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Major Bacterial Challenges Facing Nile Tilapia (*Oreochromis niloticus*) in Egyptian Fish Hatcheries

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ABSTRACT

Nile tilapia is the most farmed freshwater fish species across vast areas of the African continent. Although Egypt is considered the largest African producer of Nile tilapia, this fish has been faced with frequent episodes of mortality at both hatchery and farm levels. In the present study, we conducted field screenings to identify the major bacterial pathogens responsible for these mass kills among broodstocks and seeds in Egyptian hatcheries. *Aeromonas hydrophila* and *Streptococcus agalactiae* were determined to be the major bacterial threats to hatchery-reared Nile tilapia. The bacterial isolates were presumptively identified using conventional biochemical tests and the API 20 NE miniaturized test. The final identities of the retrieved bacterial isolates were molecularly confirmed using PCR and sequencing of 16S rRNA genes. *A. hydrophila* and *S. agalactiae* isolates were found to be sensitive to florfenicol, while the two isolates exhibited resistance to novobiocin and ampicillin. The *A. hydrophila* isolates were confirmed to be sensitive to oxytetracycline, whereas the *S. agalactiae* isolates were sensitive to erythromycin. Histopathological examination of the livers of infected fish revealed vacuolar degeneration and necrosis of hepatocytes. Remarkably, infiltrations of Gram-positive cocci were documented within hepatic parenchyma and brain tissues. Further, the infected fish exhibited edematous brains with inflammatory cell infiltration through the meninges. Severe retinal pathology, including collagen fibre disorganization, oedema, and inflammatory cell infiltration was also detected. The current study emphasizes the deleterious impacts of some ubiquitous bacterial pathogens on the health status of Nile tilapia broodstocks and their seeds. Ultimately, we affirm that regular monitoring of water quality, feed quality, proper handling of broodstocks, and accurate diagnosis is a crucial asset in preventing disease spread and mass fish kills in Egyptian hatcheries.

Keywords: *Aeromonas hydrophila*, Aquaculture, Broodstocks, Nile tilapia, *Streptococcus agalactiae*, Summer mortalities.

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INTRODUCTION

Egypt's aquaculture sector has grown significantly during the past three decades. When aquaculture was recognized as a strategically significant business in the 1980s, investments in this sector resulted in a remarkable aquaculture success story **(Shaalan *et al*., 2018).**

Nile tilapia (*Oreochromis niloticus*) is an African native fish that has been widely produced in tropical and subtropical nations due to its unique characteristics, including a rapid growth rate, palatable taste, and high economic value (**McAndrew, 2000).** It is recognized as the most widely spread freshwater cultured fish. Nationally, a total of 1.6 million tonnes of tilapia were produced in 2020 **(FAO, 2020**). Early in 2012, a survey of the value chain for fish seeds showed that the tilapia hatchery sector had grown (**Saleh, 2007; Nasr-Allah *et al*., 2014**). According to a 2012 survey created by the Improving Employment

and Incomes through Development of Egypt's Aquaculture Sector (IEIDEAS) initiative, there are around 440 hatcheries for tilapia in Egypt (**Nasr-Allah *et al*., 2014)**.

Although tilapia fish can resist challenging climatic conditions, bacterial disease outbreaks continue to be a significant obstacle to tilapia production in intensive farming (**Hasimuna *et al*., 2020).** Stressful environmental factors impair an organism's immune system, increase the body's susceptibility to infectious invaders, and cause significant mortality, such as hot or low water temperatures, a high stocking rate, starvation, and infectious diseases (**Shourbela *et al*., 2021**). Heavy metals of agricultural origin can also be an immunocopromizing factor that triggers cascades of immunological damage among freshwater fish, including Nile tilapia **(AL Taee *et al*., 2020).** The immunocompromized broodstocks are usually potential givers of immunosuppressed juveniles **(AL Taee *et al*., 2020).**

One of the biggest problems facing fish farming is fish disease, particularly bacterial ones. Naturally occurring bacterial fish pathogens can be found in the fish's surroundings; however, in specific stress situations, which could result in significant financial losses in fish farms, 80% of deaths occur (**Austin and Austin, 2016**).

Streptococcal infection is one of the most important disease issues with direct negative impacts on the global aquaculture industry (**Abdelsalam *et al*., 2013; Eissa *et al*., 2021)**. Numerous *Streptococcus* species are considered significant as fish pathogens to farmed *O. niloticus* such as *Lactococcus garvieae*, *S. agalactiae*, *S. dysgalactiae*, *S. iniae*, and *E. faecalis* (**Abdelsalam *et al*., 2013; Osman *et al*., 2017**). Regardless of the causative streptococci, affected fish show similar clinical signs such as loss of appetite, skin darkness, corneal opacity, eye haemorrhages, unilateral or bilateral exophthalmia (popeye), erratic swimming, nervous signs and stiffness, ascites and haemorrhages at the base of fins (**Abu-Elala *et al*., 2016**).

