



Molecular Identification and Histopathological Alterations Associated with *Prohemistomum vivax* Encysted Metacercariae Infection in Farmed African Catfish (*Clarias gariepinus*)

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DIGENETIC trematode infections pose a significant threat to the African catfish aquaculture industry in Egypt. This study investigated the prevalence, morphological characteristics, molecular composition, and host responses to *Prohemistomum vivax* encysted metacercariae (EMC) infecting farmed African catfish (*Clarias gariepinus*). A total of 160 fish were collected from farms in Kafi-elsheikh Governorate between 2022 and 2023. Parasitological examination revealed an overall prevalence of 55% (n = 88) of infection with EMC of *P. vivax*. The prevalence of infection exhibited seasonal variations, with the highest rates in summer (100%), followed by spring (75%), autumn (30%), and winter (15%). Parasitic intensity showed significant major seasonal differences, with the lowest mean intensity in winter (1.6 ± 2.3 EMC per field) and the highest in summer (14.4 ± 9.3 EMC per field). Molecular sequencing and phylogenetic analysis of the internal transcribed spacer 2 (ITS2) gene unambiguously identified the metacercariae as *P. vivax*, overcoming limitations of traditional morphology-based techniques that cannot differentiate between digenetic species when present as encysted stages. Histopathological examination revealed the presence of multiple parasitic cysts between hepatocytes, with diffuse vacuolar degeneration of hepatocytes. Muscles showed the presence of multiple parasitic cysts embedded in muscle bundles, with observed tissue reaction, inflammatory cell infiltration, oedema of interstitial tissue, and hyaline degeneration of muscle fibers. These findings highlight the importance of implementing effective control strategies to minimize the impact of *P. vivax* EMC infection on the Egyptian catfish aquaculture industry.

Keywords: *Prohemistomum vivax*; EMC, internal transcribed spacer 2 (ITS2) gene, African catfish.

Introduction

The cultivation of African catfish (*Clarias gariepinus*) holds a significant role in food security and economic stability in Egypt [1]. Recognized for its adaptability to diverse environmental conditions, rapid growth, and high consumer demand, African catfish stands as a cornerstone of the Egyptian aquaculture industry [2]. However, this industry faces challenges, particularly in the form of

parasitic infections by digenetic trematodes [3]. Multiple digenetic trematodes, specifically fish-borne zoonotic trematodes (FBZT), infect second intermediate hosts, including *C. gariepinus*, in the form of encysted metacercariae (EMC), causing significant pathological alterations [4-5]. FBZT pose a significant public health risk as they can be transmitted to humans through the consumption of inadequately cooked or raw fish harboring active

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metacercariae [6]. These digenetic trematodes, including heterophyids, diplostomatids, and cyathocotylidae, pose significant threats to human health and the farming sustainability of catfish [7].

Metacercariae of various digenean trematode species frequently infected *C. gariepinus*, forming cysts in the eyes, cranial cavity, musculature, and internal organs. Diplostomatid metacercariae belonging to genera such as *Tylodelphys*, *Diplostomum*, *Neodiplostomum*, and *Posthodiplostomum* often embed in the cranial and ocular tissues as oval cysts ranging 300-1300 x 90-250 µm in size [8]. These infections can cause blindness, abnormal swimming, and mortality in juvenile catfish. Heterophyid metacercariae including species of *Haplorchis*, *Heterophyes*, and *Pygidiopsis* typically reside within the musculature and integument of catfish, eliciting focal necrosis and tissue damage [9].

Cyathocotylid metacercariae of genera such as *Mesostephanus* and *Prohemistomum* frequently encyst in the internal organs and muscles, contributing to severe pathological changes [7]. Of particular concern is *Prohemistomum vivax*, an emerging parasitic threat for fish, mammals, and humans causing substantial impacts in Egyptian aquaculture with catfish infection rates over 80% [10]. The metacercarial stage of *P. vivax* embeds within the muscles and internal organs of catfish tissues, resulting in stunted growth, diminished quality, and economic losses [11].

