Egyptian Journal of Veterinary Sciences

https://ejvs.journals.ekb.eg/

Pathophysiological and Molecular Characterization of *Streptococcus agalactiae* in Farmed Nile Tilapia

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Abstract

THIS study investigated an outbreak of *Streptococcus agalactiae* infection in Nile tilapia *(Oreochromis niloticus)* farmed in Egypt, with a focus on pathogen identification and the resulting pathophysiological impacts. The investigation was carried out at a private aquaculture facility in the Manzala region, Dakahlia Governorate, during the summer of 2021. Clinically, affected fish showed signs of exophthalmia, skin haemorrhages, and marked congestion of internal organs. Bacteriological and molecular characterization using 16S rRNA gene sequencing confirmed *S. agalactiae* as the causative agent, with an overall prevalence of 15% among examined specimens. Experimental infection trials demonstrated significant oxidative stress responses, evidenced by increased levels of nitric oxide and malondialdehyde, and decreased antioxidant biomarkers including catalase (CAT), superoxide dismutase (SOD), glutathione (GSH), and glutathione peroxidase (GPx). Histopathological lesions were observed in multiple organs, including hepatic degeneration, renal tubular necrosis, splenic congestion, and meningoencephalitis. These findings highlight the systemic nature of *S. agalactiae* infection and underscore the urgent need for preventive strategies, early diagnostic protocols, and improved biosecurity measures to mitigate fish losses and safeguard aquaculture sustainability.

Keywords: *Streptococcus agalactiae*, Nile tilapia, Molecular diagnosis Oxidative stress, Histopathology.

Introduction

Aquaculture expanded rapidly over the past decades as a vital source of high-quality animal protein [1-3]. Nile tilapia *(Oreochromis niloticus)* is one of the most significant freshwater fish among cultured species due to its low production costs, adaptability to poor water conditions, high consumer demand, and rapid growth rate. In Egypt, tilapia accounts for over 65% of total fish production, making it a cornerstone of the national aquaculture industry [4, 5].

However, intensive aquaculture systems posed significant risks, especially the outbreak of bacterial diseases, which caused considerable economic losses [6-8] . Among the major bacterial pathogens affecting tilapia, *Streptococcus* species, particularly *S. agalactiae*, play a central role in disease outbreaks.[9, 10]. These infections were often associated with environmental stress, high water temperatures, poor handling, and suboptimal water quality [11-13].

S. agalactiae is a gram-positive, β-hemolytic bacterium that affects a wide range of freshwater and marine fish species [14] and has also been recognized as a zoonotic agent. In fish, it causes septicemia, meningoencephalitis, and high mortality rates, especially under stressful conditions [15, 16]. The disease was reported globally in tilapia farms across countries such as Thailand, Brazil, Malaysia, and Egypt [12, 17, 18].

In Egypt, several outbreaks of *S. agalactiae* infections were documented in recent years, particularly during the summer. Despite this, limited studies explored the combined pathological, molecular, and oxidative stress effects of this pathogen in naturally and experimentally infected tilapia [11, 19-22].

Although outbreaks of *S. agalactiae* in Egyptian tilapia farms have been increasingly documented,

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(Received 20 June 2025, accepted 30 August 2025)
DOI: 10.21608/ejvs.2025.395469.2912

few studies have explored the combined pathological, molecular, and oxidative stress responses associated with this infection. This study aimed to characterize an outbreak of *S. agalactiae* in Nile tilapia in the Manzala region by identifying the pathogen through molecular techniques, assessing histopathological changes, and measuring oxidative stress biomarkers following experimental infection. The goal was to provide comprehensive insights into the disease mechanisms and support improved management strategies in Egyptian aquaculture.

Material and Methods

Study site and water quality assessment

This study was conducted at a private aquaculture facility in Dakahlia Province, Egypt, where Nile tilapia (*Oreochromis niloticus*) was raised in traditional earthen ponds, each approximately 1 hectare in area and 1.5 meters in depth. Ponds were filled with water from adjacent agricultural drainage canals and operated under a semi-intensive system utilizing natural productivity alongside commercial pelleted feed. Routine water exchange was implemented to maintain optimal water conditions.

Water samples (n = 3, 1 L each) were collected from the affected pond using a column sampler. Physicochemical parameters were measured as follows: salinity using a handheld salinometer, water temperature using a glass mercury thermometer, and dissolved oxygen (DO) with a portable oximeter. Additional parameters, pH, un-ionized ammonia, nitrite, nitrate, and hydrogen sulfide, were assessed using the LaMotte® Aquaponics Kit (Code 3637, LaMotte Co., MD, USA), according to [23] .

Fish sampling and clinical examination

Fish sampling was conducted during a mortality outbreak that occurred in the summer of 2021 (June–August). Daily mortalities were recorded over three consecutive days, peaking during periods of elevated ambient temperatures. A total of 300 Nile tilapia (100–120 g) were collected, including freshly dead specimens transported on ice and live moribund fish transported in aerated tanks and euthanized upon arrival to minimize stress.

All samples were transferred immediately to the Microbiology Laboratory at the Animal Health Research Institute (Mansoura Branch) for further analysis. Clinical signs were assessed following the criteria of [24], while postmortem findings were recorded according to the methods of [25, 26].

Bacterial isolation and phenotypic identification

To isolate the causative bacterial agent, moribund and Moribund and freshly dead fish were externally sterilized with 70% ethanol. Under aseptic conditions, internal tissues (liver, kidney, spleen, brain, and eyes) were sampled using sterile swabs and inoculated into tryptic soy broth (TSB; Oxoid, UK). Tubes were incubated at 28°C for 24 hours to enrich bacterial growth.

Enriched samples were streaked onto Modified Edward agar (Oxoid, UK) supplemented with 5–7% defibrinated sheep blood, and blood agar plates (Oxoid, UK)[27], [28]. Plates were incubated aerobically at 28°C for 24 hours.

Colonies were subcultured for purification and subjected to phenotypic identification, including Gram staining, hemolysis pattern assessment, and biochemical tests (catalase, oxidase, and esculin hydrolysis).

Molecular confirmation via PCR and sequencing

DNA extraction

Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Germany, Cat. No. 51304) following the manufacturer's instructions. Bacterial pellets were lysed, precipitated with ethanol, purified using spin columns, and eluted in 100 µL of elution buffer. DNA was stored at −20°C.

PCR amplification of the 16S rRNA gene

Amplification was carried out using species-specific Amplification was carried out using species-specific primers targeting the 16S rRNA gene:

Forward: 5′-AGAGTTTGATCMTGGCTCAG-3′
Reverse: 5′-TACGGYTACCTTGTTACGACTT-3′ [29].

Each 25 µL reaction mixture contained 12.5 µL EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µL of each primer (forward and reverse) (20 pmol), 5.5 µL of nuclease-free water, and 5 µL of template DNA. Reactions were performed in an Applied Biosystems 2720 thermal cycler (Biometra, USA). with the following conditions: initial denaturation at 94°C for 5 min; 35 cycles of 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min; final extension at 72°C for 10 min.

