products were separated by electrophoresis on 1.5% agarose gel (Thermo Scientific™, UK) and visualized using TVG-SYS- Vari gel MIDI documentation SYSTEM (Sci-Plas Ltd, UK). The PCR products were sent for sanger sequencing to Color Lab, Cairo, Egypt using the same primers used for PCR assays. The identification and sequence homology percentages of the different sequences were performed by using the basic local alignment search tool (BLASTn-suit) that is available from the National Center for Biotechnology Information (https://bla st.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn& PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

Table 1. Primer used for identification of the recovered bacterial pathogens using PCR

Name of primer	Primer sequence	Amplicon size	Reference
gyrB -F	5′-GCCGAGCCCGACCATCTTCAG-3′	875bp	**(Zhang *et al.*, 2014)**
gyrB -R	5′-AGATCATCTTGTCGAAACGGGC-3′		
Sin 1	5′-CTAGAGTACACATGTAGCTAAG-3´	300bp	**(Zlotkin *et al.*, 1998)**
Sin 2	5′-GGATTTTCCACTCCCATTAC-3´		

2.7 Antimicrobial susceptibility

Antimicrobial susceptibility testing was performed by disc diffusion method on Mueller-Hinton's agar following the method described by **Eissa (2016)**. The following antibiotics discs (Oxoid, UK) were tested, including ampicillin (10µg), amoxicillin (10µg), ciprofloxacin (5µg), enrofloxacin (5µg), doxycycline (30µg), oxytetracycline (30µg), streptomycin (10µg), erythromycin (15µg), spectinomycin (10µg), neomycin (30µg), levofloxacin (5µg), amikacin (30µg), lincomycin (10µg), erythromycin (15µg), and spiramycin (100µg).

2.8 Histopathological examination

Samples from the hepatopancreas, posterior kidney, spleen, brain and gills of the diseased fish were collected for histopathological examination. Tissues were initially fixed in Davidsons's fixative solution for 24hr at room temperature (RT, ~25°C) and then incubated at a series of gradient ethanol concentrations before processing and staining with Hematoxylin and Eosin (H&E, Sigma, CA, USA), following the protocol of **Roberts (2012)**.

RESULTS

1. Clinical findings and post-mortem lesions

The moribund fish showed exophthalmia with ocular haemorrhages, skin lesions that ranged from detached scales to skin ulcers of variable depth, pustules on the operculum, hemorrhagic areas on the skin surface and at fin bases, redness on the caudal peduncle, and abdominal dropsy (Fig. 1A- D). Internally, fish revealed accumulation of bloody ascitic fluids in the abdominal cavity, congestion of the gills and spleen, enlargement, paleness, and appearance of petechial haemorrhages on the hepatopancreas, distended gall bladder, enlargement and congestion of spleen, as well as posterior kidney and brain (Fig. 1E, F).

2. Water analysis

The water quality of samples collected from the different farms was within the normal range. The average values were as follows: general hardness was 180± 10ppm; carbonate hardness was 240± 20ppm; pH was 8± 0.5, NO_2 was 0+ 1ppm, and NO_3 was 0+ 1ppm, except for TAN which was higher than normal in some farms (reach 6ppm). The water temperature was around 25- 30± 2°C.

3. Viral and fungal screening

All hepatopancreas and brain samples collected from the diseased tilapia tested negative for TiLV by RT-qPCR. In addition, no fungal growth was observed on SDA from all inoculated samples.

4. Bacterial isolation and identification

Morphologically, the inoculated samples showed round, convex or smooth, shiny, and yellowish opaque, creamy or whitish, flat or pin-point colonies on the TSA plates. Gram-stained

smears of the dominant colonies showed two types of bacteria. The first type was Gram-negative, rod-shaped bacteria (the flat, yellowish opaque colonies), while the other type was Gram-positive cocci arranged in pairs and chains (pin-point round smooth colonies). The biochemical tests' results are summarized in Table (2).

The PCR amplification of the *gyrB* gene of the tentative *Aeromonas* sp. resulted in an amplicon of 875bp (data not shown). The sequencing of the PCR amplicon showed that the isolates shared an average nucleotide identity (ANI%) of 99.71% to *A. veronii* (n= 2), 99.80% to *A. enteropelogenes* (n= 1), 99.90% to *A. hydrophila* subsp. *dhakensis* (n= 1), and 99.80% to *A. sobria* (n= 1). For suspected *Streptococcus* sp., PCR amplification of the 16s rRNA gene showed a 300bp band (Data not shown), and sequencing of this amplicon showed that the isolates are 100% similar to *S. iniae* (n= 3).

Table 2. Biochemical characterization of dominant bacterial cultures recovered from diseased tilapia from 3 provinces in Egypt

Province	Farm	Isolation period	No. of fish sampled	Average weight (g)	Viral Isolation	Fungal isolation	Morphological and biochemical chatacterisation					PCR and sequencing result
							G	Cat	Ox	H_2S	TSI	
Kafr-Elsheikh	1	5/2019	3	75 – 100	-ve	-ve						
Kafr-Elsheikh	2	6/2019	3	45 -60	-ve	-ve						
Kafr-Elsheikh	3	6/2019	3	80 - 100	-ve	-ve	-	+	+	+	A/C	*A. veronii*
Port Said	4	6/2019	3	40 -50	-ve	-ve						
Kafr-Elsheikh	5	6/2019	3	35 - 60	-ve	-ve						
Port Said	6	6/2019	3	70 - 95	-ve	-ve	+	-	-	-	-	*S. iniae*
Kafr-Elsheikh	7	7/2019	3	80 - 120	-ve	-ve						
Kafr-Elsheikh	8	7/2019	3	100 - 155	-ve	-ve						
El-Behera	9	7/2019	3	85 -140	-ve	-ve						
Port Said	10	7/2019	3	165 - 200	-ve	-ve	+	-	-	-	-	*S. iniae*
Port Said	11	7/2019	3	85 -105	-ve	-ve						
Port Said	12	7/2019	3	80 – 95	-ve	-ve						
El-Behera	13	8/2019	3	100 -140	-ve	-ve	-	+	+	-	-	*A. enteropelogenes*
Kafr-Elsheikh	14	8/2019	3	140 - 220	-ve	-ve						
Port Said	15	8/2019	3	90 -130	-ve	-ve						
Port Said	16	8/2019	3	340 - 430	-ve	-ve	-	+	+	+	A/C	*A. sorbia*
Port Said	17	8/2019	3	180 - 230	-ve	-ve						
Port Said	18	8/2019	3	210 - 290	-ve	-ve						
Kafr-Elsheikh	19	9/2019	3	200 – 310	-ve	-ve	-	+	+	+	A/C	*A. veronii*
Port Said	20	9/2019	3	165 - 200	-ve	-ve						
Port Said	21	9/2019	3	150 - 210	-ve	-ve						

