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Research Article

Molecular Characterization and Immunological Approaches Associated with Yellow Grub Trematode (Clinostomid) Infecting Nile Tilapia

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Yellow grub disease is caused by clinostomid metacercariae (CM) infecting freshwater fish and has a potential impact on human health by causing Halzoun disease. This study aimed to combine both molecular and immunological approaches to characterize CM naturally infecting Nile tilapia. Two hundred and fifty Nile tilapia, *Oreochromis niloticus*, were collected from Giza Governorate and screened for the presence of CM. Samples were collected from buccal cavities and blood of 20 highly infected fish (≥10 cysts/fish) under aseptic conditions. Partial sequencing of the ITS rRNA region was performed to identify and distinguish the causative agents from closely related species. The cellular immune response between infected and noninfected *O. niloticus* was investigated by quantitative real-time-PCR targeting tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). Results showed significant increases in the levels of both TNF-α and IL-1β ($p<0.01$) in fish infected with *Clinostomum phalacrocoracis* when compared to those infected with *Clinstomum complanatum* and negative controls (3.6 ± 0.2). Furthermore, the assessment of the levels of lysozyme activity in infected fish sera showed high levels versus noninfected ones.

1. Introduction

Clinostomid metacercariae (CM) are digenetic trematodes with genetic heterogeneity and complex life cycle, including two hosts: snails and fish [1–3]. Their larval stages significantly affect the aquaculture industry, especially due to infection with encysted metacercariae [4, 5]. *Oreochromis niloticus* (Nile tilapia) is considered an important cheap protein source in Egypt [6–8]. The metacercarial stage of *Clinostomum complanatum* has zoonotic importance by causing "Laryngopharyngitis or Halzoun" disease that can cause death from asphyxiation in infected humans [9]. Furthermore, because of the high morphological similarity among different clinostomid species, advanced molecular techniques have been employed to effectively distinguish between these species [10].

In Egypt, several recent studies have been conducted on the morphological and molecular characterization of different CM that infect freshwater fish, including *C. phalacrocoracis*, *C. complanatum*, and *C. cuteanum* [2, 3, 11]. However, the immunological response elicited by clinostomid infection in fish is rarely investigated. Immunological proteins, including interleukins (ILs) and interferon cytokines, are generated by various immunological cells, such as macrophages, lymphocytes, granulocytes, and epithelial cells. Several pathogens, such as parasites, have the capability to provoke high expression of these immune genes [12]. Understanding the immune-related response postinfection with these parasites may facilitate the development of effective control strategies against further infection in cultured fish species.

Tumor necrosis factor-α (TNF-α) and IL are proinflammatory cytokines produced in the early stages of infection and have an important role in parasitic infection pathogenesis [13, 14]. IL-1β and TNF-α play a major role in restricting parasite proliferation and causing the chronic latent infection

through cyst formation, as reported in *Neospora caninum* and *Toxoplasma gondii* infections [15].

Due to a lack of immunological information regarding fish infected with yellow grub trematode, this study aimed to employ both molecular and immunological approaches to identify the parasites and evaluate the immune status of infected fish. Analysis of TNF-α and IL-1β gene expression and oxidative stress in *O. niloticus* infected with CM and histopathological investigations of infected tissues were performed.

2. Material and Methods

2.1. Collection of Fish. From May to August, 250 Nile tilapia, *O. niloticus*, were randomly collected from Elbaher Alazam and Elmoneb localities of Giza Governorate, Egypt. Fish were transported alive to the parasitology lab using aerated plastic buckets. Fish samples were investigated for clinical signs and for external lesions. In addition, fish specimens were subjected to parasitological and molecular examination as well as immunological investigations.

2.2. Blood and Tissue Sampling. Blood was collected from the caudal vein of each fish specimen to obtain serum samples while Nile tilapias were still alive. Furthermore, fish samples were euthanized by an overdose of tricaine (Sigma A5040), their heads were removed, and the buccal and pharyngeal tissues, as well as the surrounding muscles, liver, and kidneys, were thoroughly inspected for parasite infection using the naked eye and a magnifying lens [11]. Bacteriological examination was done on all the collected tissues by inoculation of aseptically collected tissue swabs from the organ into tryptic soy broth (Divco, Detroit, MI, USA) and incubated at 30°C for 24 hr, then subcultured in tryptic soy agar. For fungal infection, loopful from different tissues were inoculated on Sabourauds dextrose agar (Divco, Detroit, MI, USA) and incubated at 25°C for 2 weeks. Organs with no bacterial or fungal infection were stored at −20°C for RNA extraction and subsequent gene expression.