Gel electrophoresis

PCR products were resolved on a 1.5% agarose gel (AppliChem, Germany) in 1× TBE buffer at 120 V for 90 min. Gels were stained with SYBR Safe, visualized under UV light, and analyzed using a gel documentation system (Alpha Innotech, Biometra). Product sizes were compared to a 100 bp DNA ladder (Qiagen, Germany).

Sequencing and phylogenetic analysis

Amplified products were purified using the QIAquick PCR Extraction Kit (Qiagen Inc., Valencia, CA, USA) and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (PerkinElmer, Foster City, CA). Sequencing reactions were cleaned using Centrisep spin columns

(Cat. CS-901) and analyzed with the Applied Biosystems 3130 Genetic Analyzer (HITACHI, Japan). Sequences were compared to GenBank entries using BLAST® (Basic Local Alignment Search Tool) for species confirmation [30]. Multiple sequence alignments and phylogenetic tree construction were conducted using the CLUSTAL W version 1.83 program within the MegAlign module of the Lasergene software (DNASTAR Inc.) [31]. The genetic similarities among different species or strains were analyzed using MEGA 6 software. applying the maximum likelihood method with 1000 bootstrap replications [32].

Experimental infection trial

Fish acclimatization and experimental design

Ninety healthy Nile tilapia (average weight ~50 g) were obtained from a commercial hatchery and acclimated for two weeks in six 300-liter aquaria under controlled water conditions (26 ± 1°C, DO: 6.5–7.5 mg/L, pH: 7.1–7.5). Fish were fed a commercial pellet diet at 1.5% of body weight twice daily [33]. Water quality was maintained by daily partial renewal. Following acclimation, fish were randomly divided into six groups (15 fish per group). Five groups were challenged by intraperitoneal injection with 0.5 mL of *Streptococcus agalactiae* suspension at different concentrations (1.0×10^7 to 5.0×10^8 CFU/mL). The sixth group received sterile phosphate-buffered saline (PBS) as a negative control. All procedures were performed under anesthesia using tricaine methanesulfonate (MS-222, 100 mg/L; Sigma-Aldrich, USA). Each group was maintained in triplicate.

Fish were monitored for 7 days. Moribund and dead individuals were sampled for re-isolation of *S. agalactiae* from kidney, liver, and brain tissues on tryptic soy agar to confirm infection.

Calculation of median lethal dose (LD_{50})

The median lethal dose (LD_{50}) of *Streptococcus agalactiae* was determined by intraperitoneal injection of Nile tilapia with serial tenfold dilutions of bacterial suspensions ranging from 1.0×10^7 to 5.0×10^8 CFU/mL. Each group included 15 fish, and mortality was monitored daily for 7 days. To estimate the LD_{50}, mortality percentages were plotted against the logarithmic bacterial doses to construct a dose-response (mortality) curve. The Reed and Muench method [34] was used to interpolate the LD_{50}, defined as the dose causing 50% mortality. The formula used was: log LD_{50} = log D_lower + ((50 − M_lower) / (M_higher − M_lower)) × log (D_higher / D_lower). Where: (D_lower = Dose below 50% mortality, D_higher = Dose above 50% mortality, M_lower = Mortality (%) at D_lower, M_higher = Mortality (%) at D_higher).

To visualize the dose-response relationship, a mortality curve was plotted by graphing mortality percentages against the logarithm of the bacterial doses (CFU/mL) using GraphPad Prism.

Histopathological examination

Liver, spleen, kidney, brain, and muscle tissues were collected from both infected and control groups on days 3 and 7 post-infection and fixed in 10% neutral buffered formalin for 24 h. Fixed tissues were routinely processed, embedded in paraffin, sectioned at 5 µm thickness, and stained with hematoxylin and eosin (H&E). Sections were examined microscopically (Olympus CX 31, Tokyo, Japan), and representative lesions were photographed [35].

Oxidative stress and antioxidant enzyme assays

Liver and kidney tissues were collected from both infected and control fish at days 3 and 7 post-infection. Approximately 10 mg of each tissue was rinsed with cold saline, homogenized in phosphate-buffered saline (PBS; pH 7.5), and centrifuged at 8000 rpm for 5 minutes. The resulting supernatants were used for biochemical analyses. Concentrations and activities of nitric oxide (NO), reduced glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) were quantified using commercially available colorimetric assay kits (Bio-Diagnostics Co., Giza, Egypt) following the manufacturer's protocols. The corresponding catalog numbers are listed below.

Nitric oxide (NO) concentration was determined according to Griess reaction methodology and expressed as µmol/g tissue. Briefly, 100 µL of tissue homogenate was mixed with an equal volume of Griess reagent (2.5% phosphoric acid, 1% sulfanilamide, 0.1% N-naphthylethylenediamine), incubated at 27°C for 10 minutes in a 96-well microplate, and the resulting azo dye was measured at 540 nm. The concentration of NO was calculated using a sodium nitrite standard curve (Catalog No. CA 25 33) [36].

Reduced glutathione (GSH) was assessed using the DTNB method, based on the formation of the yellow-colored 5-thio-2-nitrobenzoic acid (TNB), and expressed as mmol/g tissue. Absorbance was measured at 412 nm following reaction with DTNB, phosphate buffer (pH 7.4), and NADPH (Catalog No. GR 25 11) [37] .

Glutathione peroxidase (GPx) activity was measured using an NADPH-dependent coupled enzymatic assay and reported as U/g tissue. The reaction involved phosphate buffer, glutathione, glutathione reductase, and NADPH, with H_2O_2 added as the substrate. GPx activity was determined by the rate of NADPH oxidation, measured at 340 nm (Catalog No. Gp 25 24) [38] .

Superoxide dismutase (SOD) activity was estimated following the method described by [39], expressed as U/g tissue. The assay is based on the inhibition of phenazine methosulfate-mediated reduction of nitro-blue tetrazolium (NBT) by SOD. The reaction mixture (50 mM phosphate buffer, 1 mM NADH, and 1 mM NBT) was combined with 100 µL homogenate and terminated using 0.1 mM phenazine methosulfate. Absorbance was read at 560 nm after 5 minutes of incubation at 25°C, and activity was calculated using a multiplication factor of 3.75 (Catalog No. SD 25 21) [39] . Catalase (CAT) activity was measured using a colorimetric assay [40] and expressed as U/g tissue. The rate of H_2O_2 decomposition was monitored by mixing tissue homogenate with phosphate buffer (pH 7.0) and H_2O_2 and stopping the reaction with ammonium molybdate. The resulting yellow complex was measured at 405 nm (Catalog No. CA 25 17) [40] . Malondialdehyde (MDA) concentration was assessed as a marker of lipid peroxidation using the thiobarbituric acid reactive substances (TBARS) method and reported as nmol/g tissue. Homogenates (200 µL) were incubated with TBA reagent at 95°C for 30 minutes, cooled, extracted with n-butanol, and measured at 534 nm (Catalog No. MD 25 29) [41] .

Statistical analysis

Data were analyzed using SPSS v20.0 (IBM Corp., Armonk, NY, USA). One-way ANOVA followed by Duncan's multiple range test was used to compare the control and infected groups. Results are expressed as mean ± standard error (SEM), with statistical significance set at $p < 0.05$.