Accurately diagnosing digenetic trematode metacercariae infecting catfish is challenging due to morphological similarities between genera such as *Prohemistomum*, heterophyids, diplostomatids, and cyathocotylids when present in the encysted metacercarial stage [7]. Species-level classification is particularly difficult due to the absence of genitalia in metacercariae, and this organ is crucial for the identification of adult trematodes [12]. Therefore, molecular techniques like sequencing of the internal transcribed spacer 2 (ITS2) enable more reliable detection and confirmation of metacercarial species identity [7-13]. ITS2 sequencing also permits earlier diagnosis and improved sensitivity compared to traditional microscopy, given the high interspecific variability of this genetic marker [14-15]. Overall, integrating morphological, molecular, and histopathological approaches is vital for prompt and accurate diagnosis of these widespread digenean parasites to better manage the catfish aquaculture industry.

A comprehensive understanding of the prevalence, morphological characteristics, molecular composition, and host responses to these parasites is essential for developing effective control strategies. To address this need, this study aimed to determine the seasonal and overall prevalence of metacercariae infection in farmed African catfish. Additionally, this study involved detailed morphological and molecular characterization of the retrieved metacercariae to accurately identify the digenetic trematode species involved. Finally, this study investigated the histopathological responses of the host to this digenetic infection, providing insights into the impact of EMC on catfish health.

Material and Methods

Ethical Approval

This study, approved by the Ethics Committee of the Faculty of Veterinary Medicine at Cairo University adhered to institutional, ethical, and animal welfare guidelines, as outlined in the Laboratory Animal Care and Use Guide (Ethics approval code: VET-CU-IACUC-09092023797).

Fish sampling

A total of 160 African catfish (*C. gariepinus*) were systematically and randomly collected from farms in Kafrelsheikh Governorate, Egypt, between 2022 and 2023. The fish were delicately transported to the laboratory in oxygen-supplied, water-filled plastic bags. Upon arrival, a comprehensive examination was conducted to identify EMC of digenetic trematode infecting *C. gariepinus*. The sampled fish exhibited a size range, with total lengths ranging from (30-28) cm and weights varying from (120-150) gm. Clinical examinations were performed, and any abnormal external and internal gross lesions were meticulously recorded and documented. Type the main text in 10-point Times New Roman, single-spaced with single line spacing and fully justified right and left. Do not use double-spacing. The manuscript must be formatted in two columns.

Parasitological examination

To thoroughly examine the fish for the presence of EMC parasites, a comprehensive parasitological examination was conducted. Each fish was euthanized using a clove oil solution and then dissected. Various tissues and organs, including the gills, skin, eyes, fins, muscles around the abdomen, muscles around the head, and internal organs, were

examined under a light microscope (Olympus CX41 microscope; Japan). Additionally, muscle samples were prepared by compressing them between two slides and subsequently examined under a stereoscopic microscope to identify the presence of EMC [16-17].

ITS2 Region Sequencing

To further characterize the EMC parasites, genomic DNA was extracted from metacercarial parasites obtained from different infected fish collected from the same locality during various seasons of the year. The DNeasy Blood & Tissue extraction Kit (QIAGEN, USA) protocol was followed to ensure efficient DNA extraction. The quantity and quality of the extracted DNA were assessed using an Implen NP80 NanoPhotometer (Munich, Germany). Subsequently, PCR amplification of the ITS2 region of the parasite DNA was performed. The universal primers AP102-F:5'-AGAGCGCAGCCAACTGTGTGA-3' and AP102-R:5'-TGCCACGTCCTAGCATCAGCC-3', were employed for this amplification [18].

The PCR reactions were carried out in 25 µl aliquots using Maxima® Hot Start PCR Master Mix (Thermo Fisher Scientific, USA). The PCR conditions involved an initial phase at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for 38 seconds, extension at 72°C for 42sec, and a final step at 72°C for 7 minutes. The amplified PCR products were visualized by UV light after agarose gel electrophoresis. Purification of the amplified DNA fragments was performed using the GeneJET™ PCR Purification Kit (Thermo Fisher Scientific, USA).

Sequencing of the purified DNA was carried out using the Big Dye Terminator v3.1 cycle sequencing kit with the same PCR primers. The sequences were analyzed using an ABI prism 3730XL (Applied Biosystems, USA) automated sequencer. Careful sequence assembly was facilitated using BioEdit software v.7.2 (Hall, 1999) [19]. The edited sequences were matched against the public genetic sequence database using BLAST programs. The final sequences were submitted to GenBank for public access programs. The final sequences were submitted to GenBank for public access.