2.3. Parasitological Investigation. The collected fish were visually examined using a handheld magnifying glass to detect the CM in the skin, gills, fins, and buccal cavities. Infected fish with CM were divided into two groups: 1st group, fish infected with less than 10 cysts/fish, and 2nd group, fish infected with more than 10 cysts/fish. In addition, a dissecting microscope was used to collect mucous secretion surrounding the buccal cavity of both infected ($N = 20$) and noninfected fish ($N = 5$). Collected CM from buccal cavities were measured using a ruler and dissecting light microscope, where all measurements were expressed as mean ± standard error (SE). Cysts were compressed between two slides and inspected under a dissecting microscope, according to Paperna [16]. In addition, images of any detected parasites were recorded using a digital camera for further identification. The morphological analysis included ten specimens ($n = 10$) of two species of CM. The collected CM were classified as encysted or exocysted and measured in millimeters unless specified otherwise [17]. The parasites were identified based on the international keys for clinostomid, according to Caffara et al. [18–20] and Mahdy et al. [2].

2.4. Estimation of Lysozyme Activity. Lysozyme activity was measured in serum samples using turbidimetrically following the procedure of Morgan et al. [21] and Chalmers et al. [22]. Each serum sample (100 μL) was mixed with *Micrococcus lysodeikticus* (at a concentration of 0.4 mg/mL in phosphate-buffered saline solution, pH 6.2 and 25°C) in a flat-bottomed microplate. The ELISA reader was used to scan the microplate (Synergy HT, BioTek Instruments, USA) at a wavelength of 450 nm. The lysozyme activity was determined by the reduction of 0.001 per minute in the cell suspension's optical density. Lysozyme activity was compared between two groups of infected *O. niloticus*, as described below. Fish that were infected with *Clinostomum phalacrocoracis* were separated into two categories based on the degree of their infection. The first group was comprised of fish that had over 10 EMC, whereas the second group consisted of fish infected with less than 10 flukes. The estimated levels of lysozyme were determined.

2.5. Molecular Identification. Genomic DNA was extracted from two morphologically different metacercarial that were previously preserved at −20°C, using the GeneJet Genomic DNA purification kit (Thermo Scientific, Lithuania), following the manufacturer's instructions. The purity and the concentration of the extracted DNA were measured using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). Polymerase chain reaction (PCR) amplification for the ITS region was performed using the primers Ophet F1 5′-CTCGGCTCGTGTGTCGATGA-3′ and Ophet R1 5′-GCATGCARTTCAGCGGGTA-3′. The amplification of ITS region was carried out in a 50 μL reaction that included 25 μL i-Taq™ 2X PCR master mix (intron biotechnology), 5 μL DNA template, and 1 μL of both primers and double distilled water up to the volume. The PCR test was started by denaturing at 94°C for 2 min, then going through 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 10 min. After electrophoresis in 1% agarose gel and ethidium bromide staining, PCR products were visualized using UV transillumination [1, 18]. Using the DNA Clean and Concentrator TM-25 Kit (Zymo Research), PCR products were subsequently purified in accordance with the manufacturer's instructions. In order to sequence them, purified products were delivered to Macrogen (Korea). Applied Biosystems' Sequencer Model 3730XL automated DNA sequencing device was used to sequence samples in both forward and reverse directions while utilizing the same PCR primers. Bio-Edit software [23] was used to edit and validate the raw sequencing data. The assembled sequences were examined and aligned against other sequences using BLAST, which is available at (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Using MEGA 7, the phylogenetic tree was generated using the Kimura 2-parameter model and the neighbor-joining approach with 1,000 bootstrap repetitions [24].

2.6. Quantitative Real-Time PCR (qRT-PCR). Highly infected fish with CM were aseptically dissected to get the tissues and

mucous secretions that surrounded the CM in the affected buccal cavities. Samples were taken from 20 fish that were severely affected (10 cysts/fish). As negative controls, samples were taken from five fish that were not affected. β-actin gene was used as a reference gene to normalize TNF-α and IL-1β genes expression. Finally, tissue and mucosal samples from infected fish were aseptically preserved and stored at −20°C for both CM, *C. phalacrocoracis*, and *C. complanatum*. Isolation of RNA was performed from 100 mg of pharyngeal tissues using the Ambion RNA isolation kit (Applied Biosystems) following the manufacturer's instructions. Tissues were homogenized using Fast Prep 24 homogenizer (MP Biomedicals) in lysing Matrix D tubes (MP Biomedicals) in two cycles of 30 s at 6 m/s. The purity and quantity of isolated RNA were evaluated using Nanodrop (Thermo Scientific, USA). cDNA was synthesized using a high-capacity cDNA archive kit (Applied Biosystems) following the manufacturer's instructions and the method of Picard-Sánchez et al. [25]. In this study, Nile tilapia-specific primers for the TNF-α and IL-1β genes were used (Table 1). RNA from five uninfected fish samples was used as negative controls, and the β-actin gene was used as a reference gene to normalize TNF-α and IL-1β genes expression [26].