Results

Water quality assessment

Water quality parameters from the affected pond during the outbreak are summarized in (Table 1).

Dissolved oxygen was significantly reduced (3.5 ± 0.11 mg/L), falling below optimal levels for tilapia culture. Water temperature reached 32.5 ± 0.8°C, exceeding the ideal range (25–30°C). Although pH remained within acceptable limits (7.85 ± 0.67), concentrations of hydrogen sulfide (290 ± 17.2 µg/L), un-ionized ammonia (0.42 ± 0.015 mg/L), and nitrite (3.5 ± 0.05 mg/L) were markedly elevated and exceeded safe thresholds. These suboptimal conditions likely induced physiological stress and contributed to increased susceptibility to infection and mortality [42].

Clinical and postmortem findings

Infected Nile tilapia (*Oreochromis niloticus*) exhibited prominent neurological and behavioral disturbances, including erratic spiraling, loss of equilibrium, anorexia, and reduced responsiveness to external stimuli. Externally, affected fish showed extensive hemorrhagic lesions, particularly in the ventral regions beneath the pectoral and pelvic fins, as well as scale detachment over the dorsal musculature, tail fin erosion, and signs of hemorrhagic vent prolapse. Notably, ocular abnormalities such as corneal opacity and bilateral exophthalmia were also frequently observed (Fig. 1a, b).

Internal examination revealed severe congestion and hemorrhages in the dorsal musculature, spleen, head kidney, and liver, accompanied by marked gallbladder distension (Fig. 1c, d). These clinical and pathological findings are indicative of acute systemic septicemia, which is consistent with *Streptococcus agalactiae* infection in Nile tilapia.

Cultural and biochemical characterization of Streptococcus agalactiae

Colonies of *Streptococcus agalactiae* grown on Edward agar appeared as small, bluish, smooth colonies with distinctive white edges. On blood agar, the isolates formed pinpoint, white colonies surrounded by clear β-hemolytic zones, indicating hemolytic activity. Biochemical identification revealed that the isolates were Gram-positive cocci arranged in chains, non-motile, oxidase-negative, catalase-negative, and unable to hydrolyze esculin.

Prevalence of Streptococcus agalactiae in naturally infected Nile tilapia

During the disease outbreak, a total of 300 Nile tilapia (*Oreochromis niloticus*) specimens were collected and examined. Conventional biochemical identification revealed that 120 isolates (40%) were presumptively identified as *Streptococcus* spp. Subsequent molecular confirmation using PCR targeting the 16S rRNA gene and sequencing analysis validated 45 isolates (37.5% of *Streptococcus* spp. isolates; 45/120) as *S. agalactiae*. This corresponds to an overall prevalence of 15% (45/300) among the examined fish, indicating that *S. agalactiae* played a significant role in the observed disease outbreak.

PCR amplification of the 16S rRNA gene yielded a distinct band of approximately 1,485 bp, consistent with the expected size for *Streptococcus agalactiae* (Fig. 2). The amplified product was sequenced, and the resulting nucleotide sequence was deposited in the GenBank database under accession number OQ842337. BLAST analysis of the sequence revealed a 97.52% identity with previously published *S. agalactiae* sequences from multiple geographic origins, confirming the identity of the isolate.

Phylogenetic analysis was conducted using the maximum-likelihood method in MEGA version 6. The generated phylogenetic tree demonstrated that the isolate clustered closely with reference *S. agalactiae* strains (Fig. 3). The analysis revealed a high degree of genetic similarity among *S.*

agalactiae strains, indicating evolutionary conservation in the 16S rRNA gene region.

Experimental infection and determination of LD_{50}

Following intraperitoneal injection with increasing doses of *Streptococcus agalactiae* (1.0×10^7 to 5.0×10^8 CFU/mL), Nile Tilapia exhibited dose-dependent mortality, with no deaths in the PBS-injected control group. Cumulative mortality rates over 7 days were: 0% (1.0×10^7), 15% (5.0×10^7), 40% (1.5×10^8), 55% (2.0×10^8), 75% (3.5×10^8), and 90% (5.0×10^8 CFU/mL). Mortalities began within 24 hours at higher doses ($\geq 2.0 \times 10^8$), peaking between 48–72 hours.

Clinically, infected fish showed lethargy, exophthalmia, loss of balance, skin darkening, abdominal swelling, and hemorrhages. Internally, hepatomegaly, splenomegaly, and ascitic fluid were observed. *S. agalactiae* was consistently re-isolated from the kidney, liver, and brain of dead and moribund fish.

A sigmoidal dose-response curve (Fig. 4) was generated by plotting mortality against log-transformed bacterial doses. The median lethal dose (LD_{50}) was calculated as 1.82×10^8 CFU/mL using the Reed and Muench method. These findings confirm the strain's virulence and validate the model for future challenge and vaccine studies.

Histopathological findings

Histopathological examination of infected Nile tilapia revealed pronounced lesions in multiple organs, while tissues from the control group exhibited normal histoarchitecture. By day 3 post-infection, hepatocytes showed hydropic degeneration and vascular congestion (Fig. 5c). By day 7, lesions progressed to include diffuse edema, erythrocyte extravasation, and multifocal hemosiderin deposition, indicative of chronic hemorrhagic degeneration (Fig. 5e, f). On day 7, the hepatopancreas exhibited hyperplasia, vacuolar degeneration of acinar cells, and increased melanomacrophage center (MMC) aggregation, reflecting active inflammation and immune activation (Fig. 5d). Renal damage was evident as early as day 3, characterized by tubular epithelial detachment, interstitial edema, perivascular fibrosis, necrotic MMCs, and leukocytic infiltration (Fig. 6b–e). By day 7, notable MMC proliferation was observed, suggesting ongoing immune stimulation (Fig. 6f). By day 3, the spleen showed lymphoid depletion and dilated blood vessels, with increased MMC activity (Fig. 7b, c). On day 7, focal necrosis of MMCs was observed, indicating advanced tissue damage and compromised immune regulation (Fig. 7d).

Neuronal vacuolation and degeneration were noted at day 3 (Fig. 8b, c). By day 7, neurons exhibited pyknosis and cytoplasmic degeneration, indicating irreversible neuronal injury (Fig. 8d).

Early muscle pathology included widespread subcutaneous fiber vacuolation. With disease progression, loss of striation and continued vacuolation indicated advancing muscle fiber degeneration (Fig. 9).

Oxidative stress and antioxidant enzyme assays

The oxidative and antioxidative profiles of Nile tilapia post-infection were assessed by measuring NO, GSH, GPx, SOD, CAT, and MDA levels in liver and head kidney tissues (Figs. 10–15; Tables 2–3). Nitric oxide (NO) levels were significantly elevated in infected fish at both 3- and 7-day post-infection ($p < 0.05$), indicating heightened inflammatory and oxidative activity (Fig. 10). A slight decline between days 3 and 7 suggests potential modulation over time.

