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Prevalence, Morphological, and Molecular Diagnosis of Some Foodborne Encysted Metacercariae Affecting Fish and Their Control Using Some Food Safety Measures

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ABSTRACT

A total of 100 *Oreochromis niloticus*, 100 *Clarias gariepinus* and 100 *Mugil capito* of variable weights and lengths were collected from Suez and Ismailia governorates from early August 2022 to late July 2023 to detect the total and seasonal prevalence of encysted metacercariae and their distribution in different fish body parts and organs. The total prevalence of encysted metacercariae (EMC) in *O. niloticus* was 84.15%, the highest prevalence was in the muscles of the tail region (95.9%), followed by the trunk region (74.5%) and the head region (54.8%). Among *C. gariepinus*, it was 99.0% in trunk regions, 89.65% in the head region and 82.3% in the tail region. In *Mugil capito*, the highest was in the trunk region (93.9%), followed by the head region (66.95%) and the tail region (57.75%). Seasonally, the highest prevalence was recorded in winter and summer; 100.0 and 88.3% for *O. niloticus* and *M. capito*, respectively. While in *C. gariepinus*, there was no significant difference of prevalence between all seasons as it was 86.65, 85.0, 85.0 and 83.3 in autumn, summer, winter, and spring, respectively. The recovered EMC from *O. niloticus* and *C. gariepinus* were morphologically and molecularly (PCR) identified to belong to family Cyathocotylidae. These EMC were successfully advanced into adult worms after the experimental infection of Wister albino rats. The developing adult flukes were *Prohemistomum vivax*, *Mesostephanus appendiculatus* and *Mesostephanus fajardensis*, which are of public health importance. Freezing the infected muscles of *O. niloticus* and *C. gariepinus* with EMC at -17 to -15°C for 3 to 7 days was sufficient to destroy all EMC in these fish muscles. Cooking using an electric oven at 250°C for 15- 20min was sufficient only to destroy EMC in *O. niloticus* muscles. Controlling EMC using processing methods such as freezing and cooking was very important to avoid zoonosis and ensure food safety.

INTRODUCTION

Fish have a vital role as a food source of microelements, especially in the developing countries **(Hasselberg *et al.*, 2020)**. Moreover, fish are important for humans due to its bioavailability of elements. For instance, they contain vitamins, minerals, polyunsaturated fatty acids, omega-3 fatty, omega-6 fat, anti-oxidants and high biological