2.7. Histopathological Studies. The affected tissues with clinostomid cysts in the buccal cavities with the surrounding muscle and pharyngeal regions were immediately fixed in 10% neutral formalin. The preserved tissues were processed and stained with hematoxylin and eosin before being examined under a light microscope (Olympus CX41) [30].

2.8. Statistical Analysis. The observed data were analyzed using Predictive Analytics Software (PASW) Statistics, Version 18.0 software (SPSS Inc., Chicago, IL, USA). All measurements of the obtained species are expressed as mean ± SE. Furthermore, the p-value of <0.05 was considered statistically significant.

3. Results

3.1. Macroscopical Screening of Infected Fish. During this study, 175 out of 250 examined *O. niloticus* were found infected with two species of CM, identified as *C. phalacrocoracis* and *C. complanatum*, with an overall prevalence of 70.0%. Macroscopic examination of the fish buccal cavities revealed cysts containing parasitic CM that were tightly attached to the fibrous connective tissues of infected fish (Figure 1). The detected *C. phalacrocoracis* and *C. complanatum* were found in two different sizes; the large cyst measured 4.5–9.3 (8.7 ± 1.2) mm, while the smallest one ranged at 1.9–3.2 (2.2 ± 1.4) mm in diameter, respectively. The prevalence rates of *C. phalacrocoracis* and *C. complanatum* were 31.6% (79/250) and 19.6% (49/250), respectively. The mixed infection was 18.8% (47/250) in the examined tilapia. The monthly prevalence of infection with Clinostmid EMC revealed that the highest infection rate was detected in August (36/44) at 81.8% for *C. phalacrocoracis* and 28.57% (10/35) in May for *C. complanatum* (Table 2).

3.2. The Intensity Rate of Infection with CM. The macroscopic examination showed that parasites were firmly attached in the buccal cavities tissues with intensity rate assigned in three groups ranging from 1 to 5, 6 to 10, and ≤10 cysts/fish (Figure 2 and Table 3). The highest intensity rate of infection revealed that 25 of 175 CM harbored ≤10 cysts/fish (14.28%).

3.3. Morphology of CM. The cysts of clinostomid were observed as either encysted (EMC) or excysted (ExMC). The CM appeared in pea shape with yellowish-white nodules and was arranged firmly embedded in the musculature of buccal cavities of infected fish. Moreover, both detected EMC and ExMC of *Clinostomum* species were nearly stout, wider in the region of the gonads, and showed high contractility and elasticity when they were alive. ExMC were long with smooth tegument and flattened compressed dorsoventrally with rounded posterior extremities (Figure 1(a)–1(c)).

3.4. Evaluation of IL-1β and TNF-α Expression. Regardless of the type of *Clinostomum*, TNF-α and IL-1β genes show significantly high levels ($p<0.01$) in the tissues around *Clinostomum-infected* Nile tilapia when compared to negative control fish. Means of TNF-α and IL-1β in the infected tilapia were 27.45 ± 1.76, 15.21 ± 0.96, and 18.6 ± 0.73, 11.76 ± 0.41 for *C. phalacrocoracis* and *C. complanatum*, respectively. These values were higher than those detected in the noninfected ones (3.6 ± 0.2 for both species). In addition, TNF-α and IL-1β showed higher levels in *C. phalacrocoracis*-infected fish compared to those infected with *C. complanatum*.

3.5. Lysozyme Activity in Sera Specimens. The level of lysozyme in the serum of infected fish with less than 10 CM/fish was estimated at 115.0 ± 4.8 μg/mL. While in the group infected with more than 10 EMC/fish, the serum lysozyme level was 155.0 ± 5.9 μg/mL, while in the control group was 55.0 μg/mL, respectively. This study revealed that significantly higher lysozyme activities were detected in the group infected with more than 10 EMC/fish compared to fish with less than 10 CM/fish and the control group.

3.6. Molecular Identification. PCR results showed two PCR products in 448 and 743 bp for *C. complanatum* and *C. phalacrocoriasis*, respectively. All nucleotide sequences of both CM were registered in the GenBank database with accession numbers for *C. phalacrocoracis* (ON564308.1) and for *C. complanatum* (ON564307.1), respectively.