In contrast, antioxidant biomarkers were markedly suppressed in infected fish. GSH levels significantly decreased at both times, with a further reduction by day 7 (Fig. 11), indicating depletion of intracellular antioxidants. GPx activity also declined substantially over time (Fig. 12), reflecting impaired enzymatic defense. SOD activity was significantly lower in infected fish compared to controls at both times (Fig. 13). Although a mild increase was noted on day 7, values remained significantly below control levels. Similarly, CAT activity declined significantly post-infection, with continued suppression on day 7 (Fig. 14). Conversely, MDA, a marker of lipid peroxidation, was significantly elevated at both time points in the infected group ($p < 0.05$), with further increases by day 7, confirming progressive oxidative tissue damage (Fig. 15).Collectively, the results demonstrate that *S. agalactiae* infection induces substantial oxidative stress in Nile tilapia, characterized by elevated NO and MDA and concurrent depletion of GSH, GPx, SOD, and CAT, especially during the subacute phase.

Discussion

Aquaculture is a key contributor to Egypt's food security and economy, supplying an essential source of animal protein [43]. However, bacterial infections, particularly in tilapia farming, pose a significant threat by causing considerable mortalities and economic losses [5, 44]. Among emerging pathogens, *Streptococcus agalactiae* (Group B Streptococcus, GBS) is a primary causative agent of streptococcosis in both freshwater and marine fish, with reported mortality rates of 50–70% under intensive aquaculture conditions [15, 45, 46]. In Egypt, *S. agalactiae* has been repeatedly isolated from diseased tilapia (*Oreochromis niloticus*), as well as seabream, seabass, catfish, mullet, and rainbow trout during disease outbreaks [22,

47].These outbreaks are often associated with environmental stressors such as high water temperatures (>27°C), low dissolved oxygen, and elevated levels of ammonia and nitrite, which compromise the immune system of fish and increase their susceptibility to infection [5, 9, 48, 49]. In this study, water quality in the affected pond during the summer outbreak was suboptimal, with reduced dissolved oxygen (3.5 mg/L), elevated temperature (32.5°C), and increased levels of hydrogen sulfide, un-ionized ammonia, and nitrite, all exceeding the safe thresholds for Nile tilapia culture. These adverse conditions likely imposed physiological stress, impairing immune defenses and promoting the proliferation of *Streptococcus agalactiae*. Similar associations between environmental stressors and *S. agalactiae* outbreaks have been reported by a previous study [42], who emphasized that poor water quality compromises fish health and predisposes them to infectious diseases.

S. agalactiae was isolated during summer outbreaks from farmed Nile tilapia in Lake Manzala, with a prevalence of 15% (45/300 fish examined). This finding is consistent with previous reports from Egypt, which documented prevalence rates of approximately 13% [50]. Clinically, infected Nile tilapia exhibited neurological signs including erratic swimming, loss of equilibrium, and anorexia, along with external haemorrhages and ocular abnormalities. These manifestations likely reflect the neuroinvasive capacity of *Streptococcus agalactiae*, which can penetrate the central nervous system and induce meningoencephalitis and neuronal degeneration [51, 52] .The bacteria can cross the blood-brain barrier, leading to inflammation, edema, and neuronal degeneration, which explains the observed behavioral abnormalities [16]. Externally, haemorrhagic lesions and ocular signs, including exophthalmia, are attributed to septicaemia-induced vascular injury and increased capillary permeability. The systemic dissemination of bacteria and associated release of toxins and proinflammatory mediators contribute to endothelial damage, resulting in haemorrhage and tissue necrosis[20, 50, 53, 54].General septicaemic signs accompany these outbreaks and align with those of [22, 55, 56].

Internally, congestion and hemorrhages in vital organs such as the liver, spleen, and kidney reflect widespread vascular injury and inflammatory responses typical of acute systemic septicemia. These pathological changes disrupt organ function and contribute to the rapid decline in fish health. These findings may be attributed to the exotoxin released by *S. agalactiae* at high temperatures [20] Hemolysins, proteases, and other toxins rapidly come into contact with various organs, facilitating the emergence and spread of pathogens in the host [53]. Thus, the clinical signs observed in this study are the direct consequences of the invasive and systemic nature of *S. agalactiae* infection, which triggers severe inflammatory reactions and tissue damage across multiple organ systems, explaining the high morbidity and mortality during outbreaks.

The phenotypic characteristics of the isolates, including gram-positive, β-hemolytic, non-motile cocci that were oxidase-, catalase-, and esculin-negative, align well with the classical identification features of *Streptococcus agalactiae* as reported by [57]. The subsequent molecular confirmation through PCR amplification of the 16S rRNA gene, producing a ~1485 bp fragment, further validated the identity of the isolates, exhibiting a high sequence homology (97.52%) with known reference sequences [29]. This molecular approach enhances diagnostic accuracy by providing genetic evidence for species-level identification. While 16S rRNA gene sequencing is effective for species-level identification, it lacks sufficient resolution to differentiate subspecies or strain-level variants of *S. agalactiae*. This limitation highlights the need for higher-resolution molecular tools, such as multilocus sequence typing (MLST) or whole-genome sequencing, to support detailed epidemiological studies and characterize pathogenic diversity [57].Such advanced methods could provide insights into strain-specific virulence factors and transmission dynamics, which are critical for designing effective control strategies in Tilapia aquaculture.

Experimental infection demonstrated a clear dose-dependent mortality in Nile tilapia, with an estimated LD_{50} of 1.82×10^8 CFU/mL, confirming the high virulence of the isolated *S. agalactiae* strain. This reproducible challenge model is essential for assessing pathogenicity and serves as a reliable platform for vaccine efficacy trials. Comparable experimental approaches have been reported in previous studies [58, 59], which demonstrated the utility of experimental infection models in defining lethal doses and assessing vaccine protection against *S. agalactiae* in fish. The consistency of our LD_{50} estimate with previous studies further supports the reliability of the strain and the experimental design for future immunological and therapeutic investigations.

Histopathological analysis of infected Nile tilapia revealed progressive systemic lesions consistent with *S. agalactiae*-induced septicaemia. By day 3 post-infection, hepatic vascular degeneration and vascular congestion were observed, indicating early liver damage. By day 7, a marked increase in Melanomacrophage center (MMC) activity suggested a sustained immune response. Similar hepatic alterations, including hepatocellular degeneration, inflammatory infiltration, and portal vessel congestion, have been reported in naturally infected *O. niloticus* [56]. Renal tissues displayed prominent pathological

features such as epithelial detachment, interstitial edema, and early necrosis of MMCs, signifying impaired excretory and immune functions. The spleen exhibited lymphoid depletion, vascular dilation, and prominent MMC activation, collectively pointing to immune exhaustion following intense stimulation [56, 60]. The same result was observed in red tilapia *(Oreochromis sp.)* naturally and experimentally infected by *S. agalactiae* [61, 62].

Neuropathological changes, including neuronal degeneration and vacuolation in brain tissue, likely contributed to the acute neurological signs and rapid mortality observed in infected fish, which may be linked to the action of bacterial cytolysins [53, 63]. Additionally, skeletal muscle lesions such as severe vacuolation were noted at day 3, with partial resolution by day 7, suggesting initial acute tissue damage followed by regenerative processes. Coagulative necrosis and mononuclear cell infiltration observed in naturally infected fish further supported the presence of systemic bacterial dissemination and septicemic pathology [64-66]. Collectively, these findings confirm that *S. agalactiae* causes widespread organ damage and elicits robust immune responses, consistent with the pathophysiology of acute streptococcosis in tilapia.