Phylogenetic analysis

To determine the evolutionary relationships of the EMC parasites, a phylogenetic tree was constructed. The ITS2 sequences of the current EMCs were compared against 25 other accession numbers from various digenetic trematodes. The selected species exhibited over 80% similarity to the current ITS2 sequence of EMC. Multiple sequence alignment was performed, and the phylogenetic tree was established using maximum likelihood (ML) methodology in MEGA 11 [20]. The ML parameters were set based on model selection using Bayesian information criterion (BIC), corrected Akaike information criterion (AIC) scores, and bootstrap confidence values from 1,000 replicates. The general time reversible model with gamma-distributed rate variation and invariant sites (GTR+G+I) was chosen as it provided optimal accuracy. Numerous phylogenetic tree-building approaches exist, but ML analysis was deemed most suitable for aligning the current parasites at the branch terminals in this study.

Histopathological examination

To assess the pathological impacts of EMC on the fish, a histopathological examination was conducted. Samples from the muscles and internal organs were collected and fixed in 10% neutral buffered formalin. After fixation, they underwent a series of processes, including washing, dehydration, clearing, and embedding in paraffin. The paraffin-embedded blocks were sectioned to a thickness of 5 microns and stained with Hematoxylin and Eosin [21]. This histopathological examination was conducted using a light microscope (Olympus BX50, Japan).

Clinical signs

Infected African catfish displayed an array of nonspecific clinical signs indicative of parasitic infection, including loss of appetite, darkened skin pigmentation, weight loss and emaciation, excessive mucus production over the skin and gills, frayed and damaged fins, respiratory distress, and impaired growth rates compared to non-infected populations. These gross clinical manifestations likely result from the tissue migrations, feeding, and embedding of EMC in multiple organs (Figure.1).

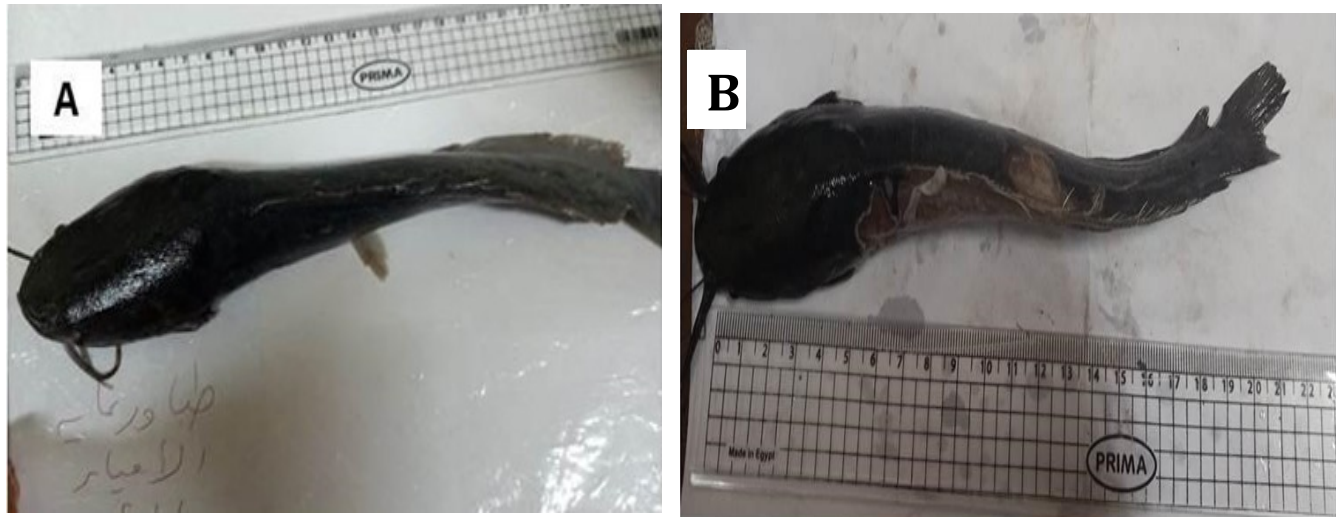


Fig. 1. Gross clinical manifestations of infected catfish showing stunted growth, skin ulceration, frayed and damaged fins.

