# Histopathological, Parasitological and Molecular Biological Studies on Metacercariae from *Oreochromis Niloticus* and *Clarias Gariepinus* Cultured in Egypt

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## ABSTRACT

*Oreochromis niloticus* and *Clarias gariepinus* collected from June 2008 to May 2009 from three different types of fish farms in Egypt and subjected for encysted metacercariae infestation microscopically, biologically and by PCR test. Infection was highest at semi-intensive farms and lowest at desert farms. *Clarias gariepinus* was more heavily infested than *Oreochromis niloticus*. Prevalence was highest during summer on the gills of *Orecochromis niloticus* and musculature of *Clarias gariepinus*. Biological experimentation using puppies, revealed *Heterophyes heterophyes, H. aequalis, Pygidiopsis genata, Procerovum varium, Haplorchis pumilio, H. taichui, Stictodora tanayensis, Prohemistomum vivax, Gelanocotyle milvi, Mesostephanus appendiculatus, M. milvi* and *M. fajardensis*. Microscopically, the affected muscles revealed encysted metacercariae surrounded by a connective tissue capsule. The encysted metacercariae induced pressure atrophy, focal degeneration or necrosis and melanomacrophage proliferation. The PCR revealed discrete bands corresponding to the expected 361-base pair fragment size in the 18S rDNA gene of the Heterophyidae genome in adult worms and metacercariae.

It could be concluded that the occurrence of metacercariae varied with aquaculture system, cultured fish species and the season of the year, many of the reported metacercariae were larval stages for digenean parasites of zoonotic importance and public health hazard. The PCR, based on two different genes, is a relevant tool for genetic characterization of Heterophyidae as a zoonotic parasite isolated from fishes in Egypt. It also has a great potential for application of the clinical epidemiological studies for the fast detection of food-borne parasites in freshwater fishes.

## INTRODUCTION

The aquaculture and fish industry is significantly threatened by digenetic trematode infections (metacercariae), fish-borne trematode infection which is a serious public health problem in several parts of the world. It is estimated that about 40 million people are affected by fish-borne trematode infections worldwide *(1, 2)*, the most important parasites being heterophyids, cyathocotylids and opisthorchioids *(3, 4)*. All have similar life cycles that involve a definitive host (man, dog or cat), with snails and fish as intermediate hosts.

Currently the identification of metacercariae is slow, involving biological experiments by infecting laboratory animals (puppies or birds) to obtain adult worms after 1-2 weeks from the onset of the experiment, or the excystation of larvae to view their morphological characteristics *(5)*. Determining the safety of fish, with respect to trematode infections, currently involves detailed physical examination for metacercariae, a time consuming procedure requiring considerable taxonomic expertise *(6, 7)*. Identification of larval stages by morphological trials is often difficult and ambiguous, and experimental demonstration of the life history is frequently unachievable because specific intermediate or definitive hosts are unknown.

The use of molecular methodologies has allowed links to be elucidated between the various developmental stages of specific trematodes *(8)*. Besides being accurate in

identification, it shortens the time from several weeks to a few days or even hours. This is in addition to the advantage of not using laboratory animals for the experimental infection to get the adult worm that is principal in the identification of metacercariae. Various ribosomal genes, especially the 18S (small subunit SSU) gene, along with the inter-specific polymorphic regions, include highly conserved regions for which universal primers can be designed for amplification of this gene from a studied species *(9)*. Polymerase Chain Reaction (PCR) based diagnostic methods have found wide application in a number of diseases as they are sensitive and fast *(10)*. The detection of metacercarial antigens in the present study, could be an initial stage for the identification of antibodies and the foundation for the preparation of fast diagnostic kits in the future.

The objectives of the current study were to screen, identify and compare the prevalence of metacercarial infection among *Oreochromis niloticus* and *Clarias gariepinus* taken from different farming systems in Egypt. Both biological studies and PCR were used for detecting and identifying the common heterophyid metacercariae, especially those of zoonotic importance and public health hazard.

## MATERIAL AND METHODS

### A- Natural infestation

Two hundred *O. niloticus* and 200 *C. gariepinus* were collected from three different fish farm types: low input (as fish reared in the Nile river and its branches), semi-intensive (farms which receive agriculture waste-water) and desert systems (farms which use uncontaminated groundwater), during the summer, autumn, winter and spring seasons of 2008-2009.

Macroscopic examination for encysted metacercariae was carried out with the aid of a magnifying hand lens to detect any changes in the liver, heart, kidneys, gonads, gills, skin and muscles and metacercariae detected were recovered *(11)*.

Small pieces of the fish samples were examined microscopically *(12)*. Encysted metacercariae were obtained by artificial tissue digestion *(5, 13)*. A detailed microscopic examination was carried out on the recovered encysted metacercariae.