This study assessed the immunopathological effects of *Streptococcus agalactiae* infection in Nile tilapia, with emphasis on oxidative stress and antioxidant defense mechanisms. Infection led to significant alterations in nitric oxide (NO) levels, suppressed activities of key antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), and elevated malondialdehyde (MDA), indicating enhanced lipid peroxidation. These findings underscore the pivotal role of oxidative stress in the pathogenesis of *S. agalactiae*, contributing to tissue damage and disease progression. Elevated NO levels in hepatic, renal, and branchial tissues indicate robust immune activation, as nitric oxide functions both as a cytotoxic effector and a marker of inflammation. Although moderate NO production contributes to pathogen clearance, excessive levels can lead to oxidative damage and tissue inflammation, consistent with previous observations [67-69]. A biphasic pattern in antioxidant enzyme activity was observed. By day 3 post-infection, activities of SOD, CAT, and GPx were significantly reduced, likely reflecting early depletion of antioxidant defenses as metabolic resources were redirected to counteract infection-induced oxidative stress [70]. By day 7, these enzyme activities rebounded, suggesting a compensatory upregulation in response to continued accumulation of reactive oxygen species (ROS) [71]. MDA levels, however, remained elevated throughout, confirming persistent oxidative damage and increased lipid peroxidation in infected tissues [45, 72]. Oxidative stress, characterized by an imbalance between pro-oxidants and antioxidants, leads to cellular dysfunction and immune compromise [73, 74] . Antioxidant enzymes are essential for neutralizing reactive oxygen species (ROS): SOD converts superoxide radicals into hydrogen peroxide (H_2O_2), which is subsequently degraded by CAT and GPx into water and oxygen [75-77]. GPx relies on reduced glutathione (GSH) to perform its function, and GSH is also crucial for general redox homeostasis and innate immune modulation [78, 79].

In this study, hepatic and renal antioxidant capacities were compromised, in line with earlier reports of reduced GSH activity in tilapia infected with *S. iniae* [79]. The sustained elevation of MDA, especially at both day 3 and day 7, indicates continuous oxidative injury. MDA is a key biomarker of lipid peroxidation that disrupts membrane integrity and cellular function [80]. These oxidative insults are closely associated with the hepatic and renal lesions observed in this and other studies involving *S. agalactiae*-infected fish species, including silver catfish and zebrafish [81] and [82] . Such damage likely contributes to the high morbidity and mortality seen in streptococcal infections, as oxidative stress not only exacerbates inflammation but also impairs the tissue recovery process [83].

Conclusion

In conclusion, pathogenic *Streptococcus agalactiae* was isolated from *O. niloticus* during a disease outbreak associated with significant mortality and economic losses in summer 2021. The identity of the isolates was confirmed by morphological, biochemical, and molecular characterization, including 16S rRNA sequencing. Histopathological analysis revealed lesions consistent with *S. agalactiae* infection in experimentally challenged fish. Biochemically, infected fish exhibited a marked decrease in CAT, SOD, GSH, and GPx activities, accompanied by increased NO and MDA levels, indicative of oxidative stress and immune activation, so future research on pathogen-host interactions and disease management strategies in tilapia aquaculture can be applied effectively.

Acknowledgments: Not applicable.

Funding statement

This study didn't receive any funding support

Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Ethics of approval

The experimental design was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Mansoura University, Egypt (protocol code: M/31).

TABLE 1. Physical and chemical analysis of water during the disease outbreak in Manzala fish farm, in pond-cultured Nile tilapia

Water parameter	Result of our investigations
Temperature (°C)	32.5 ± 0.8
Dissolved oxygen (DO) (mg/l)	3.5±0.11
pH	7.85±0.67
Salinity (g/l)	1.3±0.04
unionized ammonia (NH3) (mg/l)	0.42 ±.015
Nitrite (NO2) (mg /l)	3.5 ±0.05
Nitrate (NO3) (mg/l)	2.4 ±0.04
Hardness (mg/l)	180.2±5.8
Chloride (mg/l)	120±8.3
Hydrogen sulfide (H2S) (mg/l)	290 ±17.2

*Means with different letters are considered significant ($p<0.05$).

TABLE 2. Evaluation of hepatic oxidant and anti-oxidative stress enzymes of experimental groups of *O. niloticus*

Hepatic oxidative stress enzymes	Experimental groups		
	Control group	Infected group	
		Day 3 post-challenge	Day 7 post-challenge
NO (µmol/g tissue)	1.67 ^{c} ± 0.22	5.14 ^{a} ± 0.25	3.24 ^{b} ± 0.24
GSH (mmol/g tissue)	0.35 ^{a} ± 0.01	0.18 ^{c} ± 0.01	0.24 ^{b} ± 0.02
GPx (U/g tissue)	50.43 ^{a} ± 2.11	18.36 ^{c} ± 2.07	33.9 ^{b} ± 1.59
SOD (U/g tissue)	152 ^{a} ± 4.73	68.73 ^{c} ± 4.94	109.23 ^{b} ± 8.23
CAT (U/g tissue)	43.5 ^{a} ± 4.39	11.33 ^{c} ± 1.12	25.4 ^{b} ± 3.7
MDA (nmol/g tissue)	308 ^{c} ± 11.15	459.4 ^{a} ± 18.5	393.26 ^{b} ± 12.62

***** Means with the same letters are considered non-significant.

TABLE 3. Evaluation of renal oxidant and anti-oxidative stress enzymes of experimental groups of *O. niloticus*

Renal oxidative stress enzymes	Experimental groups		
	Control group	Infected group	
		Day 3 post challenge	Day 7 post-challenge
NO (µmol/g tissue)	0.98 ^{c} ± 0.03	2.99 ^{a} ± 0.15	1.98 ^{b} ± 0.19
GSH (mmol/g tissue)	0.23 ^{a} ± 0.01	0.11 ^{c} ± 0.01	0.17 ^{b} ± 0.01
GPx (U/g tissue)	36.3 ^{a} ± 1.96	15.1 ^{c} ± 2.62	26.73 ^{b} ± 1.88
SOD (U/g tissue)	132.13 ^{a} ± 3.16	61.66 ^{c} ± 7.35	94.76 ^{b} ± 3.69
CAT (U/g tissue)	37.53 ^{a} ± 3.94	12.53 ^{b} ± 1.32	18.73 ^{b} ± 1.91
MDA (nmol/g tissue	195.03 ^{c} ± 11.04	366.43 ^{a} ± 2.23	264.13 ^{b} ± 13.34

*Means with different letters are considered significant ($p < 0.05$) . Means with the same letters are considered non-significant

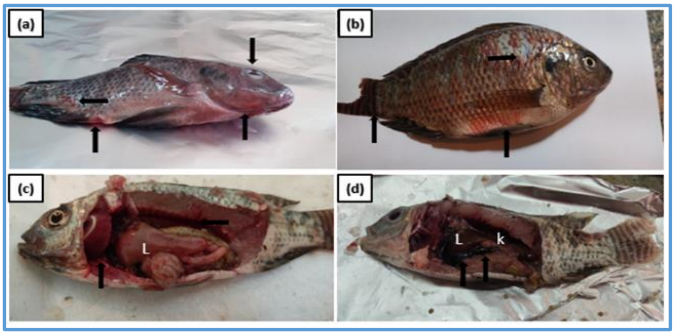

Fig. 1. Naturally infected *O. niloticus* showed (a) excess mucus secretion, ocular opacity, bleeding on the ventral aspect, and under the lower jaw with a hemorrhagic prolapsed vent. (b) Extensive hemorrhage over the body, especially at the base of the pectoral fin and on the belly, accompanied by detached scales, skin erosion progressed to the ulcerative area, and tail erosion. (c) congested gills, congested liver (L), and muscle redness. (d) Congested liver (L) with distended gall bladder, congested kidney (K), and dark, congested spleen (black arrows).