value proteins that are rapidly digested by humans. It can replace meat as a protein source and provide all necessary amino acids and iodine **(Tilami & Samles, 2018; Prabhakar *et al.*, 2020)**. The tilapia is a widespread fish species in Egypt. It has a high economic value and an increased growth rate, as well as tolerating variability of environmental conditions. Moreover, it can be cultured simply owing to its resistance to huge amounts of organic substance in the water and low O_2 level **(Arguedas *et al.*, 2017; Debnath *et al.*, 2023)**. In Egypt, another group of fish 'Mullets' are considered popular and highly consumed by the Egyptians as a result of their high taste. Furthermore, the salt-fermented *Mugil* spp. is a conventional festival food that is usually consumed throughout Easter day in Egypt **(Khalil *et al.*, 2014)**. Catfish is one of the most important and highly consumed fish species in the world. They are extremely palatable freshwater fish **(NFI, 2017)**. Fish, living in their environment, makes them susceptible to diseases and other environmental influences. Fish serve as a final host and also as an intermediate host for several parasites. One of these parasites is the encysted metacercaria of the digenetic trematodes, which can exhaust the fish. They may lower their growth, particularly young fish, increase their susceptibility to secondary infections by decreasing immunity, increase their mortality, and result in economic losses by lowering quality, marketability, and fish price **(Bhuiyan *et al.*, 2007; Abou-Eisha *et al.*, 2008)**. In Egypt, parasitic diseases represent a huge sector of fish diseases (about 80%) **(GAFRD, 2020; Eldanasory *et al.*, 2022)**. Humans become infected by fish-borne trematodes while eating raw or incompetently cooked fish that accommodated metacercariae **(Sohn, 2009; Khoa *et al.*, 2020)**. Fish-borne trematode infections influence the health of above 50 million humans worldwide **(Fürst *et al.*, 2012)**. From over 100 trematode species that influence people, there are 59 species known to be food-borne zoonotic trematode (FZT) **(Chai *et al.*, 2009; Qiu *et al.*, 2017)**. Infestation in humans is asymptomatic or unrecognized. Heavy infections might cause damage to the intestinal mucosa, abdominal pains, intermittent bloody diarrhea and colic. Moreover, eggs when entering the fluids of the circulatory system and traveling to body organs, they cause granuloma and fibrosis **(El-Sheikha, 2007; Lobna *et al.*, 2010)**. *Metagonimus appendiculatus* is of a zoonotic importance since it is reported in both man and fish **(Kuntz & Chandler, 1956; Shalaby, 1985)**. *Prohemistomum vivax* was seldom documented to harm humans or cause death **(Williams & Jones, 1976; Satour *et al.*, 2019)**. Most parasites are destroyed by different freezing degrees for different periods. The Fish and Fishery Products Hazards and Controls Guide recorded that a temperature under -20°C for 7 days or -35°C for 15 hours can destroy all parasites **(FFPHCG, 2020)**. Proper cooking of fish stimulates the decrease of viability of all metacercaria which infect fish **(Marcus *et al.*, 2012; Sripan *et al.*, 2017)**. Consequently, the current work pointed to investigate the different types of encysted metacercariae affecting some fish, their identification by ordinary and recent methods, total and seasonal prevalence, distribution and prevalence in different fish body parts and organs. The study also documented the

development of adult worms and explored methods to control zoonotic digeneans among humans through various processing techniques.

MATERIALS AND METHODS

1. Fish samples

A total of 300 fish samples (100 *Oreochromis niloticus*, 100 *Clarias garipienus* and 100 *Mugil capito*) of various weights and lengths were collected from Suez and Ismailia governorates (180 from Suez and 120 from Ismailia) from early August 2022 to late July 2023. They were transported to the laboratory of the Fish Processing and Technology Department, Faculty of Fish Resources, Suez University as soon as possible and then immediately examined.

2. Clinical picture

Moribund fish were examined for any external anomalies according to the method of **Amlacker (1970)** and **Noga (2010)**. Recently dead and sacrificed fish were then examined internally according to the method of **Conroy and Hermann (1981)**.

3. Parasitological examination

3.a. Macroscopic examination

The collected fish were examined for the revelation of any abnormalities in fish body by the naked eyes according to the method of **Syme (1966)**, **(1985)** and **Mahdy *et al.* (1995)**.

3.b. Microscopic examination

Different fish body parts and organs were examined using the compression technique; snips were taken from muscles, gills, and other tissues of each part of the fish body, mixed with a few drops of salt solution, compressed between two microscopic glass slides and examined by microscope for detection of the encysted metacercariae as outlined by **Garcia (2001)**, **Sohn *et al.* (2005)** and **Fadel *et al.* (2019)**. Infected snips of muscles with encysted metacercariae were fixed in a 10% formaldehyde solution, stained by Semichon's acetocarmine and mounted in Canada balsam **(Kruse & Pritchard, 1982)**. They were morphologically identified to the family level as detected by **Saleh *et al.* (2009)**, **Caffara *et al.* (2014)** and **Abd-ELrahman *et al.* (2023)**.

3.c. Distribution and intensity of encysted metacercariae

The fish body was divided into head, trunk, and tail region, then 10 grams from the muscles of infected parts were mechanically homogenized separately and examined microscopically. The number of EMC per gram of muscles from each part of the body was calculated as reported by **El-Naffar and El-Shahawi (1986)** and **Elsheikha and Elshazly (2008a)**.