The blast search of the resulting sequences revealed that the ITS region sequence for *C. phalacrocoracis* and *C. complanatum* from the branchial cavity metacercariae is related to the genus *Clinostomum*. The blast analysis for *C. complanatum* (ON564307.1) showed 100% similarity to *C. complanatum* (MK811210.1, MH845236.1, MF171126.1, and MT601849.1). The sequence we obtained for *C. phalacrocoracis* (ON564308.1) showed 100% similarity to that of *C. phalacrocoracis* (KP110569.1, KP110567.1, KJ786975.1, MT158303.1, and FJ609423.1). The phylogenetic tree showed that both *C. phalacrocoracis* and *C. complanatum* were grouped

Table 1: Primers used in this study.

Genes	Primers sequences forward (5′–3′)	Primers sequences reverse (5′–3′)	Reference
TNF-α	GGTTAGTTGAGAAGAAATCACCTGCA	GTCGTCGCTATTCCCGCAGATCA	Praveen et al. [27]
IL-1β	TGCACTGTCACTGACAGCCAA	ATGTTCAGGTGCACTATGCGG	Heinecke and Buchmann [28]
β-Actin	CAGCAAGCAGGAGTACGATGAG	TGTGTGGTGTGTGGTTGTTTTG	Akbari et al. [29]

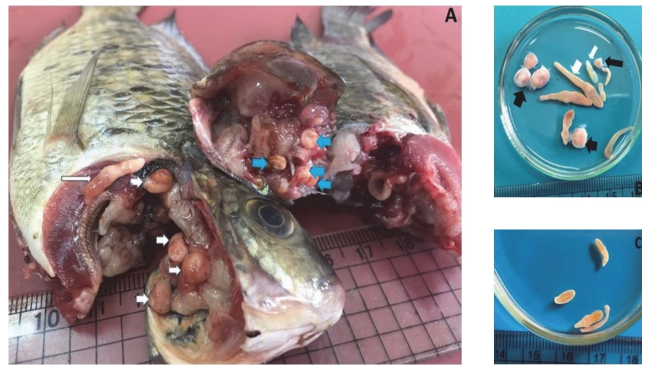

Figure 1: *Oreochromis niloticus* infected with clinostomid encysted metacercariae: (a) *C. complanatum* small-sized yellowish cysts in the buccal cavity (blue arrows) and *C. phalacrocoracis* showed large spherical cysts (white arrows); (b) ExMC and EMC of *C. complanatum* (black arrow), and ExMC and EMC of *C. phalacrocoracis* worm (white arrows); (c) excysted metacercariae of *C. complanatum*.

Table 2: Prevalence of clinostomid metacercariae (CM) in naturally infected Nile tilapia.

	No. ex. fish	No. Inf. fish	% of infection	*C. phalacrocoracis* (CM)	*C. complanatum* (CM)	Mixed infection
May	60	35	58.33	65.7 (23/35)	28.57 (10/35)	5.7 (2/35)
Jun	62	47	75.80	65.9 (31/47)	25.5 (12/47)	8.51 (4/47)
July	63	49	77.7	75.5 (37/49)	10.2 (5/49)	14.3 (7/49)
August	65	44	67.7	81.8 (36/44)	11.36 (5/44)	6.8 (3/44)
Total	250	175	70.0	79	49	47

CM, clinostomid metacercariae; No. ex. fish, number of examined fishes; No. inf. fish, number of infected fishes; % of infection, percentage of infection.

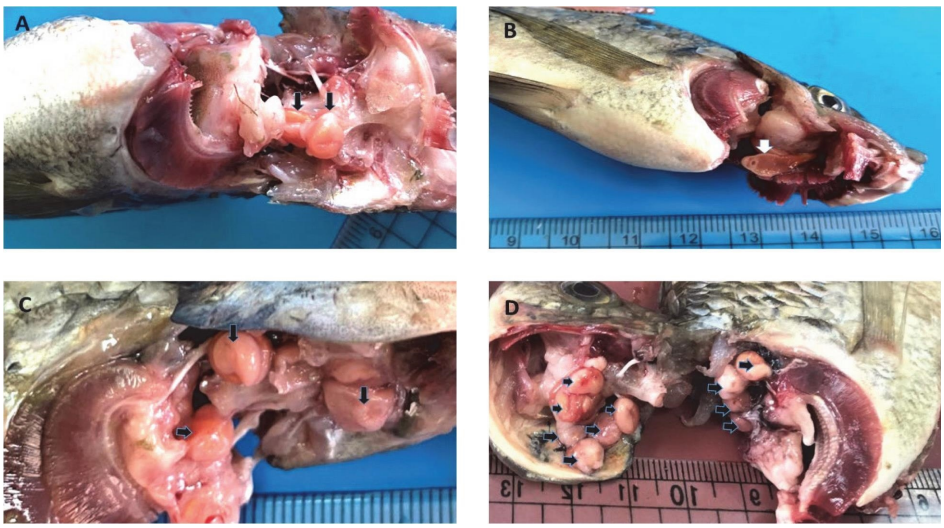

Figure 2: *Oreochromis niloticus* infected with different degrees of intensity of infection with clinostomid encysted metacercariae: (a) two cysts of clinostomid showed a yellowish cyst in the buccal cavity (black arrow); (b) single excysted metacercariae of (white arrow); (c) three cysts of *C. phalacrocoracis* infected fish showed large spherical cysts in the buccal cavity (black arrows); (d) ten cysts of *C. phalacrocoracis* that infect fish (black arrows).