Streptococcus agalactiae is the predominant pathogenic species causing streptococcosis among freshwater fish worldwide (**Bowater *et al*., 2012; Lusiastuti *et al*., 2014; Barato *et al*., 2016)**. Based on biochemical characteristics and hemolytic ability, *S. agalactiae* were classified into two biotypes, i.e., biotype-I (beta-haemolytic Group B Streptococcus) in Thailand, Malaysia, and Singapore, and biotype-II (γ-haemolytic Group B Streptococcus) in China and Indonesia (**Soto *et al*., 2015).**

Aeromonas hydrophila is a ubiquitous organism that luxuriously inhabits freshwater. The organism causes Motile Aeromonas Septicaemia "MAS," which is triggered by environmental stressors e.g., elevation of water temperature, overcrowding, high organic matter, nutritional deficiency, and parasitic infestation (**Cipriano, 2001; Elgendy *et al*., 2015)**.

Aeromonas hydrophila is well recognized as the predominant Gram-negative facultative anaerobic bacteria belonging to the family Aeromonadaceae, a waterborne pathogen that causes significant economic losses in aquaculture. This is because hemorrhagic septicemia outbreaks are associated with elevated rates of morbidity and mortality (**Marinho-Neto *et al*., 2019; Liu *et al*., 2020).**

Aeromonas hydrophila and *Streptococcus agalactiae*' linked mass kills within Egyptian Nile tilapia farms have been frequently reported through the past few decades. Yet, literature about similar mass mortalities within Egyptian Nile tilapia hatcheries were scarce. Therefore, we have investigated the major bacterial pathogens implicated in clinical diseases and mass mortalities among Nile tilapia seeds and broodstocks in some Egyptian hatcheries.

MATERIALS AND METHODS

1. Case history

In the summer of 2022, a disease outbreak among cultured Nile tilapia was reported in an earthen pond-based private hatchery in Abassa Sharkiya province, Egypt. The reported mortalities were 60% of the entire stock. The hatchery was overstocked with 400 broodstocks in 100 m2 hapa. On the other hand, a total of 100.000 seeds were stocked in a 50 m2 area. The affected broodstocks were 250 ±25 g weights on average while seeds were 5±0.2 g on average. Broodstocks were fed 45% protein sinking pellets, while seeds were fed a 40% protein powder diet. The pond's water supply was derived from an agricultural drainage known as Bahr Elbaqar. An erratic management regimen was noticeable in this hatchery where excess amounts of animal manure were used as an organic fertilizer before stocking of seed as well as delay in dead fish collection.

Another incident of mass kills among hatchery-reared Nile tilapia seeds was reported in the private hatchery at Kafr Elsheikh province during the mid-summer of 2023. A total of 300 Nile tilapia

broodstocks were stocked in 50 m2 area concrete tanks while 50 m2 hapa was stocked with 60,000 Nile tilapia seeds. The affected broodstocks were 250 ±10 g weights on average while seeds were 1±0.2 g on average. The hatchery water supply originated from an agricultural drainage. Broodstocks were fed a 40 % protein-sinking pelleted diet while seeds were fed 45 % powdered diet.

2. Sampling

A total of 100 Nile tilapia seeds were transferred in well-aerated plastic bags filled with water, and 20 broodstocks exhibiting nervous signs, bilateral exophthalmia, and skin haemorrhage were randomly selected and stored in an isothermal box filled with crushed ice until they were transferred to the lab. Concurrently, water samples were collected in sterile glass bottles utilizing the method described by **Eissa *et al.*,** (**2013**) in accordance with **APHA (1995**) guidelines. Water temperature, dissolved oxygen (DO), and pH were measured on spot using a waterproof portable logging multi-parameter water quality meter (HI9829, Woonsocket, RI, USA). Unionized ammonia (NH_3) levels were measured according to the method described by **Rice *et al.*, (2012).** Fish and water samples were transported to the Aquatic Animal Medicine and Management Laboratory (AAMML), Faculty of Veterinary Medicine, Cairo University, Egypt. Additionally, feed samples were examined for aflatoxins using **Zhao *et al.*, (2017)** methodology. Clinical examination of moribund and mortal fish was performed according to **Eissa, (2016).**

3. Bacterial isolation and identification

Fish seeds were soaked in 70 % alcohol for 10 seconds and then washed three times in 0.85% (w/v) saline **(Eissa, 2016).** Subsequently, five seeds were placed in a glass potter mixer with 1 ml of sterile saline and manually blended. An amount of 100 µL aliquotes from the homogenates were spread onto tryptic soy agar (TSA; Oxoid, UK), Streptococcus selective agar media, and Aeromonas selective agar supplemented with rehydrated ampicillin (Oxoid), and blood agar (Oxoid).

