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Insight on the potential microbial causes of summer mortality syndrome in the cultured Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

This study aimed to investigate the potential causes of the summer mortality syndrome affecting the earthen pond cultured Nile tilapia, *Oreochromis niloticus* in some Egyptian farms. A total of 100 moribund *O. niloticus* showing non-specific clinical signs of septicemia and mortality (30- 65%) were collected from five farms at Tolombat 7, Kafr El-Sheikh, Egypt, during June 2019. All the fish farms share the same water source and perform some bad management practices. Inadequate water quality measures were noticed in all the investigated farms. Molecular detection of tilapia lake virus (TiLV) using PCR confirmed the non-existence of the TiLV in all examined specimens. A total of 19 pathogenic bacterial isolates were obtained from moribund *O. niloticus*: *Streptococcus agalactiae (*14), *Aeromonas hydrophila (3), and Vibrio cholera (2).* The predominance of *Streptococcus agalactiae* infections points to its intense involvement in such mortalities. *S. agalactiae* was confirmed by 16s rRNA gene sequencing and phylogenetic analysis. The PCR detection of virulence genes showed existence of *Hyl*, *cylE*, *scpB* and *camp* genes in all *S. agalactiae* strains. Isolates of *S. agalactiae* showed variable susceptibility to the tested antibiotics with high resistance against ampicillin, amoxicillin and gentamycin. Infections were associated with several pathological alterations in naturally infected fish. The pathogenicity of *S. agalactiae* was confirmed in *O. niloticus* (90 % cumulative mortality). The data in the present study proved that the tilapia summer mortality syndrome is multifactorial. Thus, good managemental practices and water quality measures are recommended to minimize fish mortality.

INTRODUCTION

The Nile tilapia, *Oreochromis niloticus*, is one of the most significant commercial fish species in the fish farming industry worldwide. The tilapia is a major source of high-quality animal protein and income in many African nations, contributing considerably to the global food security **(Ali *et al.*, 2020)**. The global cichlid production including the tilapia accounted for 6.3 million metric tonnes in 2018 **(FAO, 2019)**. Egypt is one of the

top three tilapia producers in the world, with 1081202 MT of the cultured tilapia **(GAFRD, 2019)**.

Tilapia can thrive, reproduce in a variety of aquatic environments and accept artificial diets making it a favorable fish farming species. *O. niloticus* shows a high growth rate and can tolerate some degrees of stress conditions **(FAO, 2019)**. However recently, the Egyptian fish farms have suffered unusual tilapia mortalities during summer season. The summer mortality events impacted nearly 37% of Egyptian fish farms in Kafr El Sheikh, Beheira, Sharqia and Minya Governorates, with an average mortality rate of 9.2 % costing nearly US$100 million **(Fathi *et al*., 2017)**.

The causative agents of mortality haven't been exactly identified; however, some risk factors were suggested to predispose fish to such mortality, viz. the inferior source of water, type of culture system, higher temperature and salinity. All of the afore-mentioned predisposing factors favored the occurrence of the unusual mortality **(Ali *et al*., 2020)**. In addition, some studies suggested that bacterial infections such as *Aeromonas veronii* could be the causative agents of disease outbreaks affecting the Nile tilapia in the Egyptian farms **(Eissa *et al*., 2015)**. Other studies attributed the mass mortalities to multidisciplinary environmental and microbial causes **(Abu-Elala *et al*., 2016)**.

Overcrowding and intensification of tilapia farming operations render the tilapia fish more susceptible to numerous disease outbreaks **(Eissa *et al*., 2021)**. Infections caused by *Streptococci* spp. rank at the top list of pathogens causing severe infections and high mortalities in the tilapia aquaculture (**Abu-Elala** et al**., 2020; Legario *et al*., 2020)**. *Streptococcus agalactiae, S. dysgalactiae, S. iniae* and *Lactococcus garviae* are the main pathogenic *Streptococcus* spp. that cause infections in the tilapia farming globally **(Buller, 2004; Osman *et al*., 2017 Abu-Elala *et al*., 2019)**. Streptococcosis can occur in numerous farmed and wild fish species in fresh, brackish water and marine aquatic environments **(Mishra *et al*., 2018)**. Affected fish commonly displays lethargy, anorexia, and erratic swimming behavior. Widespread hemorrhages on the external body surfaces, abdominal distension, exophthalmia with opacity and hemorrhage in the eye are commonly seen in streptococcal infections **(Roberts, 2012; Legario *et al*., 2020)**.