3.d. Excystation of the encysted metacercariae

The detected encysted metacercariae in the fish muscles were excysted via the tissue digestion method to identify them based on the morphological details and their dimensions according to **Yokogawa and Sano (1968)**, **Elsheikha and Elshazly (2008b)** and **Sohn (2009)**.

4. Experimental infection

A total of 20 Wistar albino rats weighing 175g/ each were purchased from the Experimental Animal Center, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt. They were reared for 7 days with a daily examination of their faeces to ensure that they were free from any natural infection prior to the experimental infection, then divided into four groups (5 rats/ each) in separate cages. Each group was fed orally on fish muscles, then examined daily for detecting the digenetic trematode eggs in their feces, then sacrificed after 7 days post-infection, and the small intestines were examined for the presence of any adult digenetic trematodes according to **Hong *et al.* (1989)**. Group 1 (G1) and Group 2 (G2) were fed on 100 and 185g of healthy tilapia and catfish muscles, respectively. While Group 3 (G3) and Group 4 (G4) were fed on 100 (62EMC /g) and 185g (49EMC/ g) of infected tilapia and catfish muscles, respectively. The recovered adult trematodes were washed in the salt solution, fixed in alcohol formalin acetic acid **(Georgi & Georgi, 1992)**, stained with Semichon's acetocarmine, dehydrated, and cleaned in xylene, and mounted in Canada balsam then identified **(Abou Zaid *et al.*, 2018)**.

5. Molecular identification

5.a. DNA extraction

Accordingly, the morphological identification, the DNA of the EMC was extracted using QIAamp DNA Mini Kit (Qiagen) based on the manufacturer's instructions. DNA was kept at -20°C till usage.

5.b. Amplification of the 28S rDNA gene

Primers used for the amplification of the 28S rDNA were: AP103 F:5'AGAGCGCAGCCAACTGTGTGA3' and AP103 R:5'TGCCACGTCCTAGCATCAGCC 3'. After amplification, 8µl of the PCR yield was loaded onto a 1.5% agarose gel stained with ethidium bromide. The gel was then electrophoresed for 45 minutes and visualized using a UV transilluminator. A 5µl DNA solution was used per 50µl PCR reaction **(Arya *et al.* 2016**; **Elaswad *et al.* 2021)**.

6. Effect of electric oven temperature and freezing on metacercarial infectivity

A total of 50 Wistar albino rats weighing 175g/ each (ten groups (5/ each) in separate cages) were used for this purpose according to the method of **Mahmoud (1983)** and **Hong *et al.* (1989)**. All groups were fed on 50 grams of infected muscles as follows: G5 and G6 (control positive groups) were fed on infected tilapia and catfish muscles with a dose of 42 and 61EMC/ g, respectively. G7 and G8 were fed on infected tilapia and catfish muscles containing 22 and 39EMC/ g and were cooked in an electric oven at 250°C for 15min, respectively. G9 and G10 were fed on infected tilapia and catfish muscles containing 42 and 61EMC/ g and were cooked in an electric oven at 250°C for 20min, respectively. G11 and G12 were fed on infected tilapia and catfish muscles containing 26 and 45EMC/ g and were thawed after freezing for 3 days at 0- 5°F (-17 to -15°C), respectively. G13 and G14 were fed on infected tilapia and catfish muscles

containing 42 and 61EMC /g which were thawed after freezing for 7 days at 0- 5°F (-17 to -15°C), respectively. Examination of their feces was daily done after infection to detect the time of shedding of the trematode eggs.

7. Statistical analysis

The statistical analysis was achieved by IBM SPSS for Windows, version 22.0 (IBM Corp., Armonk, NY, USA, 2013).