Nile tilapia broodstocks were laid on left side and then sprayed with alcohol 70 % to exclude possible external bacterial contamination. Broodstocks were cut open using the standard triangular technique described by **Eissa *et al.*,** (**2016**). Loopfuls from the kidneys, spleens and brains were streaked onto plates of tryptic soy agar (TSA; Oxoid, UK), Streptococcus selective agar media, Aeromonas selective agar supplemented with rehydrated ampicillin (Oxoid), and blood agar. Culture plates were then incubated for 24 to 48 hours at 25ºC. Single colonies were taken, re-streaked on the previously described media, and re-incubated under the same conditions to obtain pure cultures. The cultural characteristics of the retrieved isolates were matched according to the standard criteria of *Streptococcus agalactiae* and *Aeromonas hydrophila* determined by Bergey's Manual of Systemic Bacteriology (**Vos *et al*., 2011; Austin and Austin, 2016**). In accordance with manufacturer instructions, biochemical assays utilizing API20-Strep and API-20E (BioMerieux, France) as well as oxidase, catalase, motility, and Gram stain were carried out (**Austin and Austin, 2016).**

4. Molecular identification

4.1. DNA extraction

Representative purified bacterial colonies were inoculated in brain heart infusion broth (BHI, Oxoid, UK) at 28 °C for 18–24 h. Bacterial cultures were centrifuged at 12 000 × g for 2 min. The genomic DNA of each isolate was then extracted using the QIAamp DNA Mini kit (Qiagen) according to the manufacturer's instructions. The extract was stored at −30 °C untill use.

4.2. 16S rRNA gene sequence analysis

Presumptively identified *Aeromonas* spp. was confirmed by PCR using 16S rRNA *Aeromonas hydrophila* primers. The primer sequences were: 16S rRNA F; 5-AGAGTTTGATCMTGGCTCAG-3 and 16S rRNA R; 5-TACGGYTACCTTGTTACGACTT-3 (**Lagacé *et al.*, 2004).** In addition, presumptive identification of *Streptococcus* spp. was confirmed by PCR using 16S rRNA *Streptococcus agalactiae* primers. The primer sequences were: 16S rRNA FD1; 5-AGAGTTTGATCCTGGCTCAG-3 and RD1; 5-TAAGGAGGTGATCCAGCC-3 **(Batdorj *et al.*, 2006)**. The PCR reactions were performed in a final volume of 25 µL, using a 1x PCR Master mix consisting of 1.0 µL of each primer, 200 mM dNTP, 1 U Taq polymerase, and 3 µL template DNA. PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen). The PCR cyclic conditions for *Aeromonas hydrophila* were as follows: 94 °C for 4 min. (initial denaturation), followed by 35 cycles of 94 °C for 1 min. (denaturation), 56 °C for 1 min. (annealing), and 72 °C for 2 min. (extension), with a final extension of 10 min. at 72 °C **(Lagacé *et al.*, 2004)).** The PCR-cyclic conditions for *Streptococcus agalactiae* were as follows: 95 ˚C for 5 min. (initial denaturation), followed by 35 cycles of 95 °C for 45 s (denaturation), 56 °C for 1 min. (annealing), and 72 °C for 1 min. (extension), with a final extension of 10 min. at 72 °C **(Batdorj *et al.*, 2006).** PCR cyclic conditions using a thermal cycler (Applied Biosystems, 2720 thermal cycler, USA). Purified PCR sequences were sequenced using an ABI 3730xl DNA sequencer (Applied Biosystems™) at Sigma. The obtained 16S rRNA

genes were analyzed and compared with the other sequences in GenBank using a BLAST® search**.**

4.3. Phylogenetic analysis

The sequences of the 16S rRNA gene of bacterial strains were deposited in GenBank. To calculate genetic distance, the phylogenetic trees were created by the neighbour-joining method **(Kumar *et al*., 2018**), and the level of confidence was verified by bootstrap analysis for each branch at 1,000 values using MEGAX 11 software **(Kumar *et al*., 2018).**

5. Histopathological findings

Kidney, brain, liver, gills, spleen, gonads, and ocular tissue specimens were fixed in 10% formol saline for 24 h. Fixed tissues were washed in tap water then dehydrated using serial dilutions of absolute alcohol. Specimens were cleared in xylene and embedded in paraffin at 56 °C in a hot air oven for 24 h. Paraffin beeswax tissue blocks were prepared for sectioning at 4 microns thickness (Leitz rotary microtonal for paraffin section). The obtained tissue sections were placed on glass slides, deparaffinized, and stained by hematoxylin and eosin (H&E) stain (**Bancroft *et al*., 2013**) for examination using low / high powers of light electric microscope (Olympus, USA).

6. Antimicrobial susceptibility assay

Antimicrobial susceptibility of the *S. agalactiae* and *A. hydrophila* isolates were tested by disc diffusion method utilizing the Kirby-Bauer method as described by **Eissa (2016).** Retrieved isolates were tested against the following antibiotics (Oxoid™): Ampicillin (AMP 10µg), oxytetracycline (OTC 30µg), Erythromycin (E 15µg), Florfenicol (FFC 30µg), trimethoprim/sulfamethoxazole (SXT 25µg), and Novobiocin (NV 5µg). Results were interpreted as susceptible (S), intermediate (I), or resistant (R) according to **CLSI (2017)**.

7. Ethics approval

The current study was approved by the Institutional Animal Care and Use Committee, Veterinary Medicine, Cairo University, Egypt. Ethical approval number (Vet CU 08072023698).