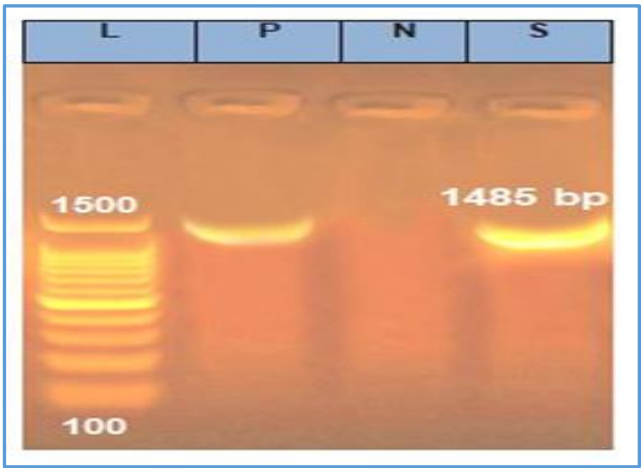

Fig. 2. PCR amplification of the 16S rRNA gene of *S. agalactiae* isolated from Nile tilapia *O. niloticus*. L: 100–1500 bp DNA size marker, Lane P: the control positive sample & Lane N: the control negative sample, Lane S: is the only positive sample. The PCR products shown correspond to the predicted molecular mass of 1485 bp

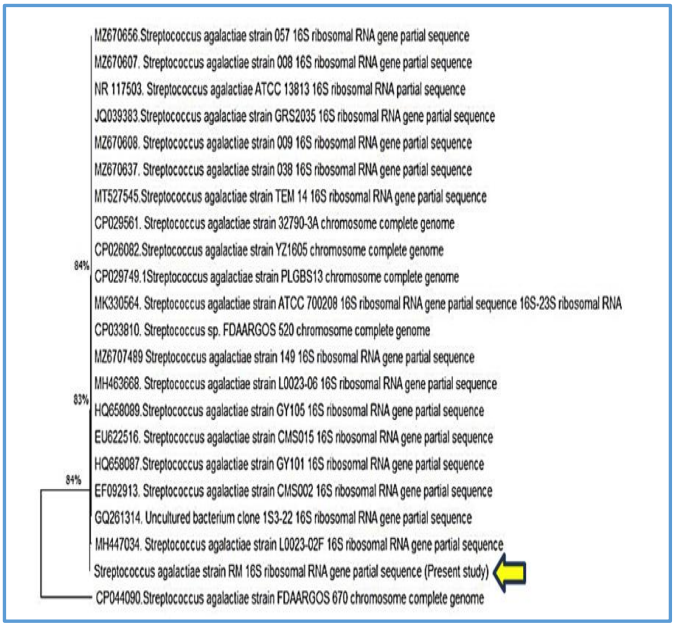

Fig. 3. Maximum likelihood phylogenetic tree showing relationships between *S. agalactiae* strain (OQ842337) isolated from diseased Nile tilapia (*O. niloticus)* and other reference strains of *S. agalactiae* based on 16S rRNA sequence. The percentages above the branches were obtained using a bootstrap approach with 1000 replicates.

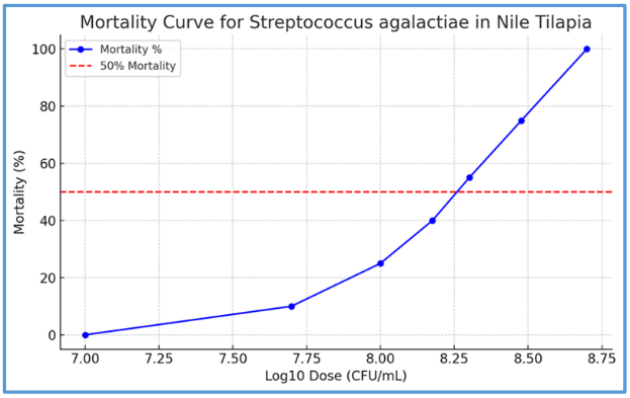

Fig.4. Mortality curve of Nile tilapia following intraperitoneal injection with serial dilutions of *Streptococcus agalactiae* ranging from 1.0×10^7 to 5.0×10^8 CFU/mL. Mortality percentages were plotted against the $\log_{10}$ of the bacterial concentrations. The curve shows a sigmoidal relationship, and the LD_{50} was calculated to be approximately 1.82×10^8 CFU/mL using the Reed and Muench method.

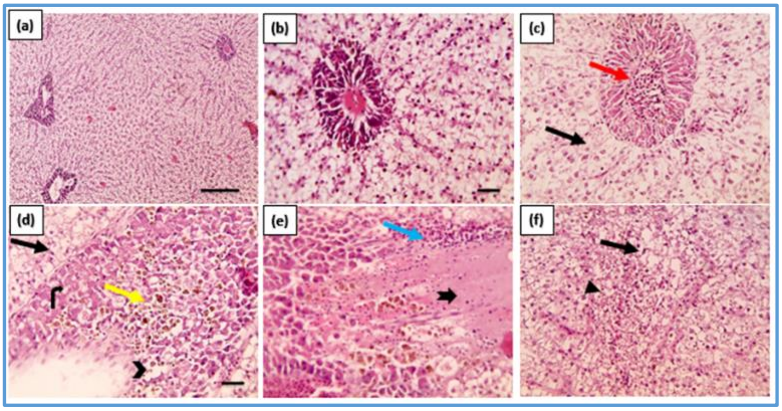

Fig. 5. (a) The photomicrograph of histological features of the *O. niloticus* liver shows normal hepatocytes and hepatopancreas, H&E, 100× (b) Liver shows normal hepatocytes and hepatopancreas, H&E, 400×. (C) Liver day 3 post-challenge shows hydropic degeneration of hepatocytes (black arrows) and congestion of blood vessels (red arrow), H&E, 400×. (d) Liver day 7 post-challenge shows hyperplasia of hepatopancreas (curved arrow) with vacuolation of pancreatic cells (open arrowhead) and aggregation of MMCs (yellow arrow), H&E, 400×. (e) Liver day 7 post-challenge shows extravasation of a few RBCs (blue arrow) with edema (thick arrow), H&E, 400×. (f) Liver day 7 post-challenge shows brown pigment in hepatocytes (closed arrowhead), H&E, 400×.