Kafr-Elsheikh	22	9/2019	3	180 - 250	-ve	-ve	+	-	-	-	-	*S. iniae*
Kafr-Elsheikh	23	10/2019	3	200 - 250	-ve	-ve						
Kafr-Elsheikh	24	10/2019	3	340-400	-ve	-ve	-	+	+	+	A/C	*A. hydrophila* subsp. *dhakensis*

Cat= Catalase test, Ox= Oxidase test, H_2S = H_2S production, TSI= Triple sugar iron, A/C= Alkaline/acid, G= Gram stain.

5. Antimicrobial susceptibility

The *in-vitro* antimicrobial susceptibility test on *Aeromonas* isolates showed that all isolates tested were susceptible to levofloxacin, amikacin, and neomycin and were also moderately susceptible to ciprofloxacin, enrofloxacin, and neomycin except *A. veronii*. *Aeromonas* isolates that were resistant to amoxicillin, ampicillin, oxytetracycline, spectinomycin, erythromycin, and lincomycin. On the other hand, *S. iniae* isolates were moderately susceptible to enrofloxacin, ciprofloxacin, doxycycline, and amikacin, while resistant to penicillin-derived antibiotics. Both *Aeromonas* sp. and *S. iniae* isolates were resistant to oxytetracycline. The results are listed in Table (3).

Table 3. The antimicrobial resistance of the bacterial isolates recovered in this study

Antibiotic	***A. hydrophila subsp. dhakensis***	***A. veronii***	***A. sobria***	***A. enteropelogens***	***S. iniae***
Amoxicillin	R	R	R	R	R
Ampicillin	R	R	R	R	R
Ciprofloxacin	I	R	I	S	I
Enrofloxacin	I	R	I	I	I
Streptomycin	R	R	R	R	R
Oxytetracycline	R	R	R	R	R
Doxycycline	S	R	S	I	I
Spectinomycin	R	R	R	R	R
Neomycin	S	R	I	R	R
Levofloxacin	S	I	I	S	R
Amikacin	I	I	I	R	I
Lincomycin	R	R	R	R	R
Erythromycin	R	R	R	R	R
Spiramycin	R	R	R	R	R

S= Susceptible, R= Resistant, I= Intermediate.

For *S. iniae (*R = Resistant ≤ 15mm, I = Intermediate 16- 21mm, S= Sensitiv ≥ 24mm) **(CLSI & CLSI, 2014)**, and *Aeromonas* spp. **(**R = Resistant≤ 13mm, I = Intermediate 14- 18mm, S= Sensitive≥ 19mm) **(Samal *et al.*, 2014)**.

6. Histopathological results

Fish infected with *A. hydrophila* showed various septicemic lesions, where the hepatopancreas showed severe diffuse degenerative and necrotic changes in both hepatic and pancreatic acinar tissues with severe congestion of main hepatic blood vessels (Fig. 2A). The posterior kidney also displayed severe diffused renal interstitial and glumerulo-tubular necrotic changes (Fig. 3A), and the spleen showed diffused congestion and moderate reduction of the white pulp and focal activation of melano-macrophage centres (MMCs) (Fig. 4A). The gills showed moderate diffused hyperplasia at the base of secondary lamellae and epithelial lining separation and occasional clubbing of the primary gill filaments (Fig. 5A). Similarly, fish infected with *A. veronii* were represented with moderate diffused hepatocytic vacuolar degeneration and severe congestion of main hepatic blood vessels and sinusoidal spaces (Fig. 2B). The kidney showed focal renal interstitial haemorrhages (Fig. 3B), while the spleen showed diffused congestion and multifocal necrotic areas of the white pulp (Fig. 4B). In contrast, gills showed moderate diffused hyperplasia at the base of secondary lamellae (Fig. 5B). For fish infected with *A. sobria*, the hepatopancreas showed moderate diffuse hepatocytic vacuolar degeneration and severe congestion of main hepatic blood vessels and sinusoidal spaces in addition to the activation of MMCs adjacent to pancreatic acini (Fig. 2C). While, the kidneys showed severe interstitial haemorrhages and diffuse renal interstitial and glumerulo-tubular necrotic changes (Fig. 3C). The **s**pleen showed diffused congestion and mild reduction of the white pulp and diffused solitary distribution of melanomacrophage cells (Fig. 4C), whereas gills showed mild diffused hyperplasia at the base of secondary lamellae and separation of the epithelial lining (Fig. 5C). Fish infected with *A. enteropelogens* showed moderate diffuse hepatocytic vacuolar degeneration and congestion of main hepatic blood vessels and sinusoidal spaces (Fig. 2D), and severe diffuse renal interstitial and glumerulo-tubular degenerative and necrotic changes, with multifocal areas of necrosis (Fig. 3D). In addition, the spleen displayed multifocal necrotic areas, diffuse congestion, white pulp depletion and focal activation of MMCs (Fig. 4-D). Furthermore, gills showed a progressive hyperplasia at the base of secondary lamellae and clubbing of some primary gill filaments (Fig. 5D).

Fish that were infected with *S. iniae* showed severe diffuse hepatocytic degeneration and multifocal circumscribed areas of necrosis, with severe congestion of main hepatic blood vessels (Fig. 6A). The posterior kidney showed severe diffused renal interstitial and glumerulo-tubular necrotic changes and diffuse interstitial haemorrhages (Fig. 6B). The spleen displayed moderate congestion and multifocal areas of white pulp depletion and pronounced activation of MMCs (Fig. 6C). The examination of the gill revealed diffused moderate hyperplasia at the base of secondary lamellae (Fig. 6D), while the brain revealed congestion of cerebral and meningeal blood vessels, which were surrounded with mononuclear cell infiltrations (perivascular cuffing) and occasional focal areas of vacuolation (Fig. 6E, F).