RESULTS

1. Clinical examination

Externally, the investigated *O. niloticus* broodstocks exhibited typical signs of septicemia including red skin patches, erosions, and ulceration on lateral body surfaces **(Fig. 1a),** unilateral or bilateral exophthalmia with corneal opacity **(Fig. 1b)** and ascites. Internally, serosanguinous fluids were found upon opening the abdominal cavity along with a congested stomach, liver, spleen, kidney, swollen liquefied brain. The Nile tilapia seeds showed remarkable nervous signs such as whirling and standing posture in some cases together with dark skin with or without patchy skin hemorrhages and bilateral exophthalmia.

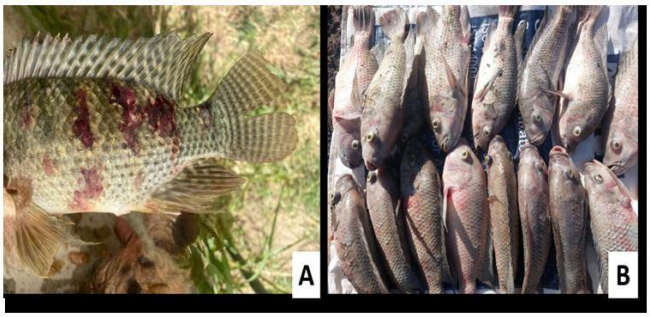

Fig. 1. a) Nile tilapia broodstock exhibiting typical haemorrhagic patches and ulceration on the external body surfaces and **b)** Nile tilapia broodstock exhibiting exophthalmia and corneal opacity.

2. Feed samples analysis

The detected levels of aflatoxins in examined 1st feed sample were 7.42, 4.82, 7.29, 0, 0 ppb for aflatoxins B1, G1, B2, B2 and G2 respectively. In the 2nd feed sample, levels were 7.29, 0, 0 and 76.7 ppb for aflatoxins B1, B2, G1 and G2 respectively. In the 3rd feed sample, levels were 0.24, 3.77, 7.05 and 0 ppb for aflatoxins G2, G1, B1 and B2 respectively. In the 4th feed sample, the levels were 35.62, 7.28, 8.40, and 0 ppb for aflatoxins G2, G1, B1, and B2, respectively.

3. Water quality samples analysis

The average water temperatures were 31±0.5°C, average pH was 9.1± 0.2, DO was 3 ppm in average, while the average of assessed unionized ammonia (NH_3) levels were 0.9 ± 0.1.

4. Bacterial isolation and identification

A total of 20 *Aeromonas hydrophila* isolates were equally retrieved from (20 seeds' pools and 20 broodstocks) with 100 % prevalence ; A total of 30 *Streptococcus agalactiae* isolates were retrieved from both broodstocks (20 isolates) with 100 % prevalence (20/20 broodstocks) and seeds (10 isolates) with 50 % prevalence (10/20 seeds' pools).

Gram-negative, motile, rod-shaped bacteria known as *Aeromonas* spp. were isolated from the kidney, liver, and spleen tissues of the affected fish. Whereas the colonies on Aeromonas selective agar medium resembled dark green to yellow, the colonies on TSA were convex, glossy, and creamy. It was also positive for oxidase and catalase. On the other hand,

Streptococcus agalactiae were isolated from the kidney and brain tissues of the infected fish. Isolates were Gram-positive cocci arranged in pairs and/or chains. The colonies emerged on TSA were translucent white, while those emerged on Streptococcus selective agar plates were red in colour. These colonies were non-hemolytic on blood agar plates.

Based upon conventional biochemical testing and semi-automated API 20 E and API 20 Strep test kits, retrieved bacterial isolates were presumptively identified as *Aeromonas hydrophila* and *Streptococcus agalactiae* (Table 1).

Table 1: Morpho-chemical characteristics of the retrieved *Streptococcus agalactiae* and *Aeromonas* isolates.

Morpho-chemical characteristic	***Streptococcus agalactiae***	***Aeromonas hydrophila***
Gram stain	Gram positive cocci	Gram negative bacilli
Oxidase	Negative	Positive
Catalase	Negative	Positive
Motility test	Non-motile	Motile
Voges Proskauer	Positive	Positive
Urease	Negative	Negative
Ornithine Decarboxylation	Positive	Positive
Lysine decarboxylase	Positive	Positive
Arginine dihydrolase	Positive	Positive
β galactosidase	Negative	Positive
Lactose fermentation	Negative	Negative
Glucose fermentation	Positive	Positive
Sucrose fermentation	Positive	Positive
Salicin fermentation	Positive	Positive
HIP (hydrolysis (Hippuric acid)	Positive	Not detected
Esculin hydrolysis	Negative	Variable
PAL (Alkaline Phosphatase)	Negative	Not detected

5. Sequencing analysis

The presumptively identified *Aeromonas* spp. and *Streptococcus* spp. were confirmed by sequencing the 16S rRNA gene. The assembled sequences were submitted and deposited in the GenBank under the accession numbers: OR822243 (Aeromonas spp.) and OR822206 (Streptococcus spp). The GenBank accession number (OR822243) was 1416 bp and showed 99.93% - 100% similarity to the accession number of *A. hydrophila* (JN400039, KX822740, MT747171, KF413421 and KF413418) **(Fig. 2a)**, while the GenBank accession number (OR822206) was 1335 bp and showed 99.93% - 100% similarity to the accession number of *S. agalactiae* (OL471408, KF826095, KP729641, KP729639, KF111277, JQ990156 and JQ990155) **(Fig. 2b)**.