### B- Experimental infection

Thirty one-week-old, puppies kept in a comfortable cage were supplied daily with the required feed and drinking water. The appropriate animal welfare protocols were followed throughout the experiment, done in the Faculty of Veterinary Medicine, Zagazig University. A single prophylactic dose of praziquantel (50 mg/10 kg B.W), as anthelmintic drug, was given to all the experimented animals, one week before the experimental infection *(14)*. Fecal samples were collected and examined daily for 2 weeks to prove that these animals were free from any parasitic infection. Each dog was orally given 200 viable collected metacercariae in a piece of minced meat *(15, 16)*. Microscopic examination of the fecal samples of all the experimental animals was daily carried out post-infection by both the direct and sedimentation techniques *(17)*, for the early detection of trematode eggs. Recovery of adult worms, from the experimentally infected puppies was done after finding the trematode eggs in their feces. The worms were collected from the intestine after sacrifice of the animals which tested positive. The sacrificed puppies were humanely disposed of in an incinerator. The worms were kept in a refrigerator at 4°C *(18)* and then identified *(16)*.

### Histopathological preparation

Specimens were collected from the internal organs of the infected fish, fixed in 10% neutral buffered formalin, dehydrated in ascending concentrations of ethyl alcohol, cleared in two changes of xylene and blocked in paraffin. Five micron thick paraffin sections were prepared and stained with hematoxylin and eosin (H & E) *(19)*.

### PCR identification

DNA extraction: application of different PCR reactions on the extracted DNA from worms and metacercariae either for general Heterophyidae (18S gene) or species examined

specific gene. All metacercariae and adult worms were preserved in 70% ethanol. Individual worms and metacercariae were washed overnight in buffer (10 mM Tris-EDTA). Following another 2 washes (1 hour each), gentle crushing of the parasites was done in a 0.2 ml PCR tube before using an extraction kit. DNA was extracted from a single adult worm using a DNeasy tissue kit (Qiagen) according to manufacturer's instructions.

*Polymerase chain reaction:* 1 X PCR buffer (20 mM Tris HCl pH 8.4 and 50 mM KCl); 1.5 mM $MgCl_2$; 0.2 mM deoxynucleotide triphosphate mixtures (dATP, dCTP, dGTP and dTTP); 100 pmol of each primer; 2.5 units (U) Thermus aquaticus (Taq) polymerase; 0.1µg of extracted DNA were mixed together and made up to 50.0 µl using nuclease-free sterile double distilled water. The resulting mixture was subjected to precise thermal profile in a programmable thermocycler as follows:

One cycle: 94°C for 2 minutes.

35 cycles: 94°C for 50 seconds 53°C for 50 seconds 72°C for 1 minute.

One cycle: 72°C for 10 minutes.

***Primers:*** Two sets of primers were used:

Set "A" The oligomer sequences based on the sequence of Heterophyidae 18S gene were designed ***(20)***

Forward primer 5\' - TCA TAT GCT TGT CTC AGA – 3\'

Reverse primer 5\' - ACG GAA ACC TTG TTA CGA– 3\'

Set "B": Two oligonucleotides (primers) were selected according to the primer design. The oligonucleotides were chosen to amplify 117 base pair in *Heterophyes nocens*cytochromec oxidase 1 (COI) gene according to [gi:47156801] nucleotide sequence in gene bank.

Forward primer 5\' -CGG AGG AAA AGA AAC TAA CC– 3\'

Reverse primer 5\' -CGA GCC AAC CTG AAC ACC AC– 3\'

**Analysis of PCR amplification products (amplicons):** The resulting PCR amplicons (10-15 µl) were analyzed by 2% agarose gel electrophoresis ***(21)***. The DNA bands were visualized using ultraviolet transillumination after gel staining with ethidium bromide (0.5 µg/ml). The PCR amplicons of the proper predicted size were about 361bp in first PCR and 117 bp in second PCR.

## RESULTS

The semi-intensive farms showed a higher rate of infestation with the encysted metacercariae, followed by the low input farms while the desert farm showed the lowest or no infection. *Clarias gariepinus* revealed a higher infection rate with encysted metacercariae than *O. niloticus* throughout the four seasons of the study. The highest prevalence of infection with encysted metacercariae in *O. niloticus* was in summer (87%), followed by spring (83%) and autumn (77%). Meanwhile, the lowest prevalence was in winter (63%). *Clarias gariepinus* also showed the highest prevalence during summer (97%) followed by autumn (87%) and spring (83%), with the lowest rate being observed during winter (65%) (Table 1).

The frequency of the recovered encysted metacercariae from *O. niloticus* was highest from both gill filaments and arches (50%) followed by skeletal muscles (45%), gonads (30%) and liver (12%). By contrast, the recovered metacercariae from *C. gariepinus* was highest in the skeletal muscles (81%) followed by the liver (40%) and then heart (14%) (Table 2).

The identification of the isolated encysted metacercariae from the examined fish was based on their morphological characters (Table 3, Figs. 1a & b). The characteristics of the recovered adult worms from the duodenum and jejunum of the experimentally infected puppies are summarized in Tables 4 & 5, Figs. 2a & b and 3a, b & c.