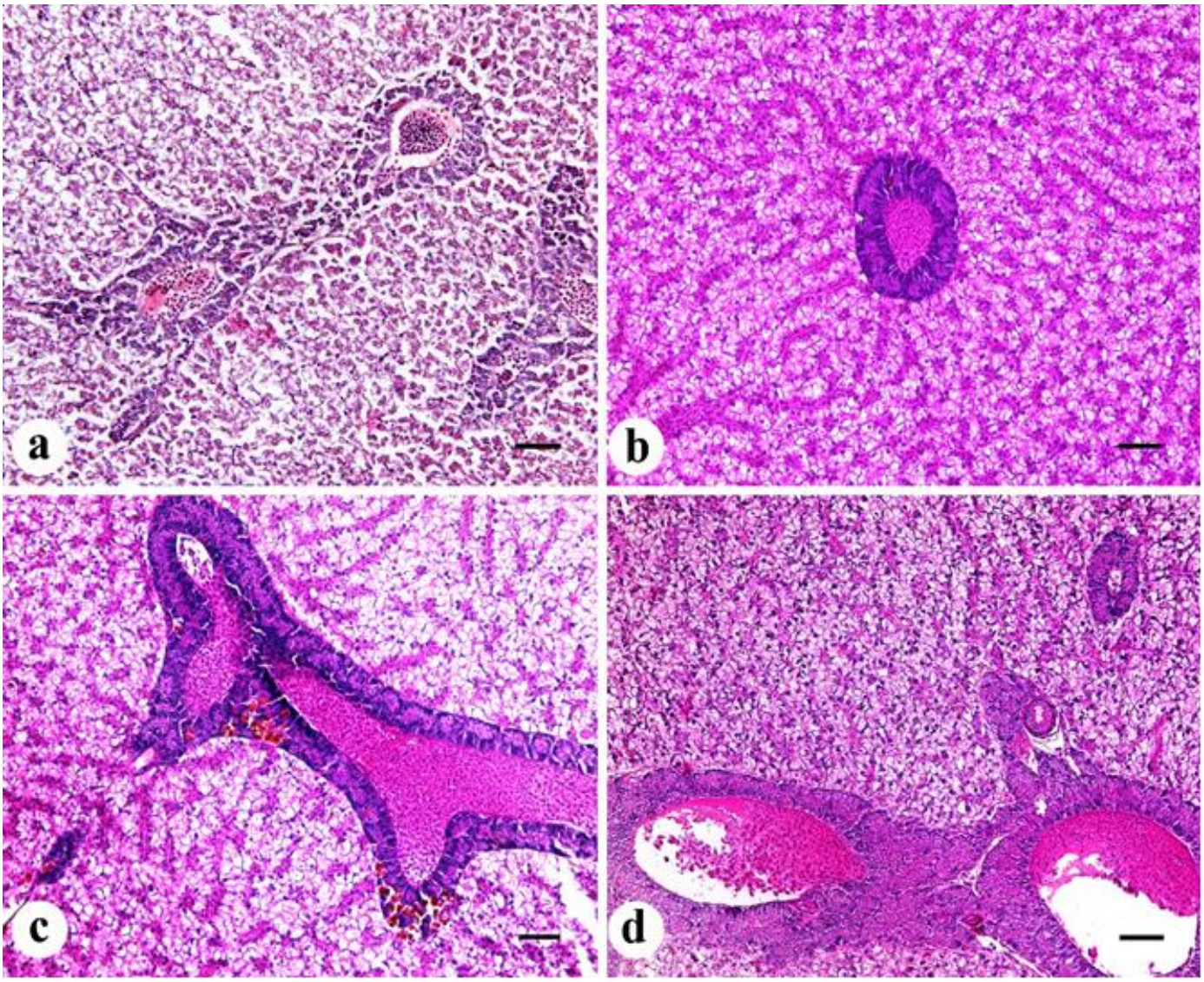

Fig. 2. Hepatopancreas of *O. niloticus* naturally infected with: **(a)** *A. hydrophila* showing severe diffused degenerative and necrotic changes in both hepatic and pancreatic acinar tissues with severe congestion of main hepatic blood vessels, **(b)** *A. veronii* showing moderate diffuse hepatocytic vacuolar degeneration and severe congestion of main hepatic blood vessels and sinusoidal spaces, **(c)** *A. sobria* showing moderate diffuse hepatocytic vacuolar degeneration and severe congestion of main hepatic blood vessels and sinusoidal spaces and activation of MMCs adjacent to pancreatic acini, and **(d)** *A. enteropelogens* showing moderate diffuse hepatocytic vacuolar degeneration and congestion of main hepatic blood vessels and sinusoidal spaces. H&E stain, bar = 50µm