RESULTS

1. Clinical examination

Most of the naturally infected fishes showed no severe clinical signs. Some cases of *Oreochromis niloticus* showed inflammations all over the body surface, with hemorrhages on the pectoral fin and scales loss (Fig. 1a), skin erosions and darkening (Fig. 1b) and excessive mucus secretion. In *Clarias gariepinus*, skin erosion and ulcerations were also detected all over the body surface (Fig. 1c), while *Mugil capito* showed black spots on the skin of the posterior part (Fig. 1d) and reddening of the fins (Fig. 1e). Some infected fish showed an abnormal behavior, such as swimming upside down, scratching against objects and gills moving rapidly.

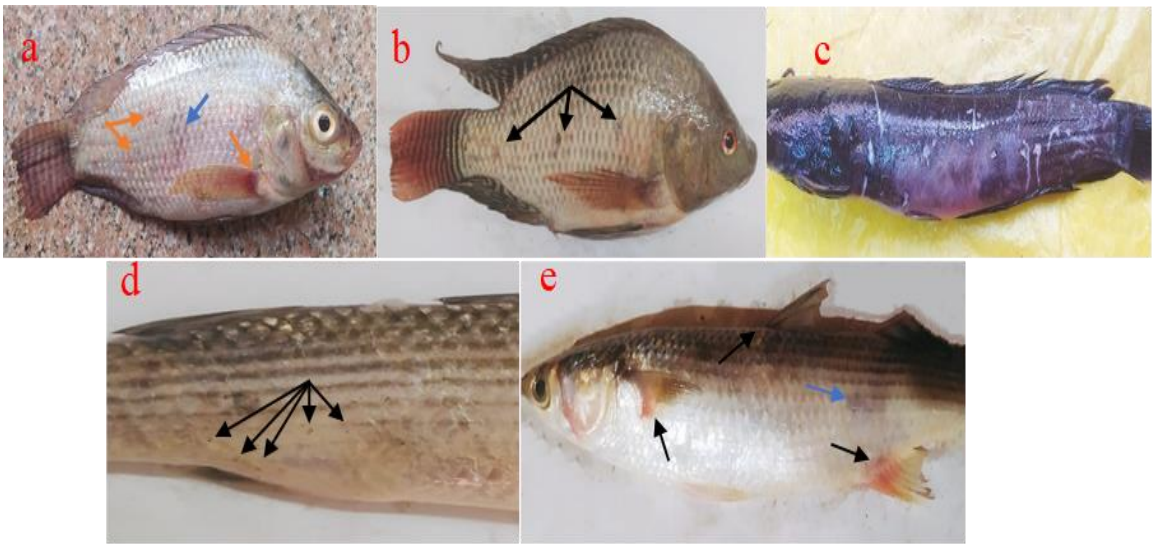

Fig. 1. *Oreochromis niloticus* showing: **(a)** Hemorrhages all over the body surface and on pectoral fin (orange arrows) with scale loss (blue arrows), **(b)** Skin erosions (black arrows), **(c)** *Clarias gariepinus* shows skin erosions and ulceration and *Mugil capito* shows **(d)** Black spots on the posterior part, and **(e)** Reddening of the fins (black arrows) and scale loss (blue arrows)

2. Prevalence of encysted metacercariae

2.a. Total prevalence of encysted metacercariae

Out of the 300 examined fish, 240 (80.0%) were infected with encysted metacercariae, 148 (82.22%) from Suez and 92 (76.67%) from Ismailia Governorate (Table 1). Table (2) exhibits no significant difference ($P > 0.05$) of prevalence among *Clarias garipienus* (85.0 %), *Oreochromis niloticus* (84.15%) and *M. capito* (69.15%).

Table 1. Total prevalence mean values % of the examined fish in Suez and Ismailia governorates

Governorate	Suez (n=180)	Ismailia (n=120)	Both (n=300)
Prevalence (mean %)	148 (82.22)	92 (76.67)	240 (79.44)

Table 2. Total prevalence mean values % in all examined fishes from both governorates

Fish species	*Oreochromis niloticus* (n=100)	*Clarias gariepinus* (n=100)	*Mugil capito* (n=100)
Prevalence (mean %)	85 (84.15^{a})	85 (85.0^{a})	70 (69.15^{a})

- n: Number of examined fish.
- Means in the same row with different superscripts are significantly different ($P \leq 0.05$).
- Means in the same raw with the same superscripts are non-significantly different ($P > 0.05$).