**Table 1. Seasonal prevalence of the encysted metacercariae from different fish farm types**

| Seasons | *Oreochromis niloticus* | | | | *Clarias gariepinus* | | |
|---|---|---|---|---|---|---|---|
| | Farm type | No. examined | Infestation | | No. examined | Infestation | |
| | | | No. | % | | No. | % |
| Summer | Low input | 50 | 41 | 82.0 | 50 | 46 | 92.0 |
| | Semi-intensive | 50 | 44 | 88.0 | 50 | 49 | 98.0 |
| | Desert | 50 | 2 | 4.0 | 50 | 3 | 6.0.0 |
| Autumn | Low input | 50 | 36 | 72.0 | 50 | 41 | 82.0 |
| | Semi-intensive | 50 | 39 | 78.0 | 50 | 44 | 88.0 |
| | Desert | 50 | 1 | 2.0 | 50 | 2 | 4.0 |
| Winter | Low input | 50 | 30 | 60.0 | 50 | 32 | 64.0 |
| | Semi-intensive | 50 | 31 | 62.0 | 50 | 33 | 66.0 |
| | Desert | 50 | 0 | 0.0 | 50 | 0 | 0.0 |
| Spring | Low input | 50 | 39 | 78.0 | 50 | 39 | 78.0 |
| | Semi-intensive | 50 | 41 | 82 | 50 | 41 | 82.0 |
| | Desert | 50 | 1 | 2.0 | 50 | 2 | 4.0 |
| Total | | 600 | 305 | 50.83 | 600 | 332 | 55.33 |

**Table 2. Comparative prevalence of encysted metacercariae among muscles and different organs of 50 *Oreochromis niloticus* and 50 *Clarias gariepinus* examined per farm type.**

| Organ | *Oreochromis niloticus* | | | *Clarias gariepinus* | |
|---|---|---|---|---|---|
| | Farm type | Infestation | | Infestation | |
| | | No. | % | No. | % |
| Gills | Low input | 10 | 20 | 4 | 8 |
| | Semi-intensive | 13 | 26 | 3 | 6 |
| | Desert | 3 | 6 | 2 | 4 |
| Muscles | Low input | 8 | 16 | 25 | 50 |
| | Semi-intensive | 13 | 26 | 25 | 50 |
| | Desert | 3 | 6 | 25 | 50 |
| Heart | Low input | 2 | 4 | 7 | 14 |
| | Semi-intensive | 3 | 6 | 11 | 22 |
| | Desert | 1 | 2 | 3 | 6 |
| Liver | Low input | 3 | 6 | 1 | 2 |
| | Semi-intensive | 3 | 6 | 1 | 2 |
| | Desert | 1 | 2 | 1 | 2 |
| Kidney | Low input | 4 | 8 | 1 | 2 |
| | Semi-intensive | 3 | 6 | 2 | 4 |
| | Desert | 1 | 2 | 0 | 0 |
| Gonads | Low input | 5 | 10 | 1 | 2 |
| | Semi-intensive | 8 | 16 | 2 | 4 |
| | Desert | 3 | 6 | 1 | 2 |
| Skin | Low input | 3 | 6 | 1 | 2 |
| | Semi-intensive | 3 | 6 | 2 | 4 |
| | Desert | 1 | 2 | 0 | 0 |

**Table 3. Morphological criteria of encysted metacercariae collected. * Posthodiplostomum, cuticola**

| Criteria | Heterophyid | Euclinostomatid | Prohemistomatid | Cyanodiplo-stomatid | Diplostomatid* |
|---|---|---|---|---|---|
| **Color** | White | Grayish opaque | Whitish | Whitish | Black |
| **Shape** | Spherical to oval | Spherical to elliptical | Rounded to oval | Oval to rounded | Elliptical to oval |
| **Host** | *O. niloticus* *C. gariepinus* | *O. niloticus* | *O. niloticus* *C. gariepinus* | *C. gariepinus* | *O. niloticus* |
| **Site of encysted metacercariae** | Gills Liver | Kidneys | Muscles | Muscles | Around eye, skin and fins |
| **Cyst wall** | Thick | Very thick | Thick | Very thick | Little thick |
| **Suckers** | Well developed | Well developed | Developed | Well developed | Difficult to see |
| **Size** | 2.7x23 µm | 4-5 mm | 2.3 x 2.1 µm | 4.5x3.3µ | 1x7 µm |
| **Pseudosuckers** | - | - | - | Present | - |
| **Black pigments** | - | - | - | - | Present |
| **Stalk-like process** | - | - | - | - | Present |

Microscopically, the encysted metacercariae were seen in the gill arch. They were surrounded by edema and some mononuclears accompanied with focal sloughing to the primary lamellae epithelium. The secondary lamellae showed hyperplasia or desquamation of its epithelial covering. Encysted metacercariae were seen attached to the skin and fins or embedded among the muscle fibers leading to pressure atrophy and focal hyaline degeneration, besides non-inflammatory edema. The parasitic cysts were frequently surrounded by a thin or thick fibrous connective tissue capsule together with or without few mononuclear leukocytes (Figs. 4 a & b). The internal organs (heart, liver, gonads and kidneys) that exhibited encysted metacercariae revealed pressure atrophy, marked degeneration, focal necrosis and proliferation of the melanomacrophages.