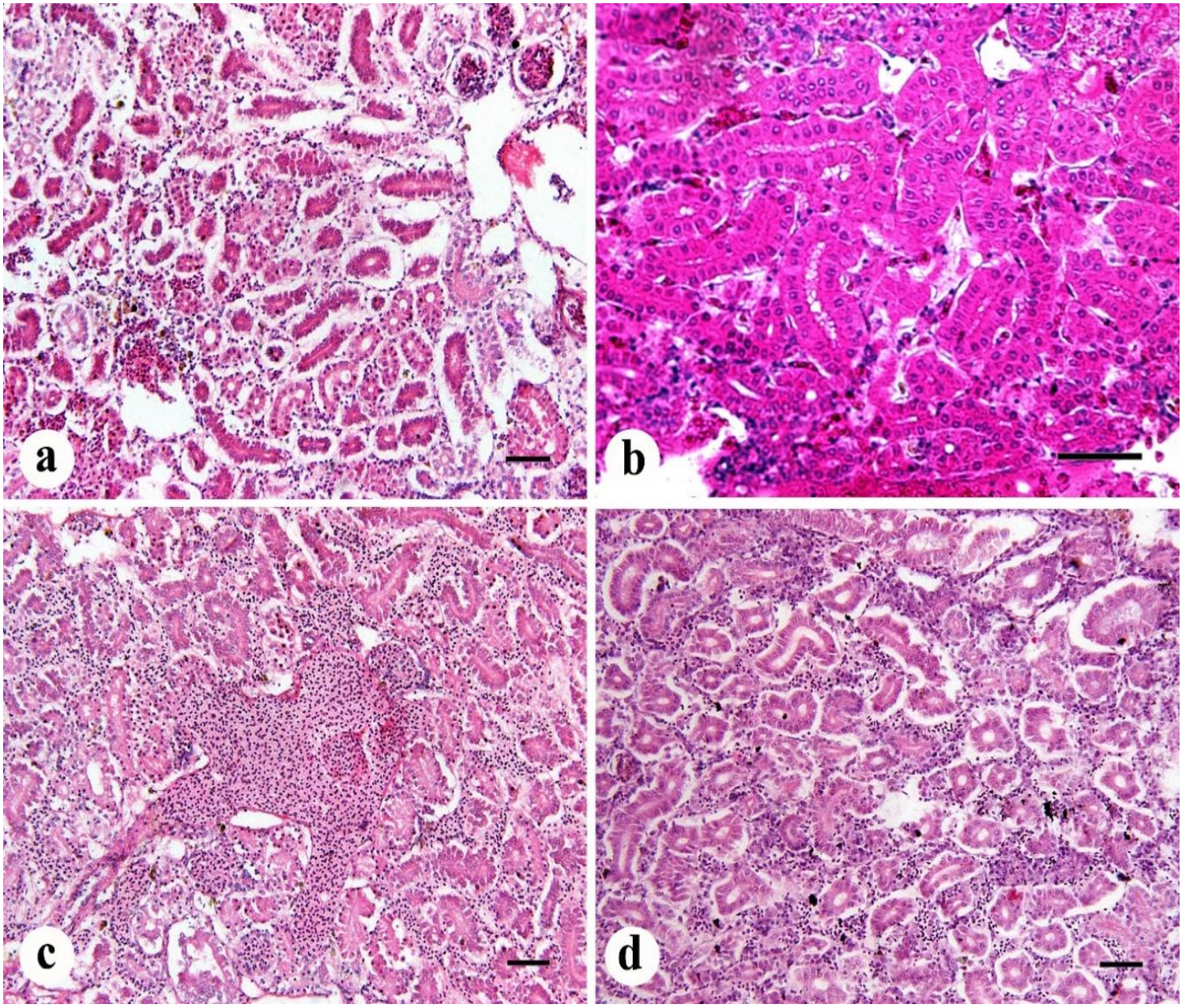

Fig. 3. Posterior kidney of *O. niloticus* naturally infected with: **(a)** *A. hydrophila* showing severe diffused renal interstitial and glumerulo-tubular necrotic changes, **(b)** *A. veronii* showing focal renal interstitial haemorrhages, **(c)** *A. sobria* showing severe interstitial haemorrhages and diffuse renal interstitial and glumerulo-tubular necrotic changes, and **(d)** *A. enteropelogens* showing severe diffuse renal interstitial and glumerulo-tubular degenerative and necrotic changes, with multifocal areas of necrosis. H&E stain, bar = 50µm