2.b. Seasonal prevalence of encysted metacercariae

Table (3) shows that there was a significant difference ($P < 0.05$) of the prevalence of encysted metacercariae in *Oreochromis niloticus* between winter and both spring and summer, however there was no significant difference of prevalence of EMC between winter and autumn. There was no significant difference of prevalence of EMC between summer, spring and autumn with the highest significant difference was in winter (100.0%) and the lowest was detected in summer (86.61%) and spring (71.65%). While, in *Clarias gariepinus*, there was no significant difference ($P > 0.05$) between all seasons, but in *Mugil capito*, there was a significant difference ($P < 0.05$) between prevalence in summer and spring with the highest prevalence in summer (88.3%) and the lowest in spring (50.0%).

Table 3. Comparative seasonal prevalence mean values % in the examined fish

Season / Fish species	Winter		Spring		Summer		Autumn	
	A	**B**	**A**	**B**	**A**	**B**	**A**	**B**
***Oreochromis niloticus* (n=25)**	25	100.0^{a}	18	71.65^{b}	19	73.3^{b}	23	91.65^{ab}
***Clarias gariepinus* (n=25)**	21	85.0^{a}	20	83.3^{a}	22	85.0^{a}	22	86.65^{a}
***Mugil capito* (n=25)**	15	58.3^{bc}	13	50.0^{c}	22	88.3^{a}	20	80.0^{ab}

- n: Number of examined fish. A= Number of infected fish. B= Prevalence of EMC.
- Means in the same row with different superscripts are significantly different ($P \leq 0.05$).
- Means in the same raw with the same superscripts are non-significantly different ($P > 0.05$).

2.c. Prevalence of encysted metacercariae in muscles and different organs of the examined fish

Table (4) displays no significant difference ($P > 0.05$) between prevalence in gills and muscles of *Oreochromis niloticus* as it was 87.44 and 82.16%, respectively, and there was significant difference ($P < 0.05$) between them and other infected tissues with prevalences of 30.13, 18.85, 17.28, 2.83, and 2.5%, respectively, in liver, heart, kidney,

gonads and spleen. While the prevalence in muscles of *Clarias gariepinus* was significantly higher (100.0%) than in other infected tissues, there was no significant difference ($P > 0.05$) between prevalence in liver and kidney and between kidney and heart, but there was a significant difference ($P < 0.05$) of prevalence between liver and heart. There was no significant difference ($P > 0.05$) between prevalence in gonads, spleen and gills, however there was a significant difference ($P < 0.05$) between them and other organs, with the highest significant difference in the muscles and the lowest in the gills. Furthermore, the prevalence in muscles of *Mugil capito*, was significantly higher (90.82%) than that in the other infected tissues, followed by gills (33.9%), while there was no significant difference ($P > 0.05$) between prevalence in the liver, heart, kidney, spleen, and gonads.

Table 4. Comparative prevalence mean values % [C] among different tissues of the examined fishes

Fish species / Infected tissue	*Oreochromis niloticus*			*Clarias gariepinus*			*Mugil capito*		
	A	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**
Muscles (n=100)	85	72	82.16^{a}	85	85	100.0^{a}	70	64	90.82^{a}
Liver (n=100)	85	28	30.13^{b}	85	50	58.8^{b}	70	3	3.4^{c}
Kidney (n=100)	85	17	17.28^{b}	85	39	44.09^{bc}	70	0	0.0^{c}
Heart (n=100)	85	20	18.85^{b}	85	20	25.49^{c}	70	2	2.25^{c}
Spleen (n=100)	85	2	2.5^{b}	85	3	2.94^{d}	70	0	0.0^{c}
Gonads (n=100)	85	3	2.83^{b}	85	0	0.0^{d}	70	0	0.0^{c}
Gills (n=100)	85	75	87.44^{a}	85	1	1.45^{d}	70	25	33.9^{b}

- n= Number of examined fish. A= Number of infected fish. B= Number of infected tissue.
- Means in the same column with different superscripts are significantly different ($P \leq 0.05$).
- Means in the same column with the same superscripts are non-significantly different ($P > 0.05$).