Table 3: The intensity of clinostomid metacercariae in naturally infected Nile tilapia.

Intensity EMC/fish	No. of inf. fish	% of infection
1–5	117	66.85
6–10	33	18.8
≤10 EMC	25	14.28
Total	175	70.0

No. of inf. fish, number of infected fishes; Intensity, intensity of infection; EMC, encysted metacercariae; % of infection, percentage of infection.

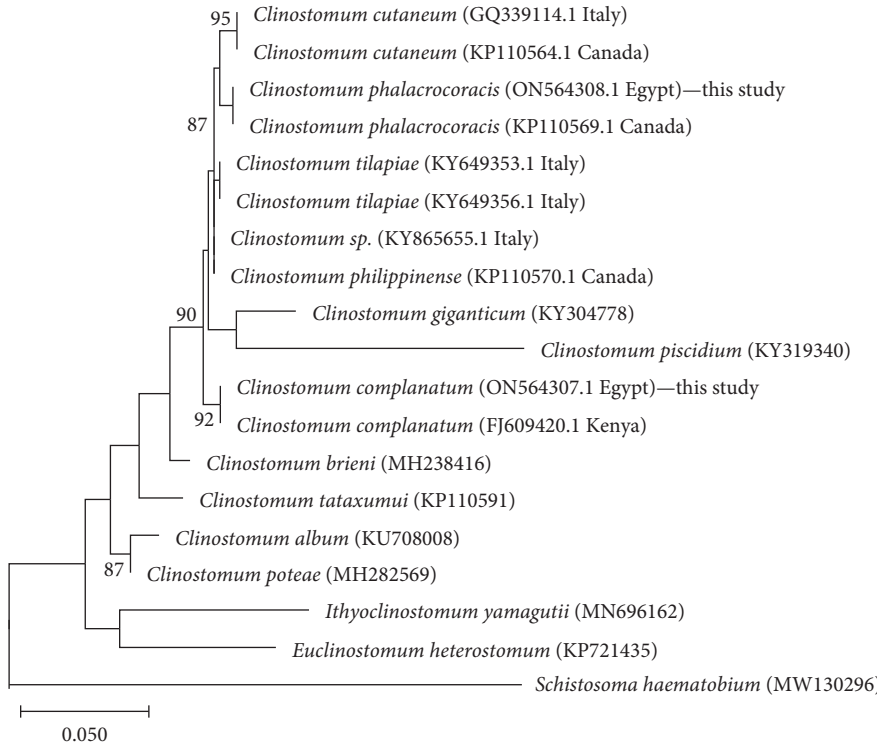

Figure 3: Phylogeny of *C. complanatum* and *C. phalacrocoracis* based on the neighbor-joining model using ITS region sequences.

with their related species and separated from other sequences of *Clinostomum* species (Figure 3).

3.7. Histopathological Examination. Microscopic examination of the buccal cavities' tissues revealed the histological structure of the clinostomid parasite (Figure 4(a)). A connective tissue capsule was noted covering the parasite, and inflammatory cell infiltrations encircling the parasite were also observed (Figure 4(b)). The cuticle, hypodermis, and large-size ventral sucker of *Clinostomum* spp. were observed in the histological section (Figures 4(c) and 4(d)). The high-power section shows the connective tissue capsule covering the worm with inflammatory eosinophilic granular cells (EGCs) infiltrations and massive muscle hyalinization encircling the parasite (Figure 5). The parasitic cysts containing metacercaria were surrounded by inflammatory reactions involving the adipose tissue of the fish. The adipose and muscle bundle tissues showed extensive necrosis with massive EGCs aggregation, macrophages, and degenerative changes spotted in the muscle fiber's structure (Figures 6(c) and 6(d)).