Prevalence of infection

Microscopic examination of wet mounts and histological sections from 160 fish specimens revealed widespread infections with encysted *P. vivax* metacercariae, with an overall prevalence of 55% (88 out of 160 fish). The prevalence of infection displayed significant seasonal variations, peaking at 100% in summer (40 out of 40 fish infected), followed by 75% in spring (30 out of 40 fish), 30% in autumn (12 out of 40 fish) and 15% in winter (6 out of 40 fish). In addition to fluctuations

in prevalence, the intensity of parasitic infection exhibited major seasonal differences. Quantification of EMC per microscopical field showed the lowest mean intensity of 1.6 ± 2.3 EMC per field occurring in the winter season. In contrast, the highest mean intensities were recorded in the summer (14.4 ± 9.3 EMC per field) and spring (8.2 ± 6.1 EMC per field) seasons. Statistical analysis confirmed these differences were significant, both between summer and spring, as well as between summer and the other seasons (Table 1).

TABLE 1. Prevalence Percentage and Parasitic Intensity of EMC Infections in Farmed *Clarias gariepinus*

Season	No. examined fish	No. infected fish	Prevalence (%)	Parasite intensity ^a
Autumn	40	12	30	3.6 ± 2.7^a
Winter	40	6	15	1.6 ± 2.3^a
Spring	40	30	75	8.2 ± 6.1^b
Summer	40	40	100	14.4 ± 9.3^c
Total	160	88	55	

^a Different small letters mean significant differences between groups.

Morphological examination

Microscopic examination of wet mount and fixed specimens enabled morphological characterization of the metacercariae. *P. vivax* metacercariae were collected from diverse organs including the musculature, skin, eyes, intestine, liver, kidney, and gills of infected catfish hosts. The metacercariae displayed typical digenean morphology, with an oval to spherical body shape. The body was surrounded by a double-layered cyst

wall, comprised of a thick outer hyaline layer and thin inner granular layer. Two lateral lobulated bladder-like sacs were observed flanking the body. Detailed morphometric analysis found the metacercariae measured 365–387 μm (mean 378 ± 1.5 μm) in length and 346–358 μm (350 ± 1.5 μm) in width. These morphological features are consistent with descriptions of the *P. vivax* metacercarial stage infecting freshwater fish (Figure 2).

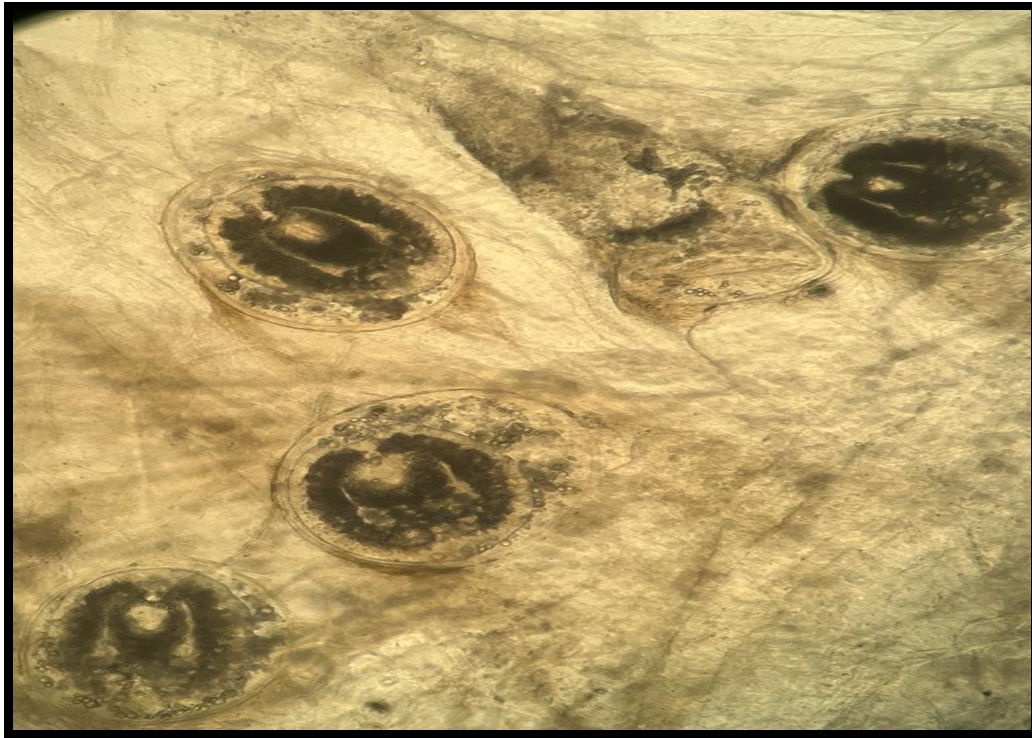


Fig. 2. Infected muscular tissues of catfish showing heavy infected with *P. vivax* EMC