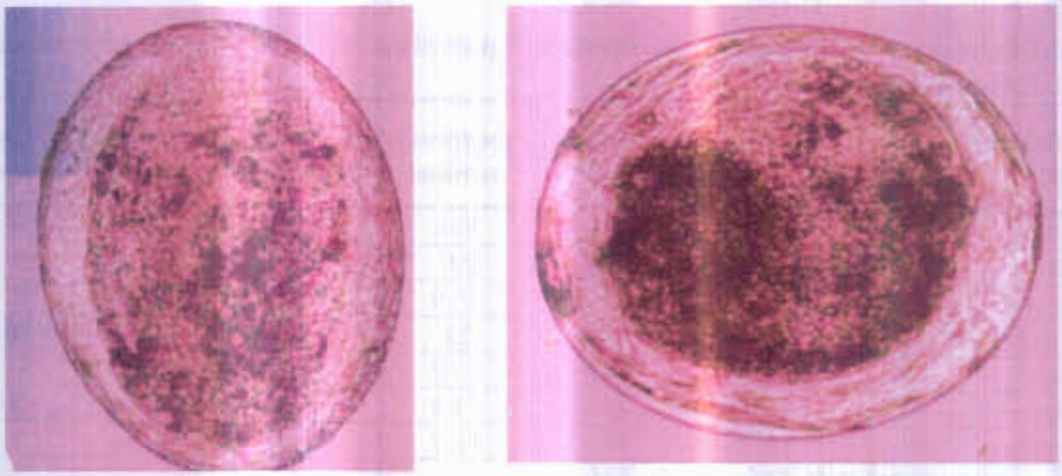
The PCR reaction analysis of the amplifed products of the extracted and purified parasitic adult worm and metacercaria genomic DNA indicated the presence of amplicon bands of the expected sizes by agarose gel electrophoresis and ethidium bromide staining. The first PCR, using primers set "A", showed discrete bands corresponding to the expected 361 base pair (bp) fragment size in the Heterophyidae 18S gene of the Heterophyidae genome, in both the adult worm and metacercaria. The second PCR reaction, with primers set "B", was used to synthesize and amplify the DNA fragment from the genomic parasitic DNA sequence. It showed a single discrete band corresponding to the expected size of about 117 base pair (bp) of the amplified DNA fragment from different metacercariae (Figs. 5 a & b).

**Table 4. Dimensions (micron) of heterophyid parasites collected from experimentally infected puppies.**

| Parasite / Morphology | *Heterophyes heterophyes* | *Heterophyes aequalis* | *Pygidiopsis genata* | *Procerovum varium* | *Haplorchis pumilio* | *Haplorchis taichui* | *Stictodora tanayensis* |
|---|---|---|---|---|---|---|---|
| **Length** | 1710 | 739 | 579 | 554 | 433 | 430 | 541.8 |
| **Breadth** | 651.2 | 431 | 303 | 291 | 252.5 | 216.5 | 251.9 |
| **Oral sucker** | 48.1 | 71.5 | 47.4 | 35.9 | 54.8 | 71.6 | 47.8 |
| **Ventral sucker** | 215.3 | 101.5 | 71.6 | 21 | 45.3 | 19.3 | 36.1 |
| **Pharynx** | 47.2 | 35.2 | 36.4 | 25.2 | 34.9 | 37.5 | 17.2 |
| **Esophagus** | 191.9 | 106.5 | 73.5 | 71.6 | 73.5 | 71.4 | 89.9 |
| **Genital sucker** | 181.1 | 104 | 49.9 | 18.2 | 51 | 44 | - |
| **Testes** | R. 181.2x143 | R. 115x102 | R. 145x56 | 108.7 x 89.1 | 93.25 | 108.7 | R. 30 x 28 |
| | L. 179.5x130 | L. 145x115 | L. 145x56 | | | | L. 35 x 31 |
| **Ovary** | 102 | 72.5 | 36.2 | 50 | 46.6 | 72.5 | 48.3 |
| **Eggs** | 29 x 16 | 24 x 16 | 16 x 12 | 26 x 13 | 24 x 11 | 22 x 11 | 35 x 21 |