4. Discussion

There is a great interest in developing freshwater fish culture and increasing awareness of the importance of yellow grub disease caused by clinostomid as one of the major detrimental factors in culturing fish in Egypt [1–3]. CM that infects freshwater fishes possesses a superior position of parasitic infection. Apart from its severe impact on fish health, it usually has a negative marketing disadvantage for fish and significant health risks to human fish consumers [2]. Moreover, when the fish body is penetrated by high quantities of *C. complanatum* cercaria leads to severe irritation in the fish tissues and higher levels of mortalities, especially in small and weak fishes during transportation than in the healthy ones [31]. The prevalence overall in this study was finding the *Clinostomum* cysts infection in examined fishes at 70.0%. The investigated *O. niloticus* in Giza revealed that the most prevalent species was *C. phalacrocoracis* 31.6%, followed by *C. complanatum* 19.6%. This result of the prevalence of CM infection nearly agrees with those reported previously by Salem et al. [11]. In contrast, the current result was higher than that prevalence reported by Taher [32], who found different species of CM (62.25%) in *O. niloticus*. The difference in the prevalence may be attributed to many factors as habitat differences, food supply, the distribution of aquatic snails (the intermediate hosts), also the distribution of aquatic piscivorous birds, which has an important role in completing the life cycles of many digenetic trematodes [33]. In recent years, the combination of morphological characteristics and partial sequencing of the ITS region has been effectively employed to identify and differentiate among digenetic trematodes [9]. This current study of *C. phalacrocoracis* isolated from Nile tilapia fishes at Giza Governorate, Egypt, was very closely positioned to *C. phalacrocoracis* isolated from Egypt fish by Mahdy et al. [2, 3], according to the blast analysis.

In addition, cytokines have a great role in the immunological response against parasitic infections. Assessment of the levels of the immunological cytokines against infectious parasites in fish. Concerning, Zhu et al. [34] reported that different cytokines, such as TNF-α secreted from macrophages in case of invasion of pathogens. In this study,

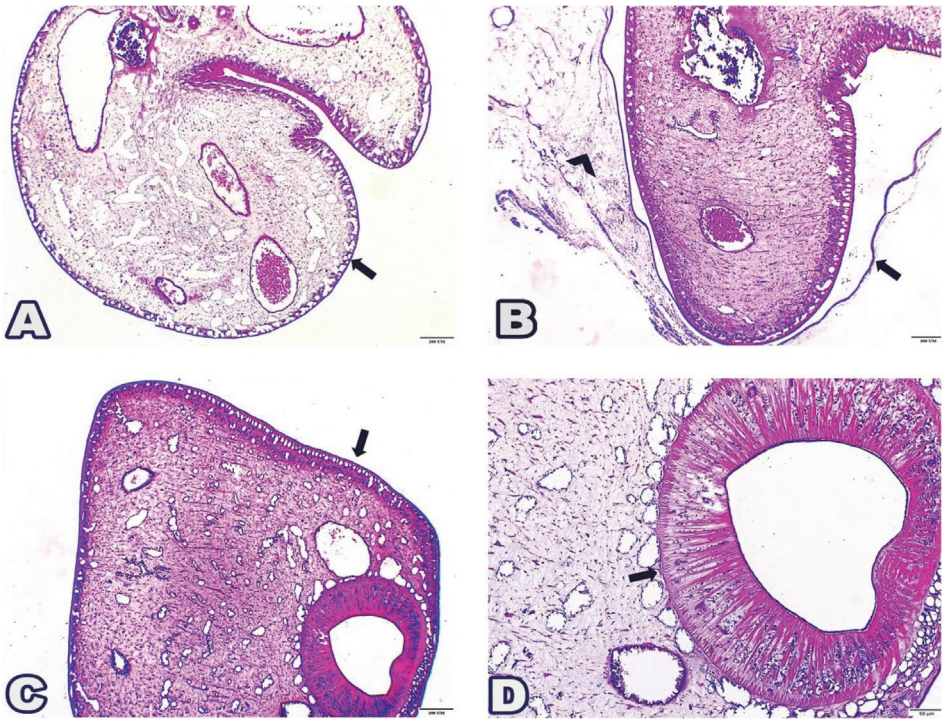

Figure 4: Photomicrographs displayed the histological structure of clinostomid cyst-infected buccal cavities of Nile tilapia: (a) section showing the encysted metacercaria of *Clinostomum* (arrow); (b) section presented the connective tissue capsule covering the *Clinostomum* spp. externally (arrow). Notice several inflammatory cells infiltrations encircling the parasite (arrowhead); (c) tissue section highlighted the cuticle of metacercaria of *clinostomum* (arrow) and hypodermis below it; (d) high power section metacercaria of *Clinostomum* spp. disclosed the composition of the ventral sucker (arrow). (H&E staining (a–c) (100× magnification, scale bar = 200 μm) (d) (400× magnification, scale bar = 50 μm)).