Genetic characterization

PCR amplification and sequencing of the ITS2 region from encysted *P. vivax* metacercariae obtained from four infected catfish, each representing a different seasonal cohort, generated a 267 base pair DNA fragment. The analysis found the ITS2 sequences from all four fish isolates were 100% identical. These sequences were deposited in GenBank under accession numbers OR826418, OR826419, OR826420, and OR826421.

Sequence alignment and phylogenetic analyses confirmed the identity of the sequenced metacercariae as belonging to the digenean genus *Prohemistomum* (Family *Cyathocotylidae*).

Furthermore, BLAST analysis against the NCBI GenBank database showed the ITS2 sequence (OR826418) exhibited 98.88-98.16% similarity specifically to others *P. vivax* (OP348897; ON775468; OR291421), 95.42% to *Holostephanus dubinini* (OM755735), 94.40% to *Mesostephanus appendiculatus* (OP377090), 91.70% to *Cyathocotylidae* sp. (MH257767), and 90.22% to *Cyathocotyle prussica* (MH521249). It also demonstrated a similarity range of 96.45-96.43% to various *Diplostomum* spp. (MW001032; MW000974; MT951904), and 87.68-87.20% to *Tylodelphys* spp. isolates (MK177841; KY432870; MW001129).

The phylogenetic tree, generated through maximum likelihood analysis, unveiled the taxonomic placement of the investigated *P. vivax* within the same clade as other *P. vivax*, supported by a robust bootstrap value of 100%. This outcome validated the molecular identification of *P. vivax*

based on ITS2 sequencing and affirmed the distinctiveness of this parasite species (Figure 3).