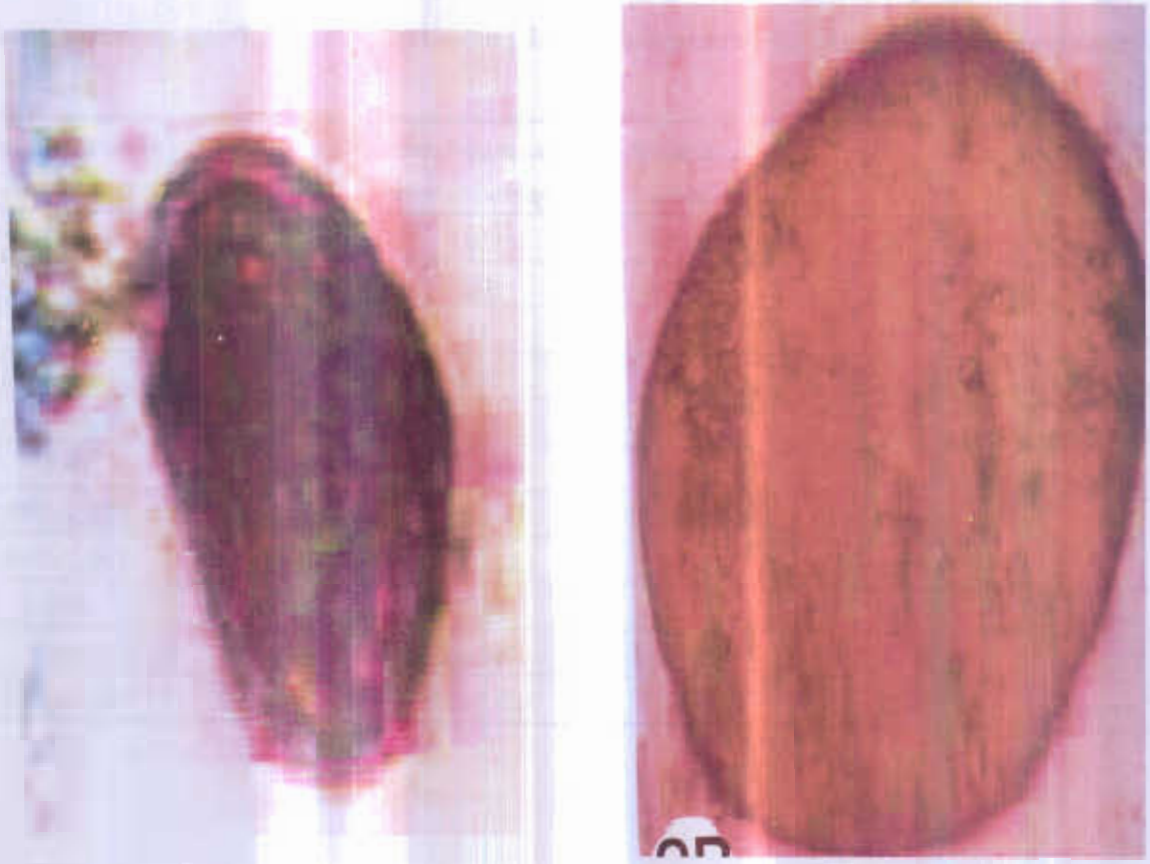
**Table 5. Dimensions (micron) of prohemistomatid parasites collected from experimentally infected puppies.**

| Parasite / Morphology | *P. vivax* | *Gelanocotyle milvi* | *Mesostephanus appendiculatus* | *Mesostephanus milvi* | *Mesostephanus fajardensis* |
|---|---|---|---|---|---|
| **Length** | 76.1 | 942.4 | 1161 | 1825 | 1087.3 |
| **Breadth** | 616.1 | 652.4 | 582 | 723 | 506.5 |
| **Oral sucker** | 39.5 | 47.2 | 51 | 53 | 71.3 |
| **Ventral sucker** | 60.5 | 35.6 | 71.6 | 71.6 | 47.5 |
| **Pharynx** | 74.9 | 44.4 | 35.2 | 47.4 | 69.8 |
| **Esophagus** | 28 | 63 | 47.8 | 95.2 | 49.9 |
| **Testes** | A. 210x170 | 228x175 | 147x158 | 160x154 | 171.1x178 |
| | P.220x181.2 | 230x193 | 182.2x205 | 145x135 | 180.2x198 |
| **Ovary** | 201.2 | 72.5 | 108.7 | 72 | 48.3 |
| **Eggs** | 109x77 | 100x47 | 110x63 | 102x60 | 93x50 |
| **Cirrus sac** | 217.5x99 | 326.2 | 507.5x102 | 326.2x82 | 181.2x72.5 |



(1A) (1B)

Fig. 1: Heterophyid metacercariae (a & b), x 100.



(2A) (2B)

Fig. 2: Heterophyid eggs (a & b), x 100.

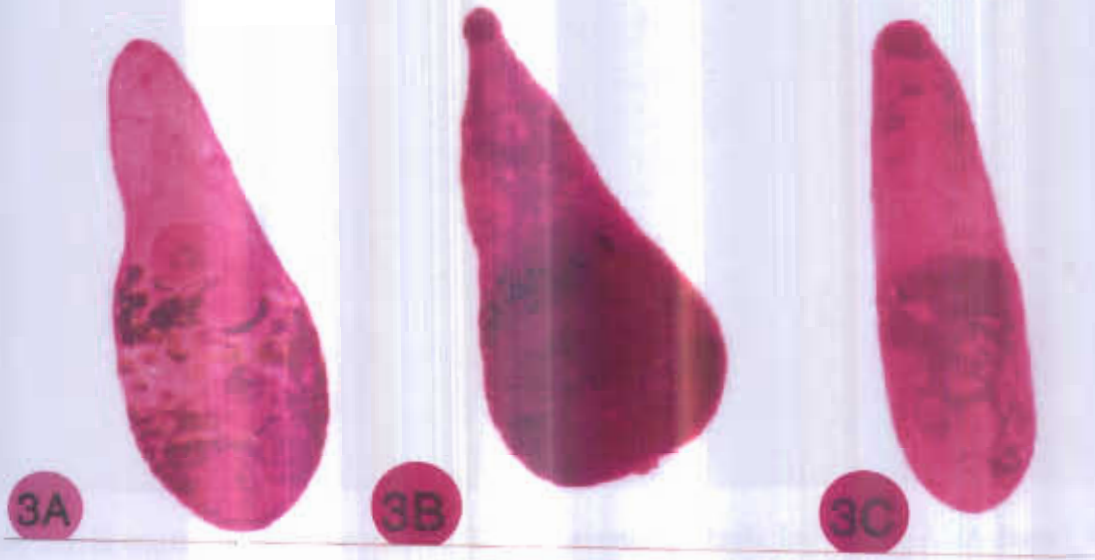



Fig. 3: Heterophyid adult worms (a, b & c), x 40.