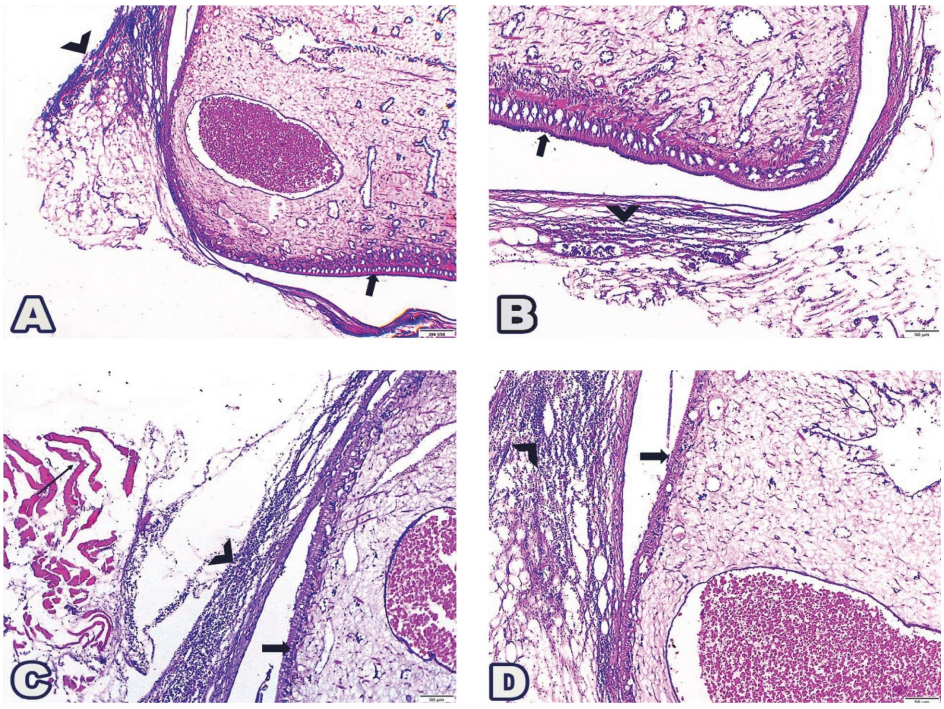

Figure 5: Photomicrographs revealed the histopathological alterations to Nile tilapia infected with *Clinostomum* spp. in the branchial tissue In all tissue sections of (a–d) encysted metacercaria of *Clinostomum* spp. are presented (arrow) and encircled by massive aggregations of inflammatory cells (arrowhead). Notice muscle hyalinization in (c) section (Thin arrow). (H&E staining (a) (100× magnification, scale bar = 200 μm) (b–d) (400× magnification, scale bar = 50 μm)).

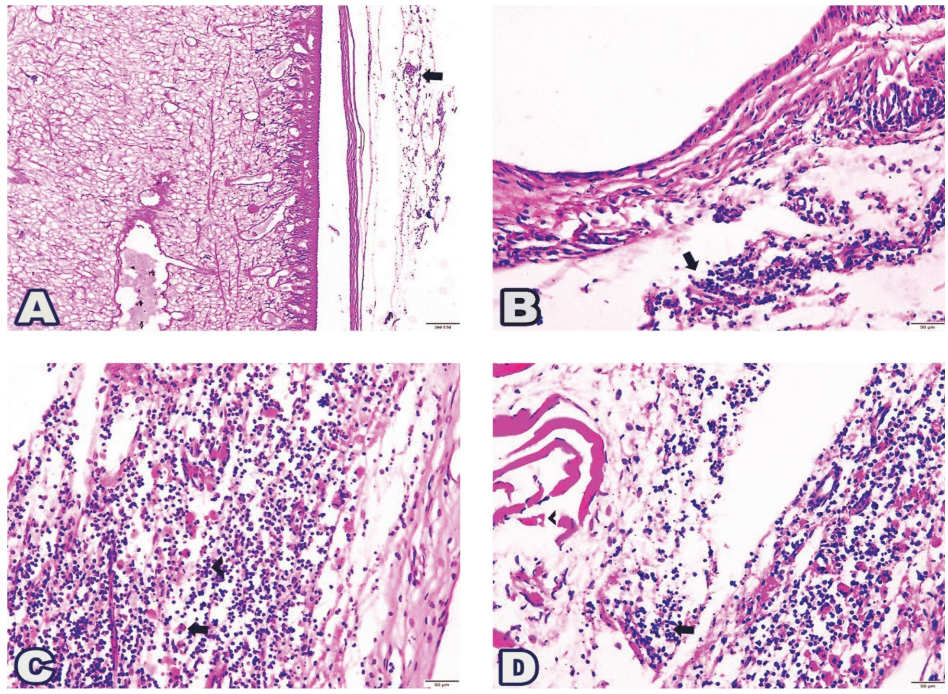

FIGURE 6: Photomicrographs exposed the effect of the *Clinostomum* parasite on *O. niloticus*. All sections of (a–d) are highlighted with the intense gathering of inflammatory cells (arrows) enclosed *Clinostomum* cyst. In the tissue section of smooth muscles of the buccal cavity (c), macrophage (arrow) and eosinophil (arrowhead) were distinguished. Also, in section (d), degenerative changes are spotted in the muscle fiber's structure (arrowhead). (H&E staining (a) (100× magnification, scale bar = 200 μm) (b–d) (400× magnification, scale bar = 50 μm)).