Therefore, the present study was planned to investigate the potential causes of *O. niloticus* summer mortality syndrome in some Egyptian farms and delineate the possible involvement of tilapia lake virus in such mortalities. Furthermore, the current work aimed to identify the bacterial pathogens in moribund fish, with special reference to the virulence, pathogenicity, histopathology, and antibiotic sensitivity of the most predominant bacterial isolates (*S. agalactiae*).

MATERIALS AND METHODS

Case history and fish sampling

Unusual mortality was detected in the earthen-pond cultured *O. niloticus* during June 2019 in 5 fish farms at Tolombat 7, Kafr El-Sheikh, Egypt. The farms share the same

water source. Mortality rate ranged between 30- 65 %. Moribund fish showed lethargy, loss of feeding reflex, and abnormal swimming behavior such as spinning and spiraling. Moribund fish showed darkening and hemorrhagic patches on the external body surfaces. Twenty moribund fish were collected from each studied farm (total number = 100) and transferred within the minimum time of delay in iceg isothermal boxes to the Hydrobiology Department Lab, National Research Centre, Egypt for further examinations. Water quality parameters (temperature, dissolved oxygen, pH and unionized ammonia) were recorded at the fish farm as follows: 30°C, 3.5 mg/L, 8 and 0.6 mg/ L.

Detection of TiLV

Fish were dissected; tissues from liver, kidney and brain were collected in RNAlater (sigma- Aldrish) and kept at -80°C.

RNA isolation, cDNA synthesis and PCR amplification:

The TiLV was tested using a PCR protocol followed in the study of **Eyngor *et al*. (2014)**. The mRNA was isolated from tissues using RNeasy Mini kit (Qiagen, Hilden Germany), following the manufacturer's instructions. Briefly, fish tissues (50mg) were homogenized in TRIzol using Lysing Matrix B and D tubes and a FastPrep-24 sample preparation system (MP Biomedicals) for 2 cycles x 40 s, at a speed of 6.5 m/s with cooling in between. The mRNA purity and quantity were measured using Nanodrop (Thermo Scientific). The purified RNA (10 ng) was reverse transcribed in a one-step RT-PCR reaction. The cDNA was synthesized using the Thermo Scientific Verso 1-Step RT-PCR Ready-Mix Kit (Thermo Fisher Scientific) following the manufacture instructions and the specific primers set in Table (1). The PCR reaction consisted of an initial denaturation step at 95°C for 2min, 35 cycles at 94°C for 30s, annealing at 58°C for 60 s, extension at 72°C for 1.30min, and a final extension for 10min at 72°C. The amplified products were electrophoresed using 1.0% (w/v) agarose gel in 0.5X TAE buffer with ethidium bromide. The gel was stained with ethidium bromide (Sigma) and visualized on a UV transilluminator. A negative control (no template DNA) and a positive control (TiLV fragment) were included in each run.

Bacteriological examination

Loopfuls prepared from brain, liver, kidney, and spleen were streaked onto tryptic soy agar (Difco Laboratories, USA) and blood agar media, supplemented with 5% defibrinated sheep blood (Oxoid™), and then incubated overnight at 30°C. Random colonies were picked, purified and identified via Gram staining, colony characteristics, biochemical methods and hemolysis on blood agar according to the method of **Buller, (2004)**. The final identification of bacterial isolates was achieved by the Vitek 2 system, version: 07.01 (bio-Merieux), according to the guidelines of the manufacturer. Isolates were stored in tryptic soy broth with glycerol at -80°C.

Molecular characterization of *Streptococcus agalactiae*

The DNA was extracted from bacterial isolates (n=5) via QIAamp DNA Mini kit (Qiagen, Germany, GmbH) following the manufacturer instructions. Briefly, a volume of 200µl of the bacterial suspension was incubated with 10µl of proteinase K and 200µl of lysis buffer at 56°C for 10min. After incubation, 200µl of 100% ethanol was added to the lysate. The sample was washed, centrifuged and nucleic acid was eluted with 100µl of elution buffer provided in the kit. The DNA concentration was measured by NanoDrop (Thermo) spectrophotometer. The extracted DNA was kept frozen at -20°C until further analyses. The 16S rRNA gene was amplified by PCR using the universal primers set (Metabion, Germany): F27(5′ AGAGTTTGATCMTGGCTCAG-3′) and R1492 (5′TACGGYTACCTTGTTACGACTT-3′) described in the study of **Lagacé *et al.* (2004)**. The PCR was performed in 25ul final volume using 1X PCR mix consisting of 250 ng genomic DNA, 200mM dNTP, 1 U Taq polymerase and 0.25µM of both primers (Metabion, Germany). The cycling conditions were as follows: initial DNA denaturation at 95°C for 4 min, 35 cycles at 94°C for 30s, annealing at 56°C for 1min and extension for 1min at 72°C, and a final extension at 72°C for 10 min. The cycling conditions were adjusted at the following settings: initial denaturation at 94°C for 7 min, followed by 35 cycles of (denaturation at 94°C for 35 sec, 55°C for 50sec and72°C for 1min), and a final extension phase at 72°C for 10min according to **Lagacé *et al*. (2004)**. The amplified PCR products were purified, sequenced in both directions by Sigma Company, using ABI 3730XL DNA sequencer and blasted against available sequences in the GenBank database.