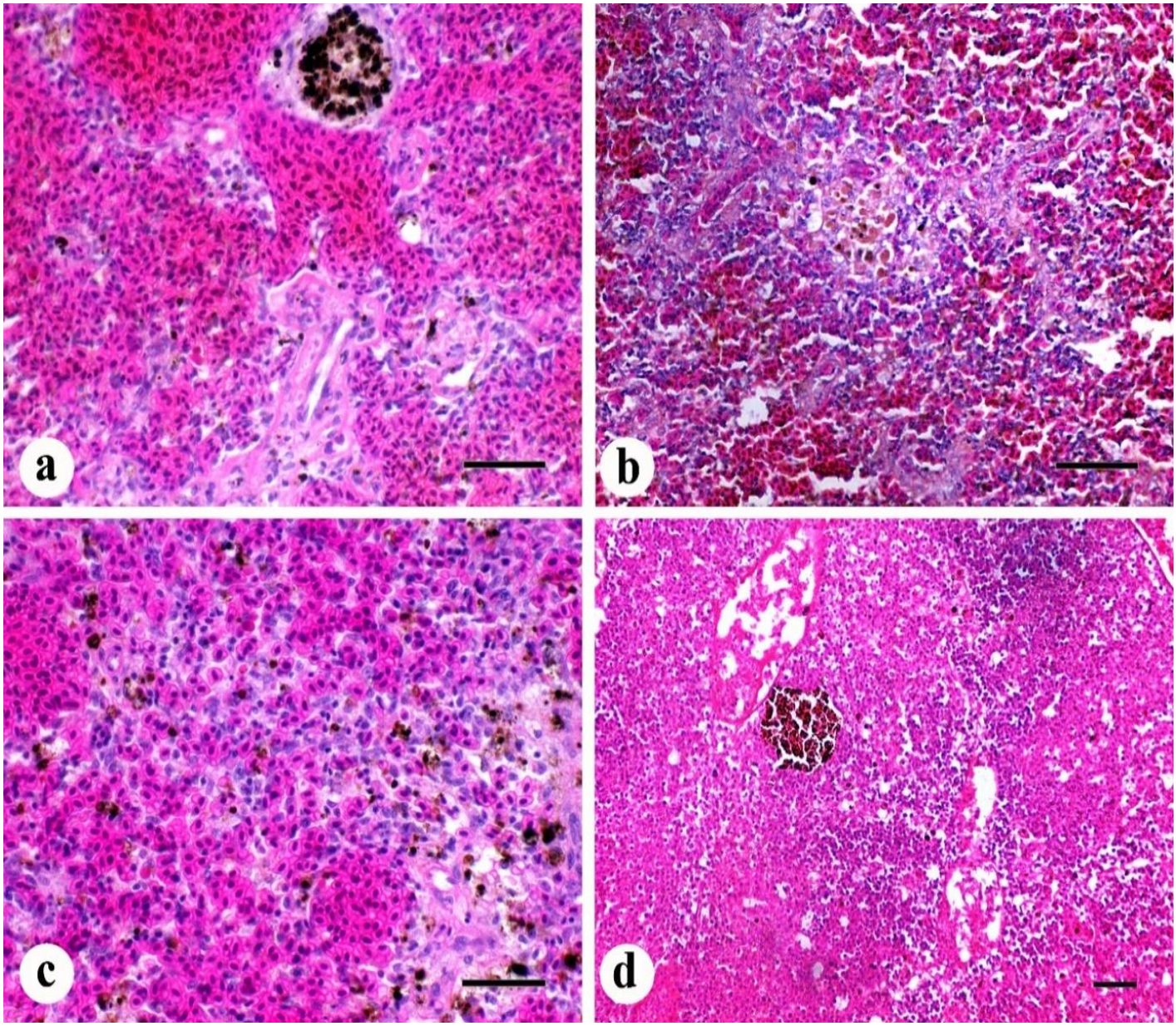

Fig. 4. Spleen of *O. niloticus* naturally infected with: **(a)** *A. hydrophila* showing diffuse congestion and moderate reduction of the white pulp and focal activation of MMCs, **(b)** *A. veronii* showing diffuse congestion and multifocal necrotic areas of the white pulp, **(c)** *A. sobria* showing diffuse congestion and mild reduction of the white pulp and diffuse solitary distribution of melano-macrophage cells, and **(d)** *A. enteropelogens* showing multifocal necrotic areas (arrows), diffuse congestion, white pulp depletion and focal activation of MMCs. H&E stain, bar = 50µm

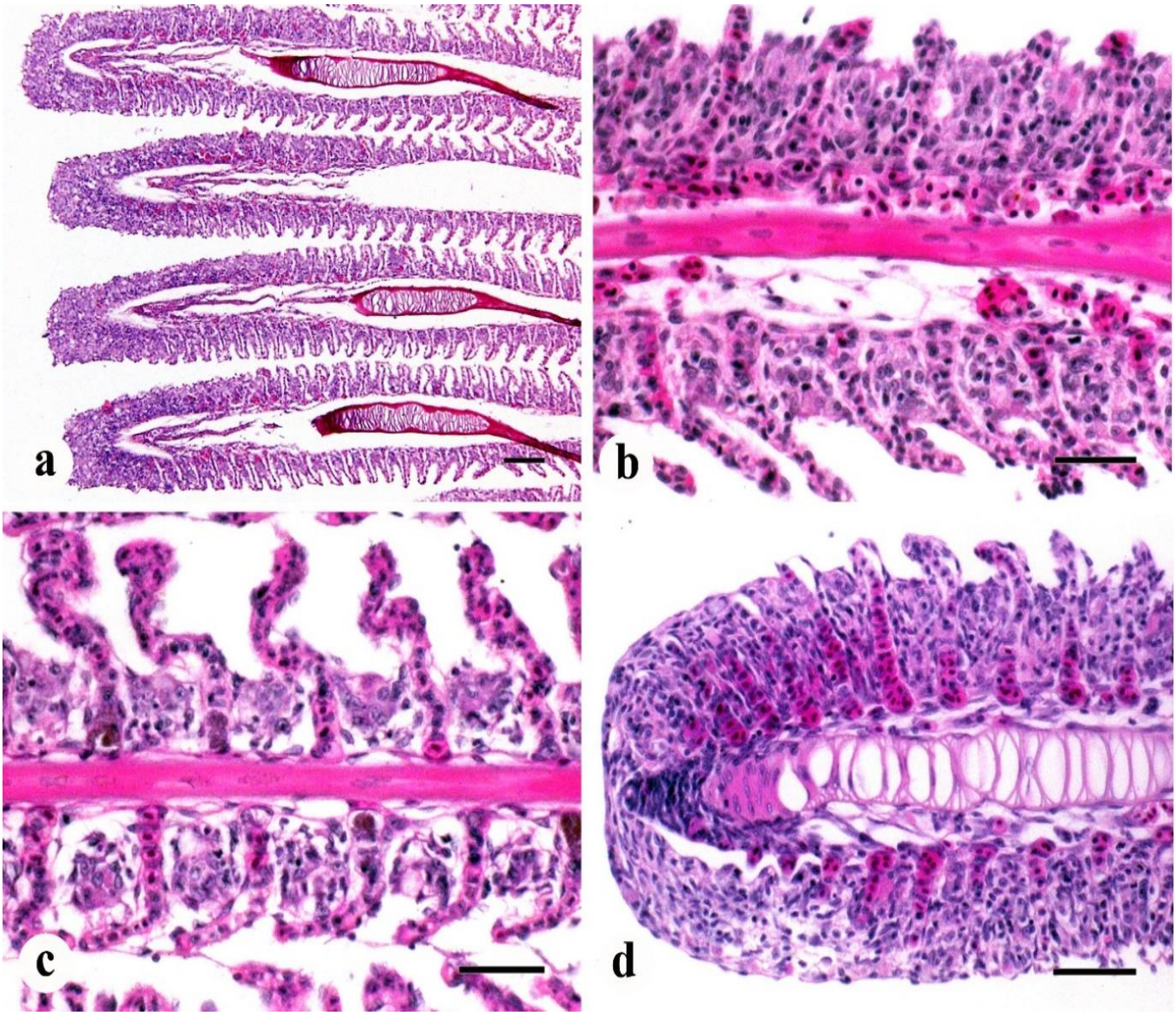

Fig. 5. Gills of *O. niloticus* naturally infected with: **(a)** *A. hydrophila* showing moderate diffuse hyperplasia at the base of secondary lamellae and epithelial lining separation and occasional clubbing of the primary gill filaments, **(b)** *A. veronii* showing moderate diffuse hyperplasia at the base of secondary lamellae, **(c)** *A. sobria* showing mild diffuse hyperplasia at the base of secondary lamellae and epithelial lining separation, and **(d)** *A. enteropelogens* showing progressive hyperplasia at the base of secondary lamellae and clubbing of the end of the primary gill filament. H&E stain, bar = 50µm