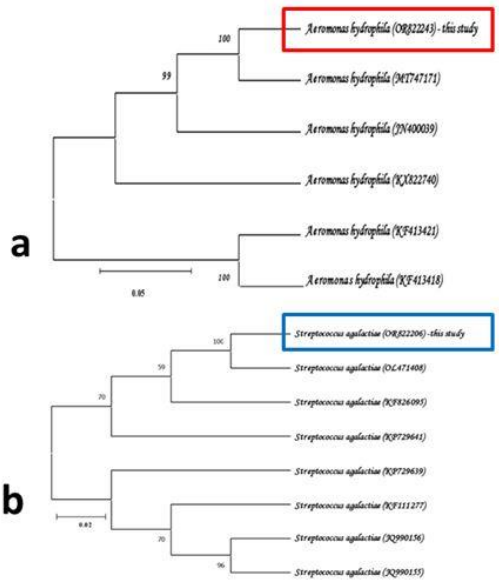

Fig. 2: a) Phylogenetic analysis based on the 16S rRNA gene sequence of *A. hydrophila* isolated in this study. The tree was constructed and analysed by the neighbor-joining method and **b)** Phylogenetic analysis based on the 16S rRNA gene sequence of *S. agalactiae* isolated in this study. The tree was constructed and analyzed by the neighbor-joining method.

6. Histopathological findings

Histopathological examination of infected fish'liver tissue sections showed vacuolar degeneration and necrosis of hepatocytes. Central hepatic veins, and blood sinusoids were severely dilated and engorged with blood **(Fig. 3 a, b)**. Some sections revealed the presence of large areas of haemorrhage **(Fig. 3c)**. The perihepatic capsule was greatly thickened by edema and inflammatory cells **(Fig. 3d)**. Interesting, the examination of liver tissue section by oil immersion lens revealed the presence of bacterial cocci within the liver tissue sections **(Fig. 3e)**. Spleen showed lymphoid depletion, activation of melanomacrophage centers (MMC)**(Fig. 3f)**, and congestion of splenic blood vessels **(Fig. 3g)**.

The brain showed edema and inflammatory cell infiltration of meninges **(Fig. 4a).** Kidneys showed vacuolar degeneration of most renal tubular lining epithelium **(Fig. 4b)** together with congestion of interstitial blood vessels **(Fig. 4c**), and hemorrhage in some areas **(Fig. 4d)**. Concerning the eyes of infected fish, the cornea showed disorganization of collagen fibers with edema and inflammatory cells infiltration, there was also a proliferation of melanomacrophages **(Fig. 4e)** and vacuolation of corneal epithelium **(Fig. 4f)**.

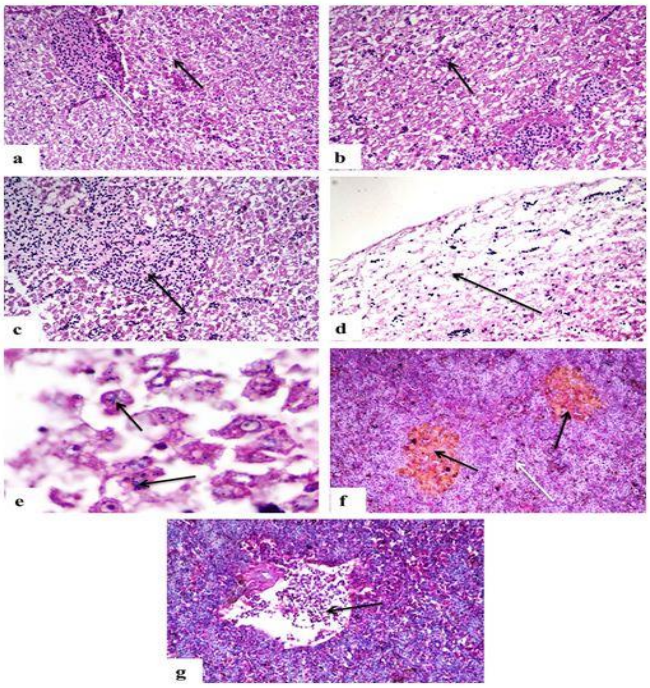

Fig. 3: Photomicrograph of **a)** Liver showing vacuolar degeneration and necrosis of hepatocytes (black arrow), congestion of central vein (white arrow) (H & E x400), **b)** Liver showing congested blood sinusoids (arrow) (H & E x400), **c)** Liver showing large area of hemorrhage (arrow) (H & E x400), **d)** Liver showing edema and inflammatory cells infiltration in perihepatic capsule (arrow) (H & E x400), **e)** Liver showing presence of colonies of cocci in liver tissue (arrows) (H & E x1000), **f)** Spleen showing areas of lymphoid depletion (white arrow) and activation of melanomacrophage centers (black arrows) (H & E x400) and **g)** Spleen showing congestion of splenic blood vessels (arrow) (H & E x400).