Phylogeny

The raw 16S rRNA sequences of *S. agalactiae* isolates were assembled using the BioEdit program, compared against other sequences available in the database of GenBank through BLASTN and deposited in the GenBank **(Hall, 1999)**. The phylogenetic tree was constructed using MEGA X, with a neighbor-joining method of the Kimura 2-parameter model, and the level of confidence was tested by bootstrap analysis at 1000 repeats according to **Kumar *et al*. (2018)**.

Detection of virulence genes in *S. agalactiae*

Streptococcus agalactiae isolates (n=5) were screened for 7 virulence genes, viz. hyaluronidase (Hyl), cytolysin activity (cylE), C5a peptidase (scpB), surface immunogenic protein (sip), resistance to protease immunity protein (rip), laminin-binding protein (lmb), and cathelicidin antimicrobial peptide (camp) using conventional PCR. The specific primers were used as shown in Table (1). The PCR was performed in a 25µl final reaction mixture containing 2.0µl template DNA, 1.5µl of 10 pmol of each oligonucleotide primer (Metabion, Germany), 12.5µl of 2X HS MyTaq MasterMix (Takara, Japan) and Milli-Q water to the volume. Detection of *Hyl*, *cylE*, and *scpB* was performed using a standard PCR protocol as the following: initial denaturation at 94°C for 10min; 30 cycles of (denaturation at 94°C for 30 s, annealing at 51 °C for 30 s, extension at 72°C for 30s), and final elongation at 72°C for 10 min according to

Sudpraseart *et al*. (2021). The cycling conditions for detection of *rip, lmb, sip,* and *camp* were initial denaturation at 94°C for 2 min, followed by 30 cycles (30 s at 94 °C, 60 s at 50°C, 60s at 72°C) and a final elongation at 72°C for 2min, coinciding with the study of **Bobadilla *et al*. (2021)**. The PCR products were electrophoresed in a 1% (w/v) agarose gel (Applichem, Germany, GmbH), prepared in 0.5X TAE buffer with ethidium bromide (0.5µg/ml) and visualized on a UV transilluminator (Alpha Innotech, Biometra).

Table 1. Primers used for genetic characterization and virulence genes in *S. agalactiae*

Genes	Primer sequence	Amplicon (bp)	Reference
TiLV RNA	F5′-GTCCAGGGCGGTATGTATTG -3′ R 5′-CTTACGGCTGACAAGTCTCTAAG -3′	834	**Mugimba *et al*. (2018)**
16S rRNA	F5′-AGAGTTTGATCMTGGCTCAG-3′ R 5′-TACGGYTACCTTGTTACGACTT-3′	1485	**Lagacé *et al.* (2004)**
Hyl	F: 5`-CATACCTTAACAAAGATATATAACAA-3` R: 5`-AGATTTTTTAGAGAATGAGAAGTTTTTT-3`	950	**Abd El-Aziz *et al*. (2021)**
cylE	F: 5`-TGACATTTACAAGTGACGAAG -3` R: 5`-TTGCCAGGAGGAGAATAGGA-3`	248	
scpB	F: 5`-ACAACGGAAGGCGCTACTGTTC-3` R: 5`-ACCTGGTGTTTGACCTGAACTA -3`	255	
lmb	F: 5`-AGTCAGCAAACCCCAAACAG -3` R: 5`-GCTTCCTCACCAGCTAAAACG -3`	397	
sip	F: 5`-ACTATTGACATCGACAATGGCAGC-3` R: 5`-GTTACTGTCAGTGTTGTCTCAGGA	266	**Krishnaveni *et al.* (2014)**
camp	F: 5`- CAAAGATAATGTTCAGGGAACAGATTATG -3` R: 5`CTTTTGTTCTAATGCCTTTACATCGTT-3`	320	
rip	F: 5`-CAGGAAGTGCTGTTACGTTAAAC -3` R: 5`-CGTCCCATTTAGGGTTCTTCC-3`	369	**Jain *et al*. (2012)**