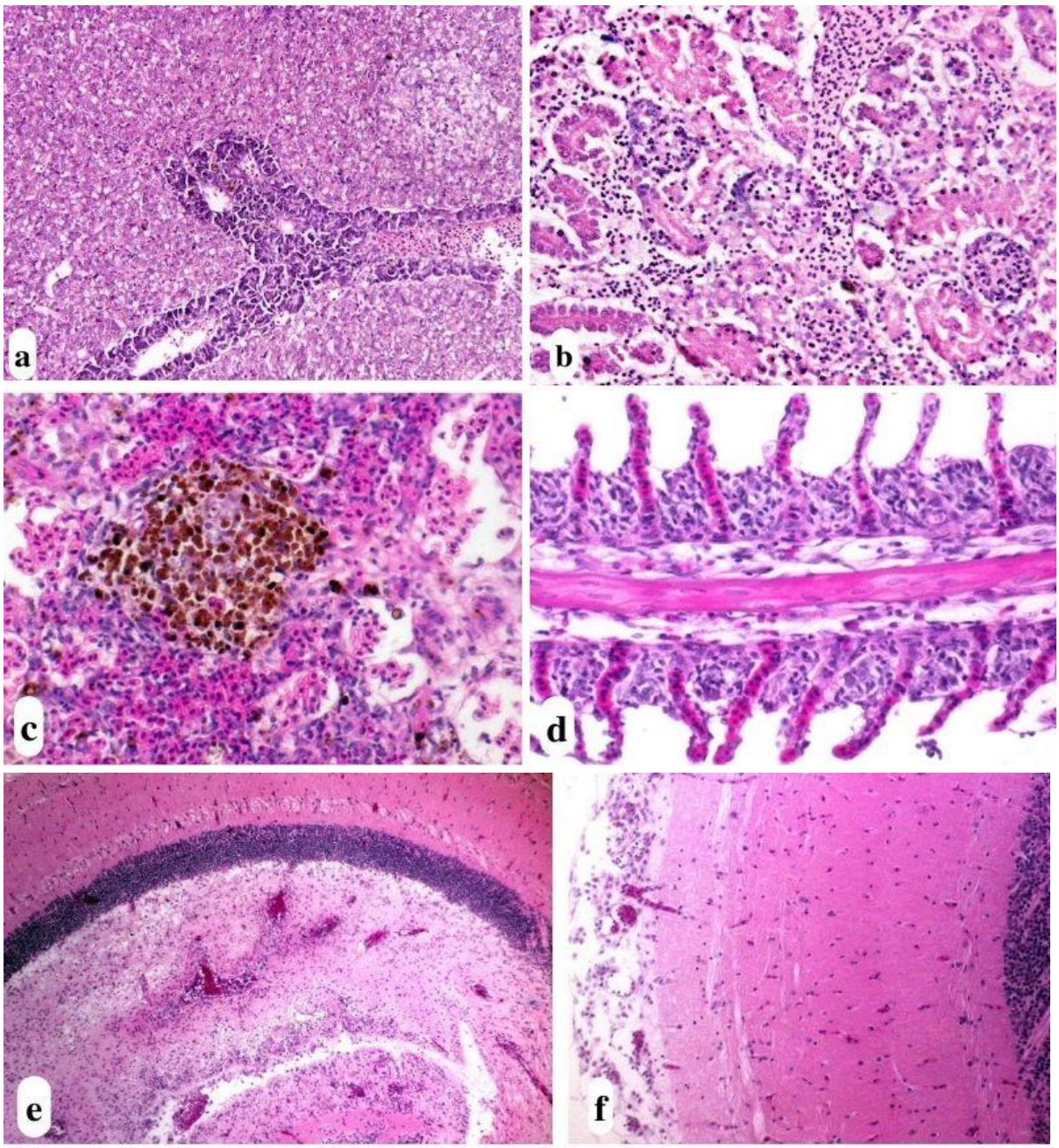

Fig. 6. Histopathological findings of *S. iniae* infection in *O. niloticus* showing: **(a)** Hepatopancreas showing severe diffuse hepatocytic degeneration and multifocal circumscribed areas of necrosis with severe congestion of main hepatic blood vessels (×200), **(b)** Posterior kidney showing severe diffuse renal interstitial and glumerulo-tubular necrotic changes and diffuse interstitial haemorrhages (×400), **(c)** Spleen showing moderate congestion and multifocal areas of white pulp depletion and pronounced activation of MMCs (×400), **(d)** Gill filaments showing diffuse moderate hyperplasia at the base of secondary lamellae (×400), **(e)** Brain showing congestion of cerebral blood vessels which are surrounded with mononuclear cell infiltrations (perivascular cuffing) with occasional focal areas of vacuolation (×200), and **(f)** Brain showing congestion of the meningeal blood vessels with mononuclear cell infiltrations (perivascular cuffing) in their vicinity(×200)

DISCUSSION

The aquaculture is the fastest-growing sector of food production around the world **(Tacon, 2020)**. Egypt ranks at the third level of the global Nile tilapia (*O. niloticus*) production, while it ranks first in northern Africa **(FAO, 2020)**.

Changes in the aquaculture environment such as polluted water or overstocking induce stressful conditions, affecting the fish health and triggering the occurrence of diseases **(Troell *et al.*, 2017)**. The occurrence of infectious or non-infectious diseases in fish farms has an impact on the net profit due to the economic losses from the treatment costs and fish mortalities **(Rodger, 2016)**. Water quality affects the health, immunity, survival, and production of the cultured fish. Any changes in water quality affect the physiological functions and immune responses of the fish which leads to an increase in their susceptibility to diseases **(Barton, 2002)**.

In the present study, there was a moderately significant elevation of ammonia levels in the examined farms. The elevation of ammonia levels is negatively correlated to the fish growth rate, performance, and disease resistance. The condition may progress to lethal effects, especially at higher temperatures and pH, that induce fish mortality **(Levit, 2010; Shin *et al.*, 2016)**.

Since 2013, many Egyptian tilapia farms have suffered from high fish mortalities during the summer seasons, resulting in what is called "summer mortality syndrome" **(WorldFish, 2015)**. While many studies have been performed to investigate this phenomenon, a definitive cause of mortality has not been obvious till date.

In the current study, fish specimens were collected from 24 tilapia farms located in 3 governorates: El-Behera, Kafr El-Sheikh and Port Saied in the course of high fish mortalities episodes during the summer season to investigate the aetiologies of mortalities. The organs collected for viral sampling were the hepatopancreas and brain of moribund fish **(Fathi *et al.*, 2017)**. **Fathi *et al.* (2017)** recorded the detection of TiLV in the Egyptian farms by rRT-PCR but failed to isolate the virus from the tissues.