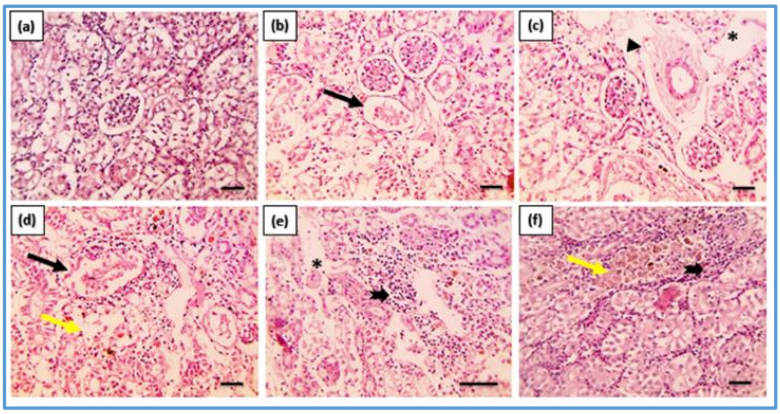

Fig. 6. (a)The photomicrograph of histological features of the *O. niloticus* kidney shows normal renal glomeruli and tubules, H&E, 400×. (b) Kidney day 3 post-challenge shows separation of the tubular epithelial lining (thin black arrows), H&E, 400×. (c) Kidney day 3 post-challenge shows interstitial edema (stars) and perivascular fibrosis (closed arrowhead), H&E, 400×. (d) Kidney day 3 post-challenge shows separation of the tubular epithelial lining (thin black arrows), and necrosis of MMCs (yellow arrows), H&E,400×. (e) Kidney day 3 post-challenge shows interstitial edema (stars) with leukocytic cells infiltration in interstitial tissue (thick arrows), H&E,400×. (f) Kidney day 7 post-challenge shows leukocytic cells infiltration in interstitial tissue (thick arrows) accompanied with aggregation of MMCs (yellow arrows), H&E, 400×.

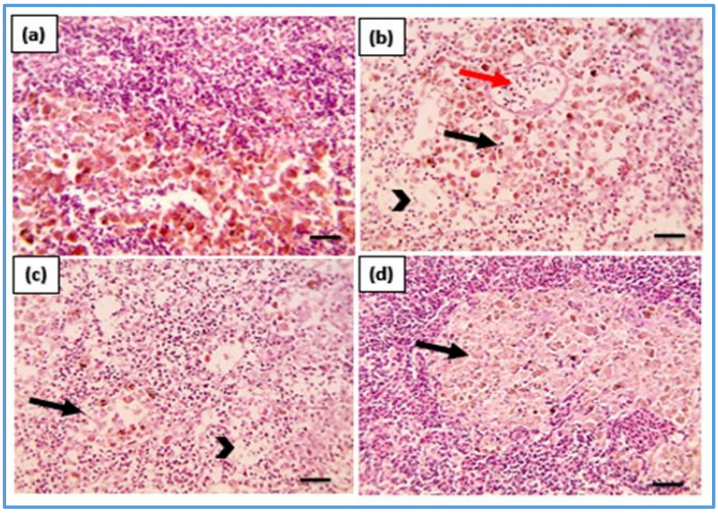

Fig. 7. (a)The photomicrograph of histological features of the *O. niloticus* spleen shows a normal lymphocytic cell population, many foci of MMCs, H&E,400×.(b) Spleen day 3 post-challenge, shows severe depletion of lymphocytic cells (black arrows) and aggregation of MMCs (arrowheads) besides dilated blood vessel (red arrow), H&E, 400×. (c) Spleen day 3 post-challenge, shows severe depletion of lymphocytic cells (black arrows) and aggregation of MMCs (arrowheads), H&E, 400×. (d) Spleen day 7 post-challenge, shows necrosis of MMCs (black arrows), H&E,400×.

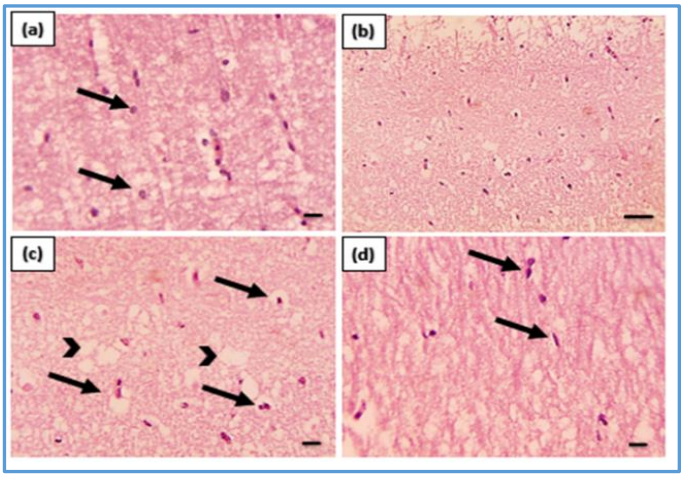

Fig. 8. (a)The photomicrograph of histological features of the *O. niloticus* brain shows normal neurons (black arrows), H&E,400×.(b) Brain day 3 post-challenge, shows many degenerated, deeply stained and shrunken neurons with prominent large vacuolation, H&E, 100×. (c) Brain day 3 post-challenge, shows many degenerated, deeply strained, and shrunken neurons (black arrows) with prominent large vacuolation (arrowheads), H&E, 400×. (d) Brain day 7 post-challenge, shows few degenerated deeply strained and shrunken neurons (black arrows), H&E,400× .

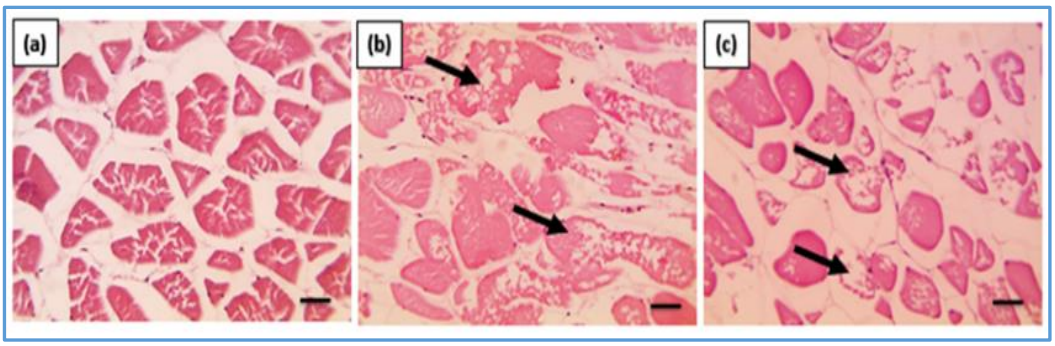

Fig. 9. (a)The photomicrograph of histological features of O. niloticus subcutaneous muscles shows normal striation of crossly sectioned muscle fibers, H&E,400×.(b) Subcutaneous muscles day 3 post-challenge, shows severe vacuolation of crossly sectioned muscle fibers (black arrows), H&E,400×. (c) Subcutaneous muscles day 7 post-challenge shows marked loss of straiation with vacuolation (black arrows), H&E,400×.