Antibiotic sensitive testing

The antibiotic susceptibility of *S. agalactiae* isolates (n=5) was investigated against 7 antibiotics as described in the works of **Bauer *et al.* (1966)** and **CLSI (2010)** via the disc diffusion method onto Mueller-Hinton agar (Oxoid™). Seven antibiotics were utilized, including gentamycin 10µg, amoxicillin 30µg, ampicillin 10µg, florfenicol 30µg, ciprofloxacin 5µg, tetracycline 30µg and trimethoprim/sulfamethoxazole 1.25/23.75µg. *Streptococcus agalactiae* isolates were cultured in tryptic soy broth and incubated overnight at 30°C. Bacterial cultures were then streaked onto Mueller Hinton agar plates in triplicates using a cotton swab, antibiotic discs that were placed onto, and plates were

incubated for 24h at 30°C. The antibiotic discs were placed, plates were incubated for 24h at 30°C, and the diameter of the inhibition zones was measured. The results of antibiotic sensitivity were interpreted as susceptible (S), intermediate (I) and resistant (R).

Pathogenicity testing

The pathogenicity of one randomly selected isolate was tested in apparently healthy *O. niloticus*. The Nile tilapia fish (50 g) were collected from a private fish farmat the Fayom Governorate Egypt. Fish were kept in aquaria (60L each) filled with dechlorinated tap water and supplied with aerators at water temperature nearly at 25°C ± 1. Fish were left to acclimatize for 2 weeks. Specimens were divided into 2 groups in triplicates (10 fish/ replica). Fish in group (1) were intraperitoneally injected with 0.1ml of 1.0×10^7 CFU/ml of *S. agalactiae,* according to the procedures of **Pereira *et al.* (2010)**. Fish in the control group were intraperitoneally injected with 0.1ml of a sterile saline solution. Fish were observed for 10 days. Clinical examination of challenged fish was noticed. Mortality and postmortem lesions were recorded. The cause of mortality was confirmed by the re-isolation of the injected bacterial isolate from succumbed fish in pure culture.

Histopathology of naturally infected fish

Tissue specimens were collected from kidney, brain, liver and spleen, fixed in Davidson's solution and then transferred to 70% ethyl alcohol. Tissues were embedded in paraffin, and then 2-6 microns thick sections were prepared, fixed on slides, and stained by Hematoxylin and Eosin stains according to the method of **Bancroft and Gamble (2008)**.

RESULTS

Clinical examination

Moribund fish showed abnormal swimming behavior. Clinical examination of fish showed darkening, exophthalmia and corneal opacity. Hemorrhagic patches were spreading on the external body surfaces with fin rot. Internally, ascites was detected with the presence of blood-tinged fluid in the abdominal cavity. Liver and spleen were enlarged and friable (Fig. 1).

Fig. 1. Photos showing the gross and P.M lesions in naturally infected *O. niloticus*: **A)** *Nile tilapia* with external hemorrhage; **B)** *Nile tilapia* with erosion and ulcers on the skin; **C)** *Nile tilapia* with bilateral exophthalmia (pop eyes) and corneal opacity, and **D)** *Nile tilapia* showing congested gills, distended gall bladder and internal hemorrhage.

Detection of TiLV

Tilapia lake virus was not detected via PCR in the collected samples (Fig. 2).

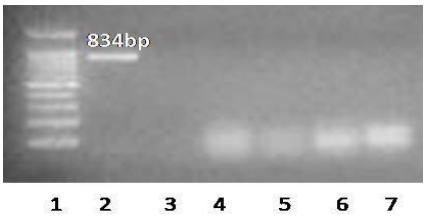

Fig 2. Agarose gel electrophoresis showing results of the PCR detection of TiLV virus. **1)** 100 bp DNA Ladder; **2)** Positive control, **3)** Negative control, and **(4-7)** Negative detection of TiLV virus in the investigated Nile tilapia samples.

Bacteriological examination

A total of 19 bacterial isolates were obtained from naturally infected *O. niloticus*. Phenotypic characteristics and Vitek 2 system (bio-Merieux) were identified bacterial isolates as *S. agalactiae (*14), *Aeromonas hydrophila (3) and Vibrio cholera (2). Streptococcus agalactiae* isolates were Gram positive cocci and catalase negative. Colonies on tryptic soy agar were pinpoint, whitish and circular. *Streptococcus agalactiae* isolates were β-hemolytic on blood agar media (Fig. 3). Some other nonpathogenic bacteria, viz. *Enterobacter aerogenes, Proteus* spp., and *Shigella* spp. were identified on agar plates.