Viral screening by qPCR showed negative results. It is worse to mention that the samples submitted for viral screening were screened three times not just in our laboratory but also in an external lab to rule out the TiLV detection and all data from both labs were negative. Thus, we hypothesize that TiLV is not associated with the current summer mortality episodes. In a previous study, TiLV was detected in tilapia farms in Egypt by qPCR, however, recovering the virus on culture media was not successful **(Fathi *et al.*, 2017)**. The inconsistency between our finding and the previous report in Egypt regarding the existence of the TiLV in Egyptian farmed tilapia warranted the necessity of performing extensive TiLV screening of farmed tilapia populations from different provinces at different stages of the production cycle.

In the current study, disease investigation showed that *Aeromonas* sp., including *A. veronii, A. enteropelogenes, A. hydrophila* subsp. *dhakensis, A. sorbia,* and *S. iniae* were the main

pathogens recovered from the screened tilapia in the three main tilapia-producing provinces in Egypt, Port-Said, El-Behera, and Kafr El-Sheikh. This finding is in agreement with previous studies which suggested that *Aeromonas* spp. and *S. iniae* are some of the most common causes of bacterial diseases affecting cultured tilapia in Egypt and highlighted their significant contribution to the mass mortalities during the summer season in recent years **(Aly, 2013; Elkemary *et al.*, 2019; Abdel-Latif *et al.*, 2020; Abdel-Latif & Khafaga, 2020)**.

In the present study, the clinical signs of diseased fish were loss of reflexes, exophthalmia, detached scales with pale skin patches, haemorrhagic areas on the skin surface and at fin bases, ulcerations, and abdominal dropsy. In addition, post-mortem lesions were congested posterior kidney, congested and enlarged spleen with haemorrhagic spots on the hepatopancreas. These results are similar to those recorded by previous research describing *Aeromonas* sp. infection **(Austin *et al.*, 2012; Aboyadak *et al.*, 2015; Dahdouh *et al.*, 2016)**. The identification of *Aeromonas* and *Streptococcus* isolates was based on morphological characterization and biochemical identification and the confirmation through molecular analysis and sequencing.

Aeromonas isolates gave distinct bands at 875bp by conventional PCR, and sequencing results identified isolates as *A. veronii*, *A. enteropelogenes*, *A. hydrophila* subsp. *dhakensis*, and *A. sobria*. These results were in accordance with the results of **Abbott *et al.* (2003)**, **Yanez *et al.* (2003)** and **Zhang *et al.* (2014)**. While, *Streptococcus* isolates gave bands at 300bp, and sequencing confirmed that the isolates are *S. iniae*. This result matches with the findings of **Zlotkin *et al.* (1998)**, **Baums *et al.* (2013)** and **Younes *et al.* (2019)**.

The bacterial invasion, replication, and production of different forms of the disease depend on the virulence factors and pathogenicity of the pathogen **(Vilches *et al.*, 2004)**. It is suggested that there is a strong relationship between the virulence factors of *A. hydrophila* and the presence of specific virulence genes, such as aerolysin, enterotoxin and protease **(Li *et al.*, 2011)**. Aerolysin and haemolysin induce cytotoxic and haemolytic activities leading to tissue destruction and haemorrhage leading to fish moralities **(Oliveira *et al.*, 2012)**. Protease, lipase, elastase, and collagenase are important exoenzymes that alter the structure of the cytoplasmic membrane of the host cells, therefore facilitating the bacterial colonization and necrosis of the tissues **(Nawaz *et al.*, 2010; Rasmussen-Ivey *et al.*, 2016)**. It is frequently reported that the posterior kidney is one of the most affected target organs by bacterial acute septicaemia, which is largely supported in the present study. These pathological changes may be attributed to various toxins produced by the invading bacteria **(Abdelhamed *et al.*, 2017)**.

A. hydrophila subsp. *dhakensis* caused remarkable lesions in various fish organs, such as the congestion of all parenchymatous organs, especially hepatopancreas, spleen, posterior kidney and gills, degenerative and necrotic changes in various tissues, and proliferative changes and the hyperplasia of the gills. Similar to the present findings, **AlYahya *et al.* (2018)** observed that the most affected organs were the hepatopancreas and posterior kidney, followed by the intestine and heart, and denoted that the profuse hepatic congestion indicated that the hepatopancreas is

possibly the target organ for haemocyte aggregation and bacterial pathogenesis. In addition, these authors found various haemorrhages in the interstitial tissues of visceral organs and some skin haemorrhages on the ventral surface of the body and anal regions. **Locke *et al.* (2007)** mentioned that β-haemolysin is a critical toxin that takes part in the pathogenesis of *S. iniae* infection, leading to the destruction of RBCs causing haemorrhage of affected tissues.

The histopathological lesions observed in the present study in naturally infected fish by different species of genus *Aeromonas* were similar to those revealed in previous studies **(Abdelhamed *et al.*, 2017; AlYahya *et al.*, 2018; Rosidah *et al.*, 2020)**. As *A. hydrophila* subsp. *dhakensis* and *A. enteropelogens* showed pronounced degenerative and necrotic changes in the hepatopancreas, posterior kidney and spleen tissues that were more profound than other isolated *Aeromonas* spp., while *A. veronii* and *A. sobria*, showed more hyperaemic and haemorrhagic tissue reactions than degenerative and necrotic one. **Hal and El-Barbary (2020)** and **Rosidah *et al.* (2020)** found that the activation of melanomacrophage centres (MMCs), hemosiderin deposition, protein deposits, lymphocyte infiltration, and congestion in various organs are various forms of damage related to the resultant haemorrhage and haemolysis due to *A. hydrophila* infection. Furthermore, hyperplasia of gill lamellar epithelial cells, causing the lamellae to stick together and blend with one another, causes a significant reduction of the active gas exchange surface. **Rahayu *et al.* (2018)** explained that the epithelial necrosis is caused by enzymatic degradation produced by *A. hydrophila*. As a result, fish will suffer from a lack of oxygen due to the deleterious gill condition and the fish's body cannot metabolize and eventually the fish will experience stress and then death. Dissimilar to our results, **Soto-Rodriguez *et al.* (2018)** observed multifocal lymphocytic infiltration in the hepatopancreas and inflammation of the brain with *A. hydrophila* subsp. *dhakensis* infection. Similar to *A. hydrophila*, *A. veronii* caused also remarkable lesions in various fish organs **(Yu *et al.*, 2010; Dong *et al.*, 2017; Raj *et al.*, 2019; Mallik *et al.*, 2020)**.