clinostomid-infected fish tissues showed a considerable rise in the initial assessment of the cytokines (TNF-α and IL-1β); this may be an immune reaction to reduce parasite infections. Additionally, cytokines are mediators that support a variety of biological processes, including fibrosis, inflammation, and the immune system [35]. Additionally, TNF-α and IL-1 levels were greater in fish infected with *C. phalacrocoracis* than they were with *C. complanatum* infection, according to the most recent study of cytokine mRNA. This result is in agreement with the recorded by Younis et al. [12], who studied the immune mechanisms of TNF-α and IL-1β from infected rainbow trout and Nile tilapia with *Ichthyophthirius multifilis*, *Trichodina* spp., and *Myxobolus* spp., respectively. Furthermore, cytokines are mediators that contribute to many biological processes; cell growth/proliferation inflammation, fibrosis, and the immune response [35]. Concerned, the lysozyme molecule is a mucolytic enzyme generated from many immunological cells such as leukocytes, monocytes, and neutrophils. These immunological cells play a significant role in pathogen invasion by the initiation of innate immune responses to protect fish. Moreover, it contributes to minimizing pathogen invasion in fish by initiating the innate immunological response, activation of the complement cascade, and phagocytosis process [36]. In the present study, the mean detected levels of lysozyme in *O. niloticus* infected with clinostomid EMC was 115.00–155.00 μg/mL. This current result recorded a high lysozyme level versus the control group (55.0 μg/mL). The same result that recorded by Attia et al. [26], who reported similar responses in infected muscles of catfish with *Cyanodiplostomum* spp. This upregulation is caused by the tissue response to infection with parasites. In a recent histopathological study at the site of *Clinostomum* cyst attachment, there was heavy infiltration of activated immunological cells and severe tissue damage in fish [37, 38]. Regarding the histopathological appraisal in tissues that surrounded the cysts of *Clinostomum* spp. exhibited parasitic cysts lodged firmly attached and incorporated into tissues [2]. Concerning, showed a degree of necrosis with massive aggregation of EGCs, as the intensive inflammatory response of surrounded host tissues as an immune response induced by clinostomid. These observations of histopathological appraisal at the site of clinostomatids' tissue infections agreed with Hamouda and Younis [1] and Mahdy et al. [3].

5. Conclusion

Both molecular and immunological methods can be utilized together to quickly detect yellow grub disease and precisely identify *Clinostomum* species, making this a valuable approach. The identification of *C. complanatum* and *C. phalacrocoracis* was accomplished through molecular characterization of their ITS region. Nucleotide sequences were registered in GenBank with accession numbers (ON564307.1 and ON564308.1), respectively. Cytokine levels measured by qRT-PCR revealed that higher levels could correlate with a positive larval parasitic infection of yellow grub trematode than the negative control. Results showed significant increases in the levels of both TNF-α and IL-1β ($p < 0.01$) in fish infected with *C. phalacrocoracis* when compared to those infected with *C. complanatum* and negative controls (3.6 ± 0.2). Furthermore, for

the infected group with more than 10 EMC/fish, serum lysozyme level was $155.0 \pm 5.9\,\mu$g/mL, while in the control group was 55.0 μg/mL, respectively. This study revealed that TNF-α, IL-1β gene expression, and Lysozyme levels of infected *O. niloticus* with clinostomid are higher than the control one. In addition, clinostomid infection has damaging effects on fish tissues and health. This study investigated the immunological response of Nile tilapia, specifically examining the expression of immune-related genes TNF-α and IL-1β in response to infection with the clinostomid MC fluke.

Data Availability

The datasets and materials used in this study are available in the manuscript.

Ethical Approval

This study was approved by the Ethics Committee of Animal Experiments of the Faculty of Veterinary Medicine, Cairo University (VET-CU-IACUC-12102021372).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

All authors share in the aim of the study; Olfat Mahdy contributed to the collection of the samples and identified the parasites. Mai Salem contributed to providing the molecular characterization and immunological tests of the specimens of infected tissues and blood. Olfat Mahdy and Mai [11] wrote the main manuscript text and prepared figures. All authors share in writing this manuscript and revising it.

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