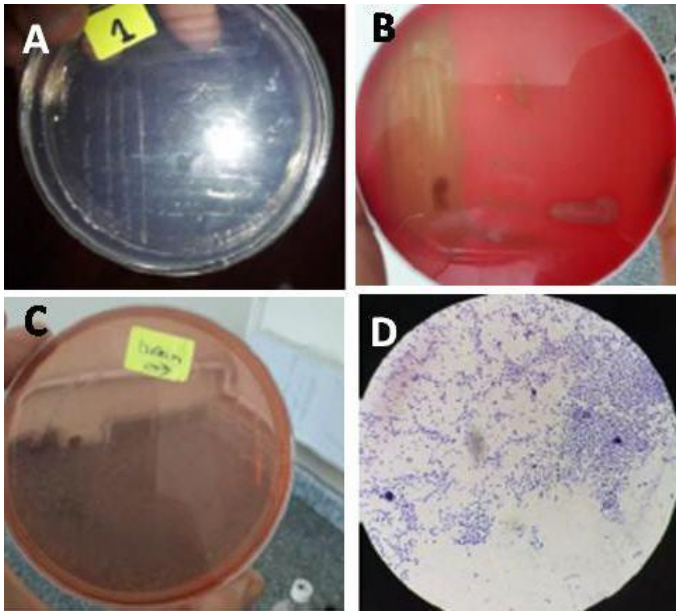

Fig. 3. Phenotypic characteristics of *S. agalactiae* showing **(A)** Pinpoint white colonies of *S. agalactiae* onto tryptic soy agar; **(B)** *S. agalactiae* group B hemolytic type colonies onto blood agar); **(C)** No growth of *S. agalactiae* onto MacConky agar, and **(D)** Gram positive cocci of *S. agalactiae*.

Molecular characterization of *Streptococcus agalactiae*

All the studied *S. agalactiae* isolates (n=5) produced nearly 1485 bp fragment (Fig. 4), and the accession number of this gene was issued as OK638442. Analysis of the bacterial 16S rRNA gene sequence confirmed to be *S. agalactiae.* The 16S rRNA sequences were identical with 100% similarity in all analyzed isolates (n=5).

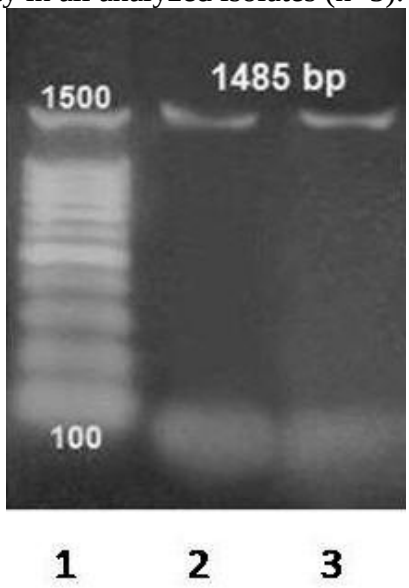

Fig. 4. Agarose gel electrophoresis showing results of the PCR detection of 16s rDNA of *S. agalactiae;* **(1)** 100 bp DNA Ladder and **(2, 3)** Positive detection of *S. agalactiae* strains

The neighbor-joining phylogenetic tree was constructed based on 16S rRNA genes of *S. agalactiae*, grouped with its relevant sequences and parted from other related species (Fig. 5). Blast results showed 96.50% sequence identity to *S. agalactiae* ATCC13813 strain (Accession:NR-040821.1).