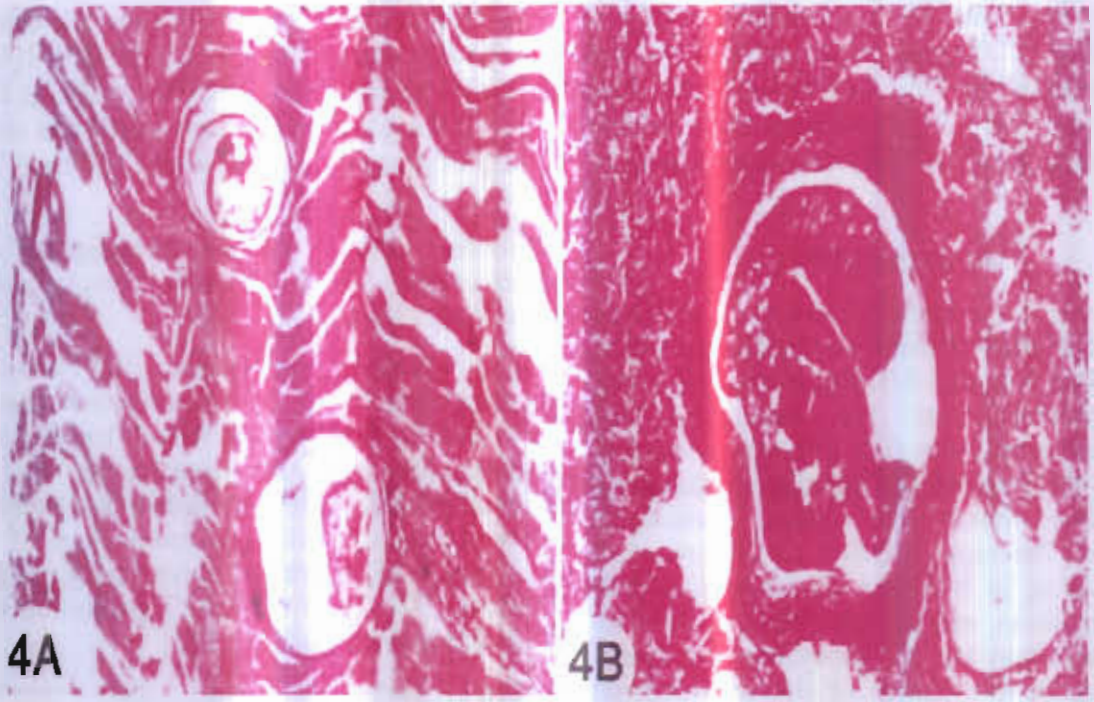



**Fig. 4:** Encysted metacercarieae in the skeletal muscles (a) and myocardium (b) of *O. niloticus* surrounded by thick fibrous capsule. H&E, x 250.