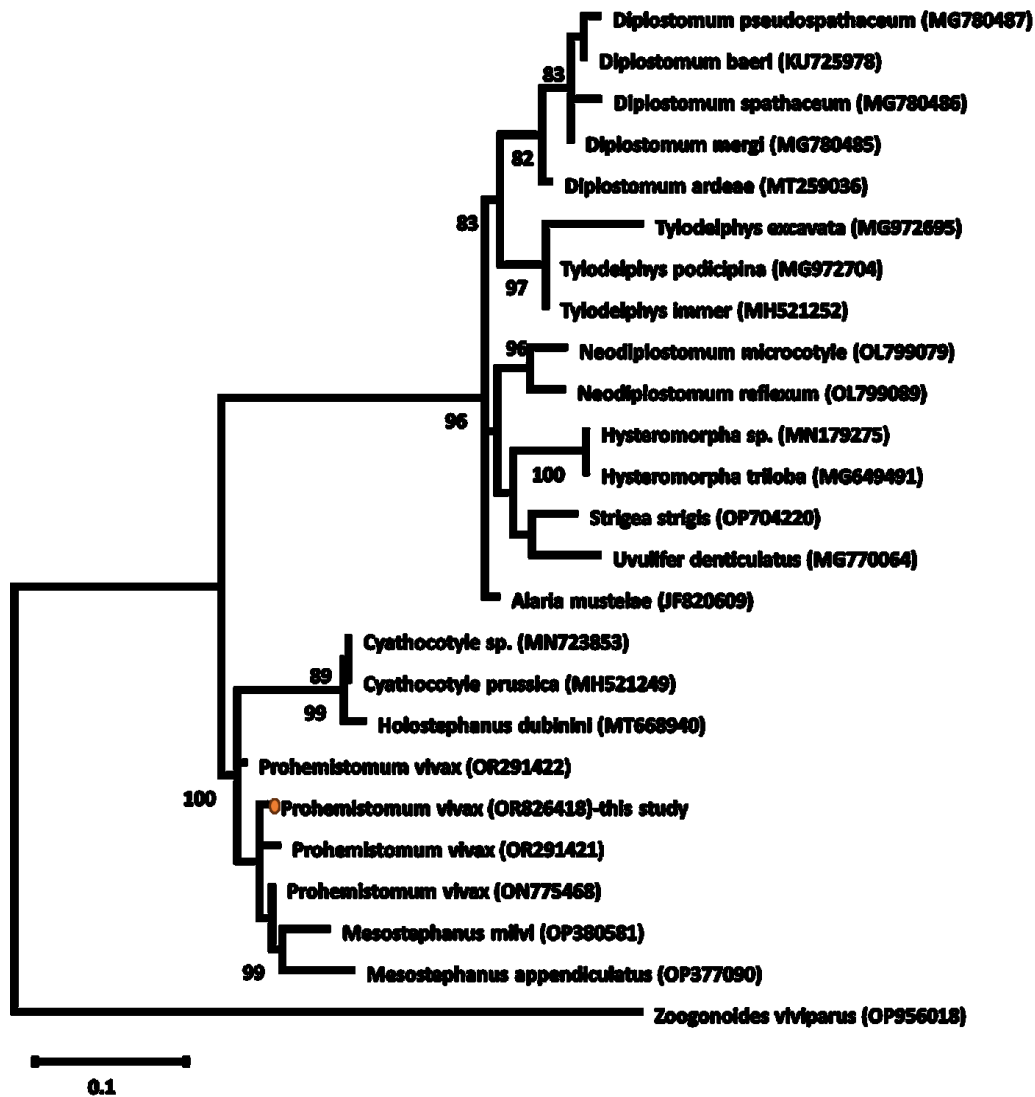


Fig. 3. The phylogenetic tree, generated through maximum likelihood analysis, unveiled the taxonomic placement of the investigated *P. vivax* within the same clade as other *P. vivax*.

Histopathological findings

Histopathological examination of liver of catfish revealed presence of multiple parasitic cysts between hepatocytes (Figs. 4 a & b) with diffuse

vacuolar degeneration of hepatocytes (Fig. 4 c), muscles of this group showed presence of multiple parasitic cysts embedded in muscle bundles (Figs. 4 d) with observed tissue reaction, inflammatory cells infiltration, edema of interstitial tissue and hyaline degeneration of muscle fibers (Figs. 4 e).