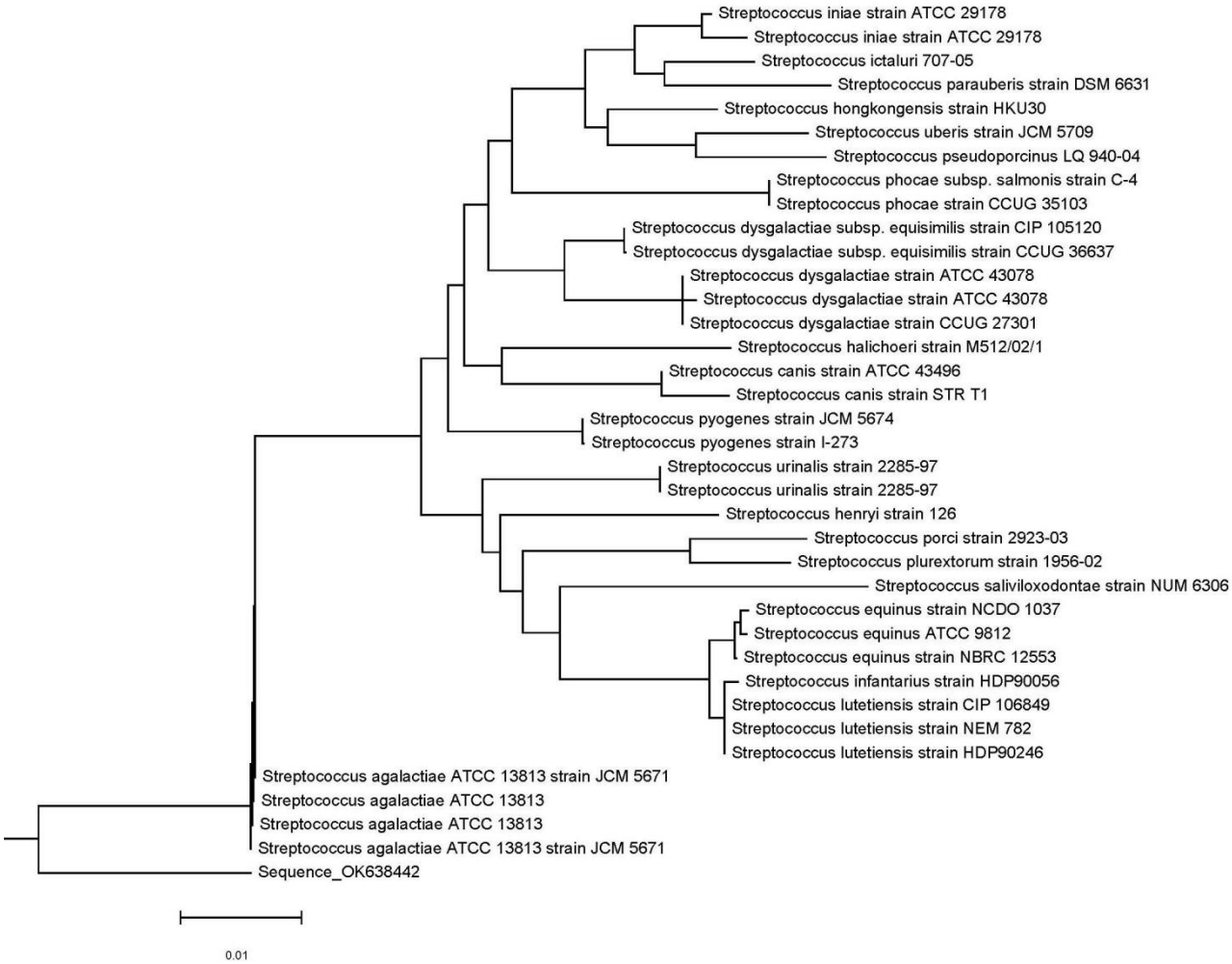

Fig. 5. The phylogenetic tree exhibiting the comparative analysis of 16S rRNA sequence of *S. agalactiae* infecting *O. niloticus*

Detection of virulence genes in *S. agalactiae*

All the investigated strains (n=5) showed positive results for hyaluronidase (*Hyl*), cytolysin activity (*cylE*), C5a peptidase (*scpB*) and cathelicidin antimicrobial peptide (camp) genes. But, none of them showed the presence of surface immunogenic protein (sip), resistance to protease immunity protein (rip), or laminin-binding protein (lmb) genes (Fig. 6).

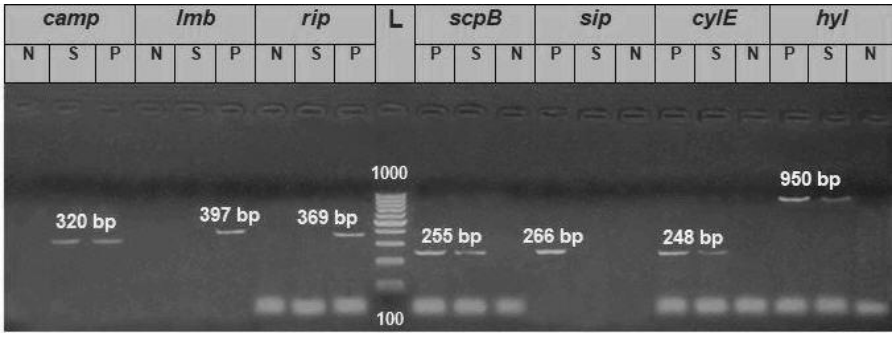

Fig. 6. Agarose gel electrophoresis showing results of the PCR detection of virulence genes with **(N)** Negative control; **(S)** Sample; **(P)** Positive control and **(L)** 100 bp ladder.