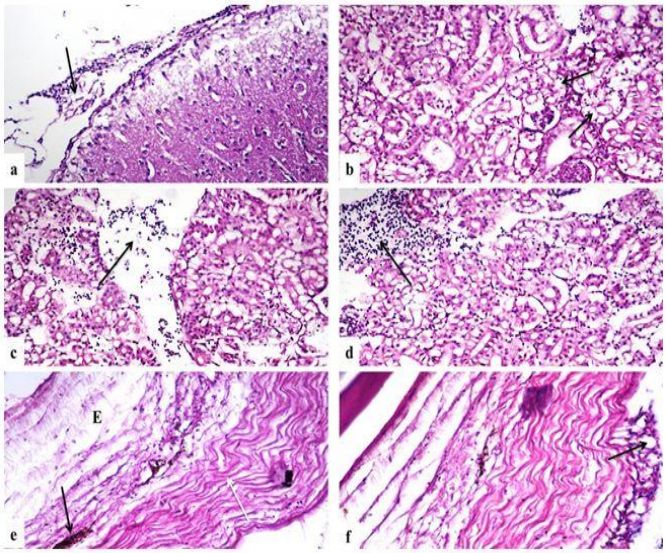

Fig. 4: Photomicrograph of **a)** Brain showing edema and inflammatory cells infiltration of meninges (arrow), **b)** Kidneys showing vacuolar degeneration of renal tubular lining epithelium (arrows), **c)** Kidneys showing congestion of interstitial blood vessels (arrow), **d)** Kidneys showing presence of area of hemorrhage (arrow), **e)** Cornea showing disorganization of collagen fibers (white arrow), edema and inflammatory cells infiltration (E) and proliferation of melano-macrophages (black arrow) and **f)** Cornea showing vacuolation of corneal epithelium (arrow) (H & E x400).

The spleen of fish showed a wide area of melanin-pigmented cells occupying the pulps **(Fig. 5a)**. The Spleen of fish showed a focal area of pigmented melanin cells in the white pulps **(Fig. 5b)**. Gills of fish showed necrosis with inflammatory cells infiltration in the arch of the filament **(Fig. 5c)**. Gills of fish were also congested **(Figs. 5c and d)**. Kidneys of fish showed degeneration of the tubular lining epithelium with cast formation and few inflammatory cells in-between tubules **(Fig. 5e)**. Atrophied oocytes were also detected **(Fig. 5f)**.

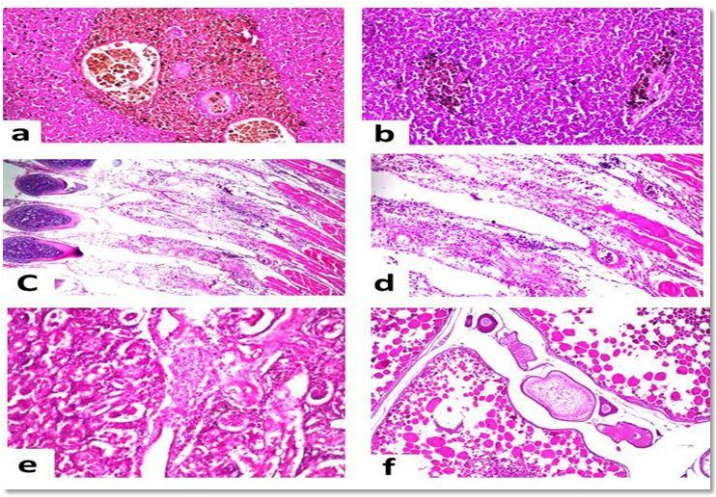

Fig. 5: Photomicrograph of **a)** Spleen of fish showing wide area of melanin pigmented cells occupied the pulps (H & E, x40), **b)** Spleen of fish showing focal area of pigmented melanin cells in the white pulps (H & E, x40), **c)** Gills of fish showing necrosis with inflammatory cells in the arch of the filament (H & E, x16), **d)** Gills of fish showing the magnification of **Fig. 3c** (H & E, x40), **e)** Kidney of fish showing degeneration of the tubular lining epithelium with cast formation and few inflammatory cells in between tubules (H & E, x40) and **f)** Female gonad of fish Showing atrophy of some individual oocytes (H & E, x40).

7. Antibiogram

All tested *A. hydrophila* and *S. agalactiae* isolates were resistant to novobiocin and ampicillin, while all *S. agalactiae* isolates were resistant to trimethoprim / sulfamethoxazole and oxytetracycline. *An eighty % of the A. hydrophila* isolates were sensitive to florfenicol and oxytetracycline. On the other hand, all *S. agalactiae* isolates were sensitive to erythromycin. Further, *all A.hydrophila* isolates were resistant to erythromycin ,while 20 % of the isolates were resistant to trimethoprim/sulfamethoxazole.