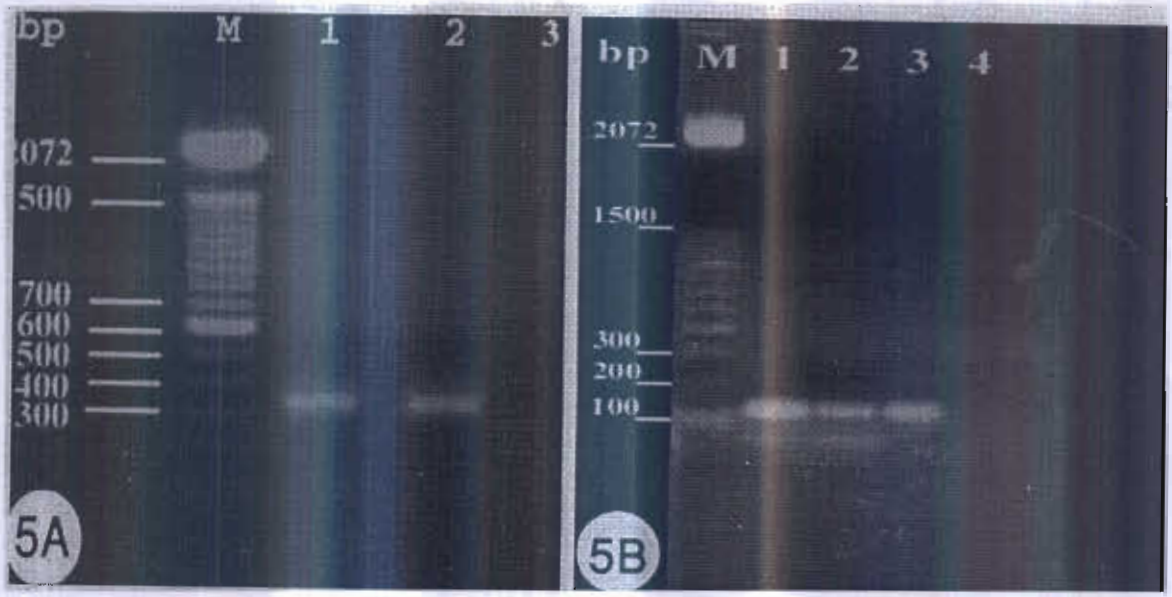



**Fig. 5: Panel A:** Agarose gel electrophoresis of PCR amplicons derived from Heterophyidea 18S gene. Agarose gel (2%) stained with Ethidium bromide. Lanes: (M) 100 bp DNA ladder (consists of repeats of 100 bp fragment size, Gibco, BRL); PCR using primer set "A", (1) single adult worm; (2) single metacercaria; (3) $H_2O$ as control negative. The size of the amplicons of about 361 bp. **Panel B:** Agarose gel electrophoresis of PCR amplicons derived from cytochrome c oxidase 1 (COI) gene. Agarose gel (2%) stained with Ethidium bromide. Lanes: (M) 100 bp DNA ladder100 bp DNA ladder (consists of repeats of 100 bp fragment size, Gibco, BRL); PCR using primer set "B"; (1) single metacercaria from, (2) single metacercaria from, (3) single metacercaria from, (4) $H_2O$ as control negative. The size of the amplicons of about 117 bp.

## DISCUSSION

Metacercarial infections have been found in fish in all studied inland water bodies in Africa and the Near East ***(22-24)***. Piscivorous birds are the definitive hosts for many of the metacercariae found in fish. The other essential factor is the presence of suitable molluscs as intermediate hosts for most trematode infections. The high infection of the encysted metacercariae, in both the semi-intensive and low input farms, ranged from 83% to 97% in both *O. niloticus* and *C. gariepinus* and could be due to the prevalence of the intermediate host (snails) and the failure of having suitable control measures. The presence of some organic matter in the water and the water temperature might also favor the survival of these intermediate hosts. The low prevalence of encysted metacercariae in the desert farms was attributed mainly to the absence of the intermediate hosts. Similar results were previously obtained ***(25, 26)***.

The higher prevalence of encysted metacercariae found in *C. gariepinus* than in *O. niloticus* may be attributed to the thin, smooth skin and soft muscles as well as absence of scales in *C. gariepinus* that probably favor the attachment and penetration of the cercariae. A high parasitic prevalence among *O. niloticus* (70 - 71.67 %) and *C. gariepinus* (80-83.6%) were reported ***(27, 28)***.

The prevalence of the encysted metacercariae in fishes depended mainly on the season and activities of the first intermediate host which disseminate infection to fish. In the present study, the seasonal prevalence of encysted metacercariae in both *O. niloticus* and *C. gariepinus* was highest in summer and

lowest in winter. Similar results were previously reported ***(29, 30)***. The warm water during summer may increases the activity of cercarial emergence and penetration. A similar observation was cited previously ***(31).*** The highest infestation rate for encysted metacercariae in summer (92%), followed by spring (60%) and autumn (28%) were reported in previous study ***(28)***.

The encysted metacercarial infections in the muscles and different organs of the examined fish were highest on the gill filaments and arches of *O. niloticus* (50%) and in the muscles of *C. gariepinus* (81%). Similar results were obtained by different authors ***(32, 33).*** The common site of infection in *O. niloticus* was the gills (74.28%) while in *C. gariepinus* was the skeletal muscles (97.5%) were observed in Egyptian aquaculture ***(28)***.

Morphological examination of the recovered encysted metacercariae from the *O. niloticus* and *C. gariepinus* samples revealed five types of encysted metacercariae; (1) Heterophyid metacercariae recovered from the gills of *O. niloticus* and liver of *C. gariepinus*. Very similar records were made ***(34).*** (2) *Euclinostomum heterostomum* metcaercariae was isolated from the kidneys of *O. niloticus,* similar to the results presented ***(35)***. (3) Cyathocotylid metacercariae were obtained from the skeletal muscles of *O. niloticus* and *C. gariepinus*, as obtained ***(36, 37)***. (4) Cyanodiplostomatid metacercariae were observed in the skeletal muscles of *C. gariepinus* ***(27, 32)***. (5) Diplostomatid metacercariae were recovered from the skin and gills of *O. niloticus* ***(38)***. Direct microscopic examination were able to identify the recovered encysted metacercariae from *O. niloticus* and *C. gariepinus* as heterophyids, clinostomatids, cyathocotylids, cyanodiplostomatids and diplostomatids ***(28)***.

Upon experimental infection of dogs with the aforementioned encysted metacercariae, many heterophyid parasites (*Heterophyes heterophyes, Heterophyes aequalis, Pygidiopsis genata, Procerovum varium, Haplorchis pumilio, Haplprichis taichui* and *Stictodora tanayensis*) and cyathocotylid parasites (*P. vivax, Gelanocotyle milvi, Mesostephanus appendiculatus, M. milvi* and *M. fajardensis*) were obtained. Very similar results were recorded in several studies ***(15, 39).*** The recovered encysted metacercariae from *O. niloticus* and *C. gariepinus* via experimental infection, and recorded two trematodes (*Prohemistomum vivax* and *Mesostephanus appendiculatus*) were identified ***(28)***.