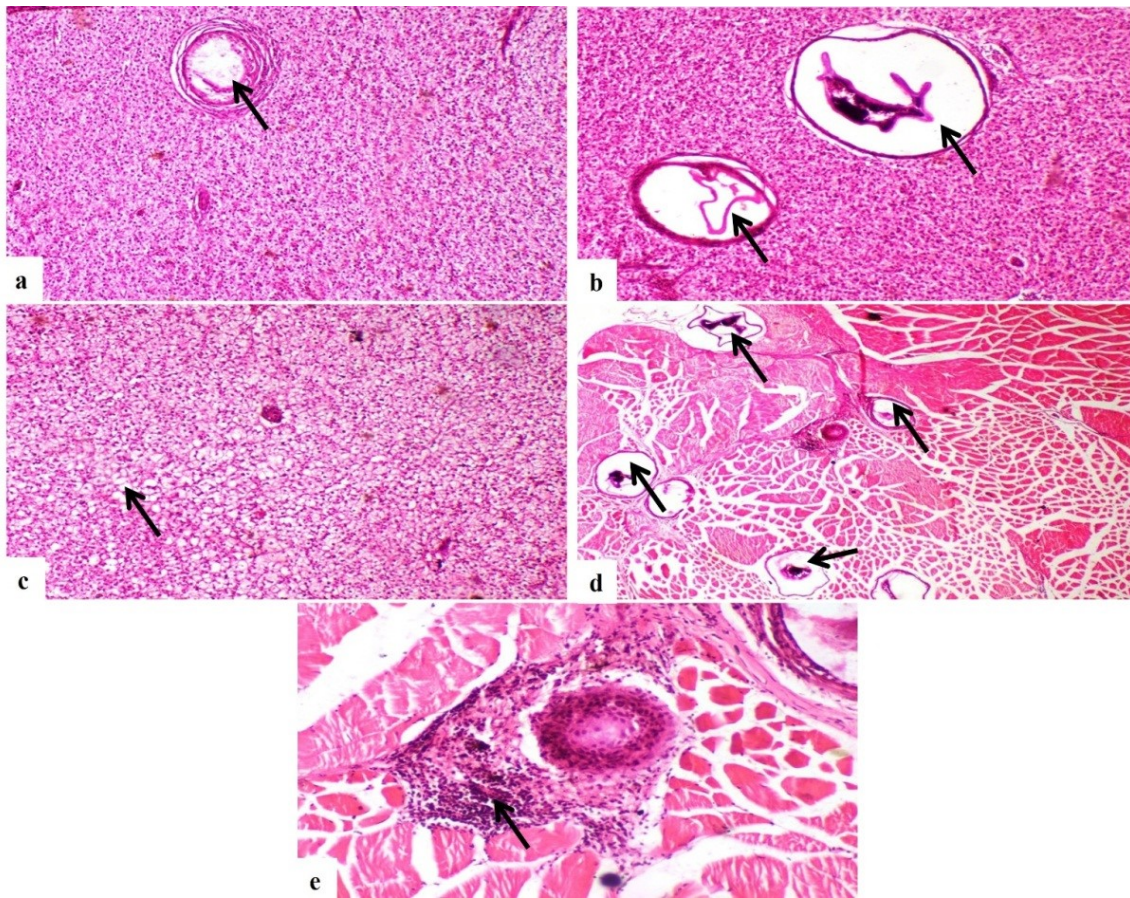


Fig. 4. Photomicrograph of the infected tissues of catfish, showing. (a) presence of parasitic cyst between hepatocytes (arrow) (H&EX100). (b) multiple parasitic cysts embedded in liver parenchyma (arrows) (H&EX40). (c) diffuse vacuolar degeneration of hepatocytes (arrow) (H&EX100). (d) multiple parasitic cysts embedded in muscle bundles (arrows) (H&EX40). (e) heavy infiltration of inflammatory cells between muscle fibers (arrow) (H&EX100).

Discussion

This study provided insights into the prevalence, morphological characteristics, molecular composition, and associated histopathology of a prominent digenetic trematode, *P. vivax*, infecting farmed African catfish in Egypt.

Poor water quality and high stocking density are ideal stress factors that are capable of hindering fish immunity and make them more susceptible to parasitic digenetic trematode infections. [22-23]. Immunocompromised fish due to digenetic trematode parasitic infestation became lethargy, off feed with abnormal skin pigmentation and excessive white mucus secretion, growth retardation, in addition to the abnormal swimming behavior [24,25] similarly to that shown in our study.

The high prevalence along with seasonal-related infection patterns have important implications for parasite transmission and control. The overall prevalence of *P. vivax* metacercarial infection in the examined catfish populations reached 55%, affirming the role of this parasite as a widespread threat to sustainable aquaculture, as described in several previous studies [6,10,11]. This study revealed pronounced seasonal variations in *P. vivax* infection levels, with summer displaying the highest prevalence (100%) and intensity (mean 14.4 EMC/field), followed by spring (75%; 8.2 EMC/field), autumn (30%; 3.2 EMC/field) and winter (15%; 1.6 EMC/field). Marked seasonal variations were evident, with summer and spring peaks coinciding with higher temperatures that likely accelerate the developmental and transmission dynamics of *P. vivax* and its snail intermediate hosts [6-10-11]. Our findings of higher

P. vivax infections during summer align with previous investigations in African catfish farms and natural habitats [26]. Additionally, immunosuppression associated with spawning stress in spring and early summer likely contributes to this seasonal pattern [6-8-11]. The synergistic effects of peak metacercarial densities and compromised host immunity promote heavy polyparasitism and morbidity during the summer months [12]. Our findings highlight the need for strategic seasonal parasite control programs, with interventions applied shortly before these projected surges in susceptibility and transmission.

The morphological analysis found that *P. vivax* metacercariae infiltrated diverse catfish organs in oval cysts ranging 365–387 x 346–358 µm in size. Metacercariae were observed within muscular tissues, skin, eyes, intestinal tract, liver, kidneys, and gills. This extensive dissemination and polyorganotropism contribute to the negative impacts of *P. vivax* infection on catfish growth and health [10-11-26]. Related cyathocotylid digeneans like *Mesostephanus* spp. similarly established in these organs as metacercariae [4-16-26]. However, examination of adult flukes with fully developed reproductive systems is ultimately required for definitive species identification, given the absence of taxonomically distinguishing characters in metacercarial forms [12-13]. Therefore, traditional microscopic examination alone is inadequate for differentiating *P. vivax* metacercariae from other morphologically similar genera. However, ITS2 sequencing enabled unambiguous molecular identification, overcoming the limitations of morphology-based techniques. The ITS2 marker displays sufficient interspecific variability to discriminate between ambiguous digenean species [14-15]. DNA sequencing and phylogenetic analyses verified the metacercariae infecting the catfish belonged to *P. vivax*, aligning with prior molecular surveys [12-16-26-27]. The ITS2 sequences exhibited 98.88% homology to other *P. vivax* GenBank entries. Further comparison against related digeneans also revealed the specificity of this marker, with the next closest matches only reaching 96% similarity for some *Diplostomum* spp. and 95% for the cyathocotylid *Holostephanus dubinini*. This investigation assists other researchers in differentiating *P. vivax* from other