DISCUSSION

Microbial diseases are the most problematic challenges facing broodstocks and their progenies in fish hatcheries worldwide **(Migaud *et al.*, 2013**). High mortalities result in significant financial losses due to decreased fish production and higher treatment costs **(Faruk *et al*., 2004**). The high mortalities among Egyptian culturedfish in farms and hatcheries were mainly linked to mesophilic bacterial diseases during the summer season (**Enany *et al.*, 2019)**. Motile Aeromonas Septicemia (MAS) and streptococcosis are common bacterial diseases affecting cultured tilapias in temperate and tropical environments **(Basri *et al.*, 2020)**.

In the current study, concurrent infection with the mesophilic *Streptococcus agalacti*ae and *Aeromonas hydrophila* was reported to cause remarkable mortalities in hatchery-reared Nile tilapias during the summer season. These results were similar to those reported by **Enany *et al*., (2019**) and **Abdel-Latif *et al*., (2020)** The retrieved *A. hydrophila* isolates were presumptively identified by conventional phenotypic characteristics. The phenotypic characteristics of *A. hydrophila* were comparable with those reported in previous studies by **Dahdouh *et al.*, (2016) ; El-Bahar *et al.*, (2019) and Liu *et al*., (2020)**. Concomitantly, *S. aglactiae* was isolated from the brain and kidney tissues of moribund and mortal *O. niloticus*. These *S. agalactiae* isolates were presumptively identified by conventional morpho-chemical tests comparable to those reported by **Abd El-Tawab *et al.*, (2017); Hardi *et al.*, (2018); Abu-Elala *et al.*, (2019) and Eissa *et al.*, (2021)**.

The presumptively identified isolates were molecularly confirmed using standard PCR utilizing the 16S ribosomal RNA gene which is a highly conserved region present in bacteria playing a major role in gene coding. The 16S rRNA sequencing approach was considered a standard marker to confirm the identity of bacterial isolates **(Nagpal *et al.*, 1998)**. Several researchers have identified bacterial species using the 16S rRNA gene such as *Aeromonas* spp., and *Streptococcus* spp. **(Lagacé *et al.*, 2004; Batdorj *et al.*, 2006; Sebastião *et al.*, 2015)**. *Aeromonas hydrophila* and *S. agalactiae* isolates were confirmed by sequencing of the 16S rRNA gene where results were concordant with those reported by **Stratev *et al.*, (2016) ; Abu-Elala *et al*., (2019)**; **Eissa *et al.*, (2021).**

The clinical pictures of moribund/mortal hatchery-reared Nile tilapias through the current study were consistent with similar clinical pictures associated with Motile Aeromonads and Streptococcal infections previously reported in mass kills' eruptions among farmed Nile tilapias during the summer season (**Aboyadak *et al.*, 2015; El-Bahar *et al.*, 2019; Abdel-Latif *et al.*, 2020; Eissa *et al.*, 2021).** The hemorrhagic patches noticeably detected on the lateral sides of mortal/ moribund fishes were highly suggestive of acute Motile Aeromonas Septicemia (MAS) (**Cipriano, 2001; Abdel-Latif *et al*., 2020**). This assumption was augmented with the reported characteristic MAS histopathology in the internal organs of affected tilapias. Congestion, degenerative changes, and inflammatory cell infiltration throughout the hepatic, splenic, and renal tissues are pathological evidence of acute septicemia induced by MAS (**Yardimci and Aydin, 2011**).

On the other hand, the typical erratic swimming behaviour and skin darkening of both Nile tilapia broodstocks and their seeds could be attributed to an acute *S. agalactiae* nfection. *Streptococcus agalactiae* is known to secrete neurolytic toxins such as streptolysins, which are capable of inducing degenerative and necrotic lesions through the nervous tissues of the brain, skin, and retina of infected fish (**Eissa *et al.*, 2021**).

Ikenaga, *et al.*, (2005) stated that eurydendroid cells, a group of neurons believed to resemble deep cerebellar nuclei in mammals, are regulated by axons sent by fish Purkinje cells. These cells are located in the molecular and granule layers of the cerebellum, and their axons extend to Nmlf in the midbrain. Therefore, these afferent connections to Nmlf, the primary regulator of the fish's swimming movements, are likely being harmed by the S. agalactiae infection. All of the infected neural tissues of the tilapia grown in the damaged hatchery had visible gram-positive cocci, suggesting that the bacteria was not completely eliminated by the fish's inflammatory response. By inducing the host cytokine cascade, extracellular materials generated by the bacteria may impede phagocytosis (**Yen-Hsi Liu and Nizet, 2004**). It has been shown that S. agalactiae can synthesize Mn++-dependent superoxide dismutase in order to protect itself from the hydrogen superoxide that macrophages release. Additionally, according to **Poyart *et al.*,**

(2001), the bacteria may outsmart the immune system or cause interference with its ability to eradicate them.