Antibiotic sensitive testing

S. agalactiae isolates showed different susceptibility patterns to tested antibiotics. All isolates (100%) were resistant to ampicillin 10µg and amoxicillin 30µg. Resistance to gentamycin 10µg, tetracycline 30µg and trimethoprim 1.25µg/sulfamethoxazole 23.75µg were (60%), (40%) and (20%), respectively. Interestingly, all isolates (100%) were susceptible to ciprofloxacin 5µg and florfenicol 30µg

Pathogenicity of *S. agalactiae*

Experimentally infected *O. niloticus* with *S. agalactiae* showed commonly noticed signs of hemorrhagic septicemia, external hemorrhages and skin darkness, in addition to congestion and enlargement of internal organs, especially the spleen. Some fish died showing no signs. Fish recorded 90 % cumulative mortality. *S. agalactiae* was reisolated from all dead fish. Fish in the control group showed 100 % survival.

Histopathology

Naturally infected *O. niloticus* showed variable histopathological changes. Circulatory, necrotic, and degenerative changes were commonly noticed. Brain showed severe hemorrhages and infiltrations of leucocytes. Kidneys showed necrotic changes in renal cells with hemorrhages in the interstitial tissue.. Liver showed congestion, cloudy swelling and degenerative changes in hepatocytes. Spleen showed activation of melanomacrophage centres and some foci of necrosis (Fig. 7).

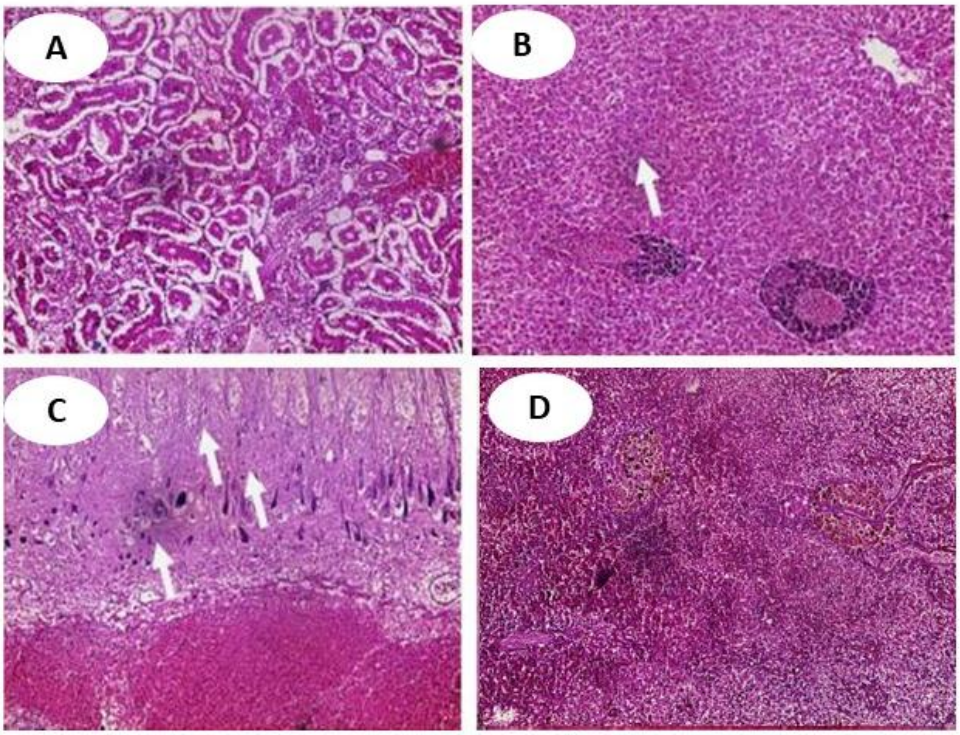

Fig. 7. Micrographs showing the histopathological alterations noticed in naturally infected *O. niloticus*: **A)** Kidney showing necrosis noted by the separation of renal tubular epithelial cells from its basement membrane with hemorrhages and necrotic changes in the interstitial tissue; **B)** Liver showing congestion, cloudy swelling and mild degenerative changes in hepatocytes; **C)** Brain showing severe hemorrhages and infiltrations of leucocytes, and **D)** Spleen showing multifocal red pulp reduction, activation of melanomacrophage centres and some foci of necrosis (H&E, Bar=50µm).

DISCUSSION

Fish diseases are one of the critical obstacles affecting the expansion of tilapia aquaculture worldwide. Combination of factors commonly works together to initiate fish infections **(Eissa *et al.*, 2021)**. In the present study, poor water quality, with respect to high temperature, low dissolved oxygen, and high unionized ammonia (NH_3) impairs immune system and renders the tilapia more susceptible to secondary bacterial infections **(Mahmoud *et al.*, 2019)**.