Although piscivorous birds are the most common definitive hosts of Diplostomatidae and other Strigeoidea, mammalian hosts, including dogs, play an important role in the dissemination of the Heterophyid *Heterophyes heterophyes,* and the strigeoid *Prohemistomum vivax*. Hyperparasitism by cysts of different species of Diplostomatidae has been recorded in *C. gariepinus* in Africa. Digenean metacercariae from fish which commonly infect man are members of the families Opisthorchiidae and Heterophyidae. The opisthorchiids include *Opisthorchis temicollis* (*Opisthorchis felineus*) and *Opisthorchis sinensis* (*Clonorchis sinensis*), are found in the bile ducts of fish-eating mammals, including man ***(40)***. A considerable number of heterophyid genera contain species which are intestinal parasites of man ***(41)***. *Heterophyes heterophyes* is a common parasite of man in the Middle East and Far East ***(42)***. Several other genera of heterophyids have occasionally been recorded from man ***(43)***.

Histopathologically, the observed connective tissue capsule represented the tissue reaction of the affected fish against the prolonged irritation of the parasitic cysts. The aggregation of melanomacrophages indicated the activated body defense and provides an explanation for the black spots observed in the infected fish. The degenerative changes observed in the affected organs could be due to the toxic product substance produced by the parasitic cysts. Similar observations were previously recorded ***(28, 44- 46)***.

This study describes the genetic characterization of the local Egyptian Heterophyidae infecting fishes, based on application of two main approaches. The first approach utilized the genomic PCR reactions

on the extracted DNA from worms and metacercariae for general Heterophyidae 18S rDNA gene. The results showed that the *Heterophyes* adult worm, isolated from experimentally infected dogs and metacercariae isolated from infected fishes in Egyptian aquaculture belonged to the family Heterophyidae. Thirteen trematode species were recorded from fishes using PCR for the detection of rDNA 18S gene and obtained 361 bp amplicons from the DNA of a single adult worm and metacercaria which belonged to Heterophyidae. The second approach relied on PCR amplicons derived from cytochrome c oxidase 1 (COI) gene as more specific PCR to the Heterophyidae species. The 117 bp amplicons of this PCR from the DNA of a single metacercaria from different localities in Egypt, were in agreement with the flanked sequence of two designed primers ***(47)*** the sequence of some helminthes by PCR product of specific genes of Heterophyidae.

The control of these digenean parasites and their larval stages should be done through public health education to persuade people not to eat raw fish to avoid human infection through proper cooking or deep freezing, which can kill the metacercariae, and the proper disposal of human fecal material which can kill the larval stages of these parasites. To limit parasitic cyst infestation in pond-reared fishes, snails and fish-eating birds either via biological control or through using molluscicides. Also, environmentally sound solutions for scaring or removing birds or use of netting, wire grids, and fencing because these devices offer producers some long-term protection.

It could be concluded that the occurrence of metacercariae varied with aquaculture system, cultured fish species and the season of the year, many of the reported metacercariae were larval stages for digenean parasites of zoonotic importance and public health hazard. The PCR, based on two different genes, is a relevant tool for genetic characterization of Heterophyidae as a zoonotic parasite isolated from fishes in Egypt. It also has a great potential for application of the clinical epidemiological studies for the fast detection of food-borne parasites in freshwater fishes.

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## الملخص العربى

# دراسات باثولوجية ، طفيلية و بيولوجية جزيئية على الميتاسيركاريا في أسماك البلطي النيلي والقراميط المزروعة فى مصر

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تم تجميع أسماك البلطي النيلي والقراميط من ثلاث أنواع مختلفة من المزارع السمكية في مصر في الفترة ما بين ٢٠٠٨-٢٠٠٩ وفحصها لوجود الميتاسركاريا المتحوصلة مجهريا ، بيولوجيا وباستخدام اختبار إنزيم البلمرة المتسلسل . لوحظ أن أعلى نسبة للعدوى كانت في المزارع النصف مكثفة واقلها في المزارع الصحراوية وكانت نسبة الإصابة عالية أثناء الصيف في خياشيم اسماك البلطي النيلي وعضلات اسماك القراميط. بالفحص البيولوجي لصغار الكلاب تبين وجود هتروفيس هتروفيس ، هتروفيس اكوالس ، بيجدوبسس جيناتا ، بروسيروفم فاريم ، هبلوركس بلمولى ، هبلوركس تاكى ، استكتودورا جيناتا،بروهيموستومم فيفكس، جيلانوستلى ملفى ، ميزوستيفنس ابندكيولاتس، ميزوستيفنس ملفى ، ميزوستيفنس فاجردنسس .بالفحص المجهرى للعضلات والأعضاء الداخلية وجد ارتشاح محاط بغطاء من النسيج الليفى بينما أدت الميتاسركاريا المتحوصلة الى ضمور وتنكس ونخر موضعي وتكثر للخلايا الحاملة للميلانين فى هذه الأعضاء. أظهر إختبار انزيم البلمرة المتسلسل خطوط منفصلة متوافقة مع القطعة بحجم ٣٦١ نيكلوتيدة من الجين 18s rDNA فى المحتوى الجيني للعائلة هيتروفيدى سواء كانت دودة ناضجة أو ميتاسيركاريا