As previously noted, the bacteria may penetrate the fish blood-brain barrier, which is crucial for the entry of germs into the fish brain parenchyma (**Daneman and Prat, 2015**). It has been demonstrated that the extracellular toxins of *S. agalactiae* bind to receptors on endothelial cells; this leads to the disruption of the blood-brain barrier (BBB) as damaged adherens and tight junctions enhance the production of interleukin-8, intercellular adhesion molecule 1, and nitric oxide from endothelial cells (**Al-Obaidi and Desa, 2018**).

Aeromonads have been suggested as either main or secondary infections in Nile tilapia (**Raj *et al.*, 2019**). Proteolytic enzymes, hemolysin, cytotoxin, dermo-necrotizing factors, gelatinase, and elastase are just a few of the virulence traits that *Aeromonas hydrophila* contains (**Aoki, 2011; Austin and Austin, 2016)**). The bacteria's increased virulence could be the result of their adaptation to the on-going climate change, which has led to higher-than-average water temperatures that are ideal for *S. agalactiae* (**Liao *et al.*, 2020**; **Sudpraseart *et al.*, 2021**).

According to **Azmai and Saad (2011**), the faecal-oral route is the primary means of transmission for streptococcus. Fish that are not ill can contract an infectious disease from bacteria found in their excrement, which can persist in water (**Nguyen *et al.*, 2002**). The virulence genes of *S. agalactiae*, such as hemolysin and cytolysin, stimulate neutrophil signalling pathways in the brain endothelium and contribute to the development of meningitis and panopthalamitis, and determine the severity of the infection (**Doran *et al.*, 2003**). Furthermore, variables pertaining to the *S. agalactiae* strain, infective dose, temperature, stocking density, and fish handling affect the severity of lesions and clinical indicators in *O. niloticus* (**Chang and Plumb, 1996**). Among the most devastating microbial diseases that affect *O. niloticus* aquaculture globally are *A. hydrophila* and *S. agalactiae* (**Basri *et al.*, 2020)**.

Optimal water quality is an integral factor for successful aquacultures. Maintaining optimal water quality conditions or parameters is a vital part of fish for optimal performance **(FAO, 2020)**. The water samples analyzed in this study indicated the presence of adverse water quality parameters, including high levels of ammonia, high water temperature, and low dissolved oxygen. Intensification of tilapia culture often results in exposing tilapia to multiple diseases, often bacterial infections **(Abdel-Latif *et al.*, 2020)**. Poor water quality, high stocking density, and erratic management practices such as using untreated poultry manures for fertilization of the earthen, using highly contaminated water sources, delaying the collection of dead fish, and unhygienic disposal of dead fish in some farming facilities. All these factors impact the health of fish and subsequently trigger the virulence of opportunistic and/or invasive pathogens, leading to disease occurrence and fish mass kills (**Small and Bilodeau, 2005; Rodkhum *et al.*, 2011; Abu-Elala *et al.*, 2016; Wamala, *et al.*, 2018; Eissa *et al.*, 2021)**.

Frequent use of antimicrobial agents eventually gives rise to antibiotic resistance among fish, leading to difficulty in controlling diseases in the future (**Aisyhah *et al.*, 2015).** In the present study, *A. hydrophila* and *S. aglactiae* isolates revealed diverse antimicrobial patterns of sensitivity. Based on the results of the antibiogram, *S. agalactiae* antimicrobial patterns were nearly similar to those reported previously by **Abu-Elala *et al.*, (2020).** *Streptococcus agalactiae* were resistant to novobiocin, ampicillin, and sulphamethoxazole/trimethoprim (Abu-Elala ***et al.*, 2020).** By contrast, *S. agalactiae* isolates in the present study were sensitive to erythromycin. On the other hand, *A. hydrophila* isolates were resistant to novobiocin, ampicillin, and erythromycin and sensitive to florfenicol and oxytetracycline and these results were comparable with those previously reported by **Dahdouh *et al.*, (2016)**. **Ahmed and Shoreit (2001)** found that *Aeromonas* sp. isolated from *O. niloticus* from Aswan fish hatcheries had a high sensitivity to oxytetracycline. In contrast, **Hamouda *et al.*, (2019)** found that most of the *Aeromonas hydrophila* isolates recovered from *O. niloticus* at the Aswan fish hatchery were resistant or moderately sensitive to oxytetracycline.

CONCLUSION

Poor water quality and bad management practices in hatcheries may predispose fish seeds and broodstocks to pathogenic infections and subsequent fish mortalities. The high modalities in Nile tilapia hatcheries during the summer season were attributed to bacterial infection by *A. hydrophila* or *S. agalactiae*. In such cases, florfenicol was the most effective antimicrobial agent based on the results of the antibiogram. Ultimately, we recommended regular monitoring of the quality of water and feed, proper handling of broodstocks, and ensuring accurate diagnosis. These measures play a crucial role in preventing the spread of diseases and large-scale mortality of fish in Egyptian hatcheries.

Availability of data and materials

All data are included in the manuscript.

Conflicts of interest

All authors declare that they have no conflict of interest.

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All authors equally contributed to the manuscript.

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