Dissimilar to our findings, **Dong *et al.* (2017)** observed a severe congestion in the brain tissue. The degree of damage observed in different organs due to various bacterial septicaemia was significantly dissimilar, this may be attributed to the specimens being collected from natural bacterial outbreaks, compared to the reviewed artificial infection experiments. Furthermore, there was no exact information about the time course of the disease or the associated stress parameters or their amplitude. In the present work, *A. hydrophila* subsp. *dhakensis* and *A. enteropelogens* showed pronounced degenerative and necrotic changes in the hepatopancreas, posterior kidney and spleen tissues that were more profound than other isolated *Aeromonas* spp., while *A. veronii* and *A. sobria* showed more hyperaemic and haemorrhagic pictures than degenerative and necrotic pictures in comparison with *A. hydrophila* subsp. *dhakensis* and *A. enteropelogens*.

The histopathological lesions observed in fish naturally infected with *S. iniae* are similar to those described in the studies of **Baums *et al.* (2013)**, **Saleh *et al.* (2019)** and **Dönmez and Cengizler (2020)**. The hepatopancreas of fish naturally infected with *S. iniae* showed a severe

diffuse hepatocytic degeneration and multifocal necrotic areas with a severe congestion of main hepatic blood vessels. Similar to these findings, **Saleh *et al.* (2019)** observed that the hepatopancreas had obvious diffused hepatic cell vacuolation and necrosis, leukocytic infiltration, nuclear fragmentation and haemorrhages. The posterior kidney showed severe diffuse renal interstitial and glumerulo-tubular necrotic changes and diffuse interstitial haemorrhages. In this study, the posterior kidney showed severe diffuse renal interstitial and glumerulo-tubular necrotic changes and diffuse interstitial haemorrhages. **Saleh *et al.* (2019)** found that the posterior kidney suffered from vacuolations of tubular epithelial cells, shrinkage of glomerular taught, increasing Bowman's space and haemorrhage. **Chen *et al.* (2007)** observed the accumulations of eosinophilic material in the cytoplasm of the tubular cells, the nuclei displaced to the side, and melanomacrophage centers were more evident in the head kidneys. These destructive changes in the hepatopancreas and kidney may be attributed to the effect of circulating bacterial toxins **(Dewi *et al.*, 2015)**. In this study, the brain revealed congestion of cerebral and meningeal blood vessels which are surrounded by mononuclear cell infiltrations with occasional focal areas of vacuolation. These findings are similar to those described by **Baums *et al.* (2013)** in addition to the presence of macrophages which were the dominating immune cells in the lesions of encephalitis and meningitis, and necrosis occurred in all four regions of the brain. Furthermore, the spleen of fish naturally infected with *S. iniae* in this study displayed a moderate congestion and multifocal areas of white pulp depletion and pronounced activation of MMCs, which coincides with the findings of **Chen *et al.* (2011)**, who observed the same alterations in the spleen of channel catfish infected with *S. iniae.* The gills pathology in this study exhibited a diffuse moderate hyperplasia at the base of secondary lamellae. **Dönmez and Cengizler (2020)** elucidated that the affected gills showed intense lymphocyte and macrophage infiltrations, epithelial cell separation and lamellar fusion with oedema, lamellar curling and aneurysms.

The utilization of antimicrobial compounds in cultured tilapia farms is accounted as a preventive measure for controlling the propagation and spread of bacterial infections **(Austin *et al.*, 2012; Zamri-Saad *et al.*, 2014)**. In this study, the results obtained from the *in vitro* antibiotic sensitivity test on *Aeromonas* isolates indicate that they were sensitive to levofloxacin, and amikacin, but resistant to amoxicillin, ampicillin, streptomycin, oxytetracycline, spectinomycin, erythromycin, lincomycin and spiramycin. The species differ in their sensitivity to ciprofloxacin, enrofloxacin, doxycycline, and neomycin. These findings are in agreement with those of **Dahdouh *et al.* (2016)** and **M. Mukwabi *et al.* (2019)**, who found that the *Aeromonas* isolates were sensitive to levofloxacin, and amikacin. However, these results disagree with the findings of **Samal *et al.* (2014)** who reported that, *Aeromonas* isolates were sensitive to amoxicillin and streptomycin. These previous studies showed that *Aeromonas* isolates were sensitive to oxytetracycline, while the isolated bacteria were resistant to oxytetracycline and the commonly used antibiotics, despite being moderately or highly sensitive to doxycycline. It may be resulting from the extensive misuse application of oxytetracycline in fish farms. The research papers

confirmed that the use of oxytetracycline did not correlate or develop resistance toward doxycycline **(Grossman, 2016)**.

The results of antimicrobial sensitivity of *S. iniae* showed their sensitivity to ciprofloxacin, enrofloxacin, doxycycline, levofloxacin, and amikacin, but resistance to amoxicillin, ampicillin, oxytetracycline, streptomycin, spectinomycin, neomycin, lincomycin, erythromycin and spiramycin. In contrast to the previous findings that *S. iniae* was susceptible to amoxicillin and ampicillin **(Chou *et al.*, 2014; Saleh *et al.*, 2017)**, our study reported resistance to both antibiotics and this matches with the result obtained by **Younes *et al.* (2019)**. The presence of amoxicillin/ ampicillin resistance may be owing to the mutations of the bacterial genome and virulence; however, this issue needs further investigation to be confirmed.

CONCLUSION

It can be concluded that the Egyptian *O. niloticus* farms under the current study are free from TiLV. While, *Aeromonas* spp. and *S. iniae* are suggested to be the most abundant pathogens recovered from the diseased fish in our study. In addition, they are suggested to take part in the summer mortalities of the Nile tilapia occurring during the summer seasons in the Egyptian aquatic farms. Further investigations using a higher number of fish from wider geographical areas and comprehensive characterization of the isolated pathogens are warranted to understand the causes of the significant high mortalities in farmed tilapia during summer. This would help in the establishment of effective management strategies to mitigate that phenomenon and improve the tilapia farming industry in Egypt.

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• CONFLICT OF INTEREST

The authors declare that they have no competing interests.

• DATA AVAILABILITY STATEMENT

The datasets used and analysed during the current study are available from the corresponding author on reasonable request. All data generated or analysed during this study are included in this published article.