species during the diagnostically challenging metacercarial stage. It also extends the known molecular data for Egyptian isolates of this emergent fluke. [15-29]

Beyond developing diagnostic techniques for these invasive parasites, understanding their pathology and impacts is key for disease management in aquaculture settings [2-7-30]. Histological analysis revealed an array of pathological changes associated with *P. vivax* metacercariae within the liver and musculature of infected catfish, including inflammation, necrosis, vacuolation, fibrosis, and organ distortion. The severe histopathological changes elicited by heavy *P. vivax* infections explain the diminished growth, poor body conditions, mortality events and resultant economic impacts widely reported among infected catfish stocks [6-11]. Hepatocellular damage disrupts metabolism while muscle necrosis directly reduces fillet quality and marketability [31]. These findings reaffirm the urgent need for improved prophylaxis, diagnostics and sustainable integrated treatment options.

Conclusions

In summary, *P. vivax* constitutes a severe obstacle for profitable and sustainable African catfish farming in Egypt due to its widespread occurrence, poly-organotropism, deleterious impacts, zoonotic potential, and the difficulty of effectively controlling complex digenean life cycles. Continued surveillance of infection dynamics and further investigation into diagnostic, preventative and therapeutic measures against this invasive parasite are warranted. Adoption of an integrated aquaculture management plan encompassing pond disinfection, farmer education, surveillance programs, and treatment regimens could mitigate the current heavy burdens of *P. vivax* plaguing one of the most economically and nutritionally valuable farmed fishes in Egypt.

Acknowledgment

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Conflict of interest

There is no conflict of interest.

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التعريف الجزيئي والتغيرات النسيجية المرتبطة بالإصابة بالميتاسيركاريا لدودة (بروهيمستوموم فيفاكس) في أسماك القط الأفريقية المستزرعة (كلارياس جاريبينوس)

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تمثل الإصابات بالديدان المفطحة الخطيرة ثنائية العائل تهديدًا كبيرًا لصناعة استزراع سمك القط الأفريقي في مصر. ناقشت هذه الدراسة مدى انتشار والخصائص الشكلية والتركيبة الجزيئية والتغيرات النسيجية للعائل المصاب بواسطة الميتاسيركاريا المتحوصلة لدودة بروهيمستوموم فيفاكس التي تصيب سمك القط الأفريقي المستزرع (كلارياس جاريبينوس). جمعت مجموعة من أسماك القراميط (160 سمكة) من مزارع في محافظة كفر الشيخ علي مدار عام كامل. أظهر الفحص الطفيلي انتشارًا إجماليًا بنسبة 55%. أظهرت انتشار الإصابة بتغيرات موسمية، حيث كانت أعلى نسبة إصابة في الصيف (100%)، تلتها الربيع (75%)، والخريف (30%)، والشتاء (15%). أظهرت الكثافة للديدان الطفيلية اختلافات موسمية رئيسية كبيرة، حيث كانت أدنى كثافة متوسطة في الشتاء والأعلى في فصل الصيف. مكن التسلسل الجزيئي والتحليل التصنيفي للجين (ITS2) من تحديد طبيعة أكياس التحوصل ك بروهيمستوموم فيفاكس بشكل صريح، أظهر الفحص النسيجي المرضي وجود العديد من الأكياس التطفلية بين خلايا الكبد، مع احتقان فجوي معمم لخلايا الكبد. أظهرت العضلات وجود العديد من الأكياس التطفلية المغروسة في حزم العضلات، مع ملاحظة تفاعل الأنسجة، وارتشاح الخلايا الالتهابية، ووذمة الأنسجة البينية، وتنكس حبيبي لألياف العضلات.

الكلمات الدالة: أسماك القط الأفريقي، العوامل الطفيلية، بروهيمستوموم فيفاكس.