2.d. Distribution of the encysted metacercariae in different muscle parts of the examined fish

Table (5) illustrates that there was no significant difference ($P > 0.05$) in the prevalence of EMC in tail and trunk regions in *Oreochromis niloticus* (95.9, 74.5%, respectively) and between head and trunk regions (54.85 and 74.5%, respectively). The lowest prevalence was found in the head region (54.85%). On the other hand, there was no significant difference ($P > 0.05$) of EMC in the trunk and head regions of *Clarias gariepinus* (99.0, 89.65%, respectively), while there was a significant difference ($P < 0.05$) between trunk and tail regions with the lowest prevalence (82.3%) in the tail region. In *Mugil capito*, there was a significant difference ($P < 0.05$) between trunk region and tail region of muscles, the highest significant difference of prevalence was recorded in trunk region and the lowest one was in tail regions and there was no significant difference ($P > 0.05$) of prevalence between head and trunk regions, and also between head and tail regions.

Table 5. Prevalence of EMC infection in different body muscle parts (mean values%) [B] in the examined fish

Different body part / Fish species	Head region		Trunk region		Tail region	
	A	**B**	**A**	**B**	**A**	**B**
***Oreochromis niloticus* (n=72)**	38	54.85^{b}	55	74.5^{ab}	68	95.9^{a}
***Clarias gariepinus* (n=85)**	76	89.65^{ab}	84	99.0^{a}	69	82.3^{b}
***Mugil capito* (n=64)**	44	66.95^{ab}	59	93.9^{a}	38	57.75^{b}

- n= Number of infected fish. A= Number of infected fish/ part of muscles.
- Means in the same row with different superscripts are significantly different ($P \leq 0.05$).
- Means in the same raw with the same superscripts are non-significantly different ($P > 0.05$).

2.e. Prevalence of encysted metacercariae in relation to the weight of the examined fish

Data in Table (6) indicate that there was no significant difference ($P > 0.05$) in the prevalence of encysted metacercariae among *Oreochromis niloticus* weights (≤50 and >50- 100g). The highest prevalence was recorded in weights ≤50g (100.0%) then 85.6% in weights were >50- 100g and the lowest was in weights >100- 150g, as it was 31.25%. In *Clarias gariepinus*, there was no significant difference ($P > 0.05$) between weights of >300- 350, >200- 250, >250- 300, >150- 200, and >100- 150g with a prevalence of 95.0, 89.5, 87.5%, 83.75, and 75.0%, respectively. However, there was a significant difference ($P < 0.05$) between them and weights of >50- 100 and >350- 400g, with a prevalence of 16.65 and 16.65%, respectively. Furthermore, in *Mugil capito*, a significant difference of prevalence was detected between weights >50- 100g (100.0%) and other weight. There was no significant difference of prevalence in weights >100- 150, >150- 200, and >200- 250g, but there was a significant difference of prevalence between weights >100- 150g and both weights of >50- 100 and >200- 250g. Furthermore, there was no significant difference of prevalence between weights >150- 200 and >200- 250g. The highest significant difference of prevalence was in weights >50- 100g, and the lowest was in weights >200- 250g.