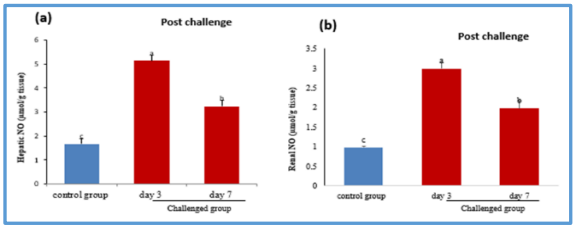

Fig. 10. (a) NO activity is significantly higher ($p < 0.05$) in the hepatic tissue of *S. agalactiae* experimentally exposed to *O. niloticus* compared to the control group. (b) NO activity is significantly higher ($p < 0.05$) in the renal tissue of *S. agalactiae* experimentally exposed *O. niloticus* compared to the control group. Data presented as mean ± SEM (standard error of the mean). Means with different letters are considered significant ($p < 0.05$).

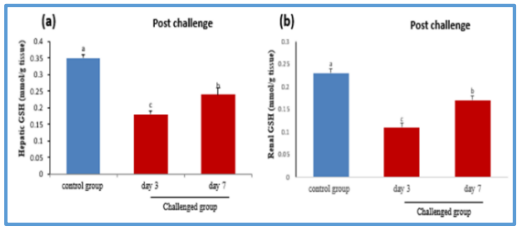

Fig. 11. (a) GSH activity is significantly lower ($p < 0.05$) in the hepatic tissue of *S. agalactiae* experimentally exposed *O. niloticus* compared to the control group. (b) GSH activity is significantly lower ($p < 0.05$) in the renal tissue of *S. agalactiae* experimentally exposed *O. niloticus* compared to the control group. Data presented as mean ± SEM (standard error of the mean). Means with different letters are considered significant ($p < 0.05$).

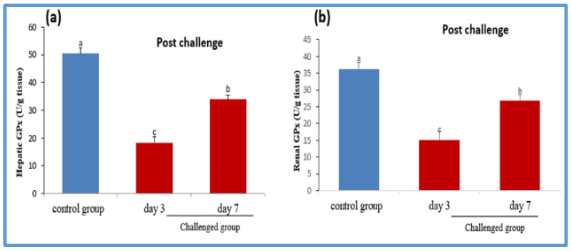

Fig. 12. (a) GPX activity is significantly lower ($p < 0.05$) in the hepatic tissue of *S. agalactiae* experimentally exposed *O. niloticus* compared to the control group. (b) GPX activity is significantly lower ($p < 0.05$) in the renal tissue of *S. agalactiae* experimentally exposed *O. niloticus* compared to the control group. Data presented as mean ± SEM (standard error of the mean). Means with different letters are considered significant ($p < 0.05$).

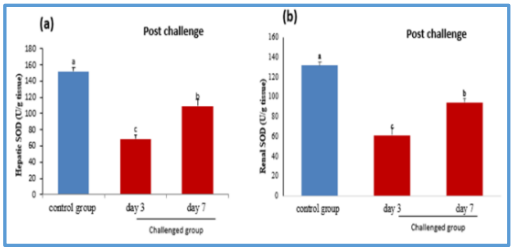

Fig. 13. (a) SOD activity is significantly lower ($p < 0.05$) in the hepatic tissue of *S.agalactiae* experimentally exposed *O. niloticus* compared to the control group. (b) SOD activity is significantly lower ($p < 0.05$) in the renal tissue of *S. agalactiae* experimentally exposed *O. niloticus* compared to the control group. Data presented as mean ± SEM (standard error of the mean). Means with different letters are considered significant ($p < 0.05$).

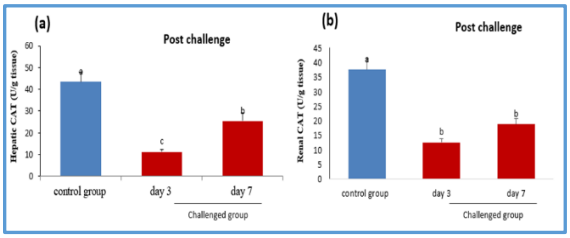

Fig. 14. (a) CAT activity is significantly lower ($p < 0.05$) in the hepatic tissue of *S. agalactiae* experimentally exposed *O. niloticus* compared to the control group. (b) CAT activity is significantly lower ($p < 0.05$) in the renal tissue of *S. agalactiae* experimentally exposed *O.* niloticus compared to the control group. Data presented as mean ± SEM (standard error of the mean). Means with different letters are considered significant ($p < 0.05$).

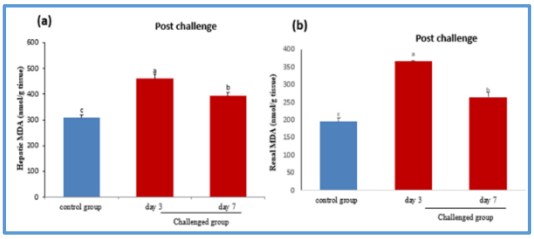

Fig. 15. (a) MDA activity is significantly higher ($p < 0.05$) in the hepatic tissue of *S. agalactiae* experimentally exposed *O. niloticus* compared to the control group. (b) MDA activity is significantly higher ($p < 0.05$) in the renal tissue of *S. agalactiae* experimentally exposed *O. niloticus* compared to the control group. Data presented as mean ± SEM (standard error of the mean). Means with different letters are considered significant ($p < 0.05$).

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دراسة ميكروبيولوجية تفاضلية على التوصيف المرضى والجزئي لبكتريا المكورات العقدية في البلطي النيلي

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الملخص

تناولت هذه الدراسة تفشى عدوى مرض المكورات العقدية في البلطي النيلي والتركيز على المسبب للمرض وكيفية التعرف عليه وتاثيره وقد أجريت هذه الدراسة في مزرعة سمكية خاصة بمنطقة المنزلة بمحافظة الدقهلية، خلال صيف عام ٢٠٢١.أظهرت الأسماك المريضة جحوظ العين، وإصابات نزيفية، واحتقان في الأعضاء الداخلية.تم التعرف الجزيئى للحمض النووى والتسلسل الجينى للمعزولات البكترية مع انتشار بنسبة ١٥٪. أدت العدوى التجريبية إلى إجهاد تأكسدي كبير، كما يتضح من المستويات المتغيرة لإنزيمات مضادات الأكسدة. وجد أن مستوى MDA وNO أعلى فى المجموعات المصابة مقارنة مع المجموعة الضابطة بينما انخفضت مستويات GPx,CAT, SOD, GSH في المجموعات المصابة مقارنة بمجموعة التحكم .ظهر مرض المكورات العقدية تأثرات هستوباثولوجية مختلفة على أنسجةالمختلفة مثل الكبد والكلى والطحال والمخ وتاثيره السلبى مؤكدةً على ضرورة تعزيز الأمن الحيوي، وأدوات التشخيص المبكر، واستراتيجيات فعّالة لمكافحة الأمراض للحد من الوفيات والخسائر الاقتصادية في تربية الأحياء المائية.

الكلمات الدالة: الاستربت كوكس ,البلطى النيلى ,التشخيص الجزيئى, الإجهاد التأكسدي,الهستوباثولوجى.