The *Tilapia tilapine* virus (TiLV) is a single stranded RNA virus, genus *Tilapine virus*, belongs to family Amnoonviridae **(Thawornwattana *et al.*, 2020)**. Tilapia lake virus is a transboundary emergent viral disease of the tilapia fish, causing colossal fish mortality **(Tattiyapong *et al.*, 2017)**. Other species in polyculture system are not affected although it was reported from river carp **(Abdullah *et al.*, 2018)**. Molecular analysis of all fish specimens in the present study confirmed the absence of TiLV in the investigated specimens. Reports on the discovery of tilapia lake virus from the Egyptian tilapia

summer mortality are contradictory **(Fathi *et al*., 2017)**, but authorities have confirmed that all data available on the existence of TiLV in the tilapia fish are not confidential.

Outbreaks of TiLV was reported in several countries including Israel, Thailand, Malaysia, Bangladesh, Ecuador, Uganda, Indonesia, USA and some others **(Subramaniam *et al*., 2019; Surachetpong *et al*., 2020)**. TiLV affects all the tilapia life stages, especially during the hot summer. It is highly contagious and can transmit through horizontal and vertical root **(Aich *et al*., 2020)**. TiLV causes severe lesions in the eyes, brain and the liver of fish. The virus causes severe pathological alterations in fish such as syncytial cell formation, and severe hepatocellular necrosis with pyknotic and karyolytic nuclei in the hepatocytes **(Subramaniam *et al*., 2019; Aich *et al*., 2020)**.

Bacteriological examination showed that some opportunistic infections are incriminated in the summer mortality that affected the investigated tilapia farms. *S. agalactiae* was the most predominant bacterial isolates (14 isolates). Some other bacterial species were also detected, viz. *Aeromonas hydrophila (3) and V. cholera (2)*. similar results were reported in the study of **Abdelsalam *et al*. (2021)**. The phenotypic characteristics of all retrieved isolates are identical to those recorded in previous studies **(Buller, 2004)**. Isolates of *S. agalactiae* were Gram positive cocci and catalase negative. The isolates producing pinpoint and circular whitish colonies on tryptic soy agar coincide with the findings of **Legario *et al*. (2020)**.

The virulence profiling assay of *S. agalactiae* isolates confirmed the existence of *Hyl, cylE, scpB* and *camp* genes. These genes are attributed to bacterial invasions and toxin production **(Maisey *et al*., 2008)**. Similarly, **Legario *et al*. (2020)** detected six virulence genes (*simA, scpI, pgm, cpsD, pdi* and *sagA*) in some strains of *S. agalactiae* that were isolated from clinical infections and mortalities in the farmed Nile tilapia, pointing to their role in the pathogenesis of streptococcal infections in fish.

The pathogenicity of *S. agalactiae* was confirmed by experimental infection in *O. niloticus.* Challenged fish showed high cumulative mortality and typical signs of streptococcosis that were noticed in naturally infected fish and are more or less similar to those of **Abu-Elala *et al*. (2020)** and **Sudpraseart et al. (2021)**. Isolates of *S. agalactiae* have several virulence gens **(Iregui *et al*. 2016; Ghetas *et al*., 2021)**. They were resistant to some antibiotics, with the uppermost resistance against ampicillin and amoxicillin. Variable resistance patterns were noticed in *S. agalactiae* against antibiotics in previous reports **(Betriu *et al*. 2003; Deng *et al*., 2019)**. This could be relevant to their excessive unregulated application in fish farming. Fish and aquatic environment could serve as reservoirs for potentially resistant pathogenic bacteria **(Rahman *et al*., 2021)**.

Naturally infected *O. niloticus* showed variable histopathological changes. Circulatory, necrotic, and degenerative changes were commonly noticed. Hemorrhages and infiltrations of leucocytes were noticed in brain which could explain abnormal swimming behavior in naturally infected fish. These alterations are nearly similar to those

described in streptococcal infections farmed tilapia **(Ortega *et al*., 2017; Legario et al., 2020)**; these alterations could be relevant to toxins produced by bacterial infections.

CONCLUSION

The summer mortality affecting the Nile tilapia is multifactorial. Inferior water quality, bacterial pathogens, and impaired immune mechanisms of fish are collectively posing impact on initiating the summer mortality. Poor water quality and bad management practices render tilapia more susceptible to secondary infections. TiLV was not isolated from tilapia summer mortality. *Streptococcus agalactiae* was the most predominant opportunistic bacterial pathogen that was isolated from the diseased *O. niloticus* reared in the studied farms. Therefore, it is strongly involved in the summer mortality events affecting the earthen pond Nile tilapia. Irresponsible use of antibiotics causes the emergence of resistant *S. agalactiae* strains. *Streptococcus agalactiae* strains have numerous virulence characteristics, and thus it is highly pathogenic to *O. niloticus* causing severe histopathological alterations and mortalities.

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