2.g. Prevalence of encysted metacercariae in relation to the length of the examined fish

Table (7) records that there was no significant difference ($P > 0.05$) between prevalence of encysted metacercariae in lengths >15- 20 and ≤15cm in *Oreochromis niloticus* with a prevalence of 87.15 and 84.75 %, respectively, but there was significant difference ($P > 0.05$) between the prevalence in the previous lengths and tha recorded in lengths >20- 25cm (30.0%). In *Clarias gariepinus*, there was no significant difference ($P > 0.05$) in lengths of >35- 40, >40- 45, >25- 30 and >30- 35cm with a significant difference of prevalence was 93.8, 87.1, 83.65, and 82.5%, respectively. Moroever, there was a significant difference ($P < 0.05$) between them and lengths >45- 50cm as they showed the lowest significant (55.0%). Additionally, no significant difference was recorded in lengths >20- 25 and >25- 30cm of *Mugil capito* with a prevalence of 79.3 and

63.3%, respectively, while there was a significant difference ($P< 0.05$) between them and lengths >15- 20cm as they showed the lowest prevalence (20.0 %).

Table 6. Comparative prevalence of encysted metacercariae (mean %) [C] in relation to weight of the examined fish

Fish species / Weight	*Oreochromis niloticus*			*Clarias gariepinus*			*Mugil capito*		
	A	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**
≤50g	15	15	100.0^{a}	0*	0*	0.0^{b}	0*	0*	0.0^{d}
>50- 100g	76	65	85.6^{a}	4	1	16.65^{b}	4	4	100.0^{a}
>100- 150g	8	5	31.25^{b}	15	12	75.0^{a}	69	52	75.1^{b}
>150- 200g	1	0	0.0^{b}	26	22	83.75^{a}	23	12	51.9^{bc}
>200- 250g	0*	0*	0.0^{b}	22	20	89.5^{a}	4	0	25.0^{cd}
>250- 300g	0*	0*	0.0^{b}	13	12	87.5^{a}	0*	0*	0.0^{d}
>300- 350g	0*	0*	0.0^{b}	17	17	95.0^{a}	0*	0*	0.0^{d}
>350- 400g	0*	0*	0.0^{b}	3	1	16.65^{b}	0*	0*	0.0^{d}

- A= Number of examined fish. B= Number of infected fish. *: Not available.
- Means in the same column with different superscripts are significantly different ($P\leq 0.05$).
- Means in the same column with the same superscripts are non-significantly different ($P> 0.05$).

Table 7. Comparative prevalence of encysted metacercariae (mean %) [C] in relation to length of the examined fish

Fish species / Length	*Oreochromis niloticus*			*Clarias gariepinus*			*Mugil capito*		
	A	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**
≤15cm	29	25	84.7^{a}	0*	0*	0.0^{b}	0*	0*	0.0^{b}
>15- 20cm	65	57	87.15^{a}	0*	0*	0.0^{b}	8	2	20.0^{b}
>20- 25cm	6	3	30.0^{b}	2	0	0.0^{b}	48	38	79.3^{a}
>25- 30cm	0*	0*	0.0^{b}	21	18	83.65^{a}	44	30	63.3^{a}
>30- 35cm	0*	0*	0.0^{b}	14	12	82.5^{a}	0*	0*	0.0^{b}
>35- 40cm	0*	0*	0.0^{b}	37	35	93.8^{a}	0*	0*	0.0^{b}
>40- 45cm	0*	0*	0.0^{b}	17	15	87.1^{a}	0*	0*	0.0^{b}
>45- 50cm	0*	0*	0.0^{b}	9	5	55.0^{b}	0*	0*	0.0^{b}

- A= Number of examined fish. B= Number of infected fish. *= Not available.
- Means in the same column with different superscripts are significantly different ($P\leq 0.05$).
- Means in the same column with the same superscripts are non-significantly different ($P> 0.05$).

3. Microscopic examination

The examined encysted and excysted metacercariae from muscles and organs of infected *Oreochromis niloticus* and *Clarias garipienus* were identified to the family Cyathocotylidae, which is oval to rounded in shape. The cysts had a rigid inner wall and a brittle double wall on the external layer, which was dark brown. The ventral

sucker and oral sucker were well developed, but pseudosuckers were absent. Fig. (2) illustrates the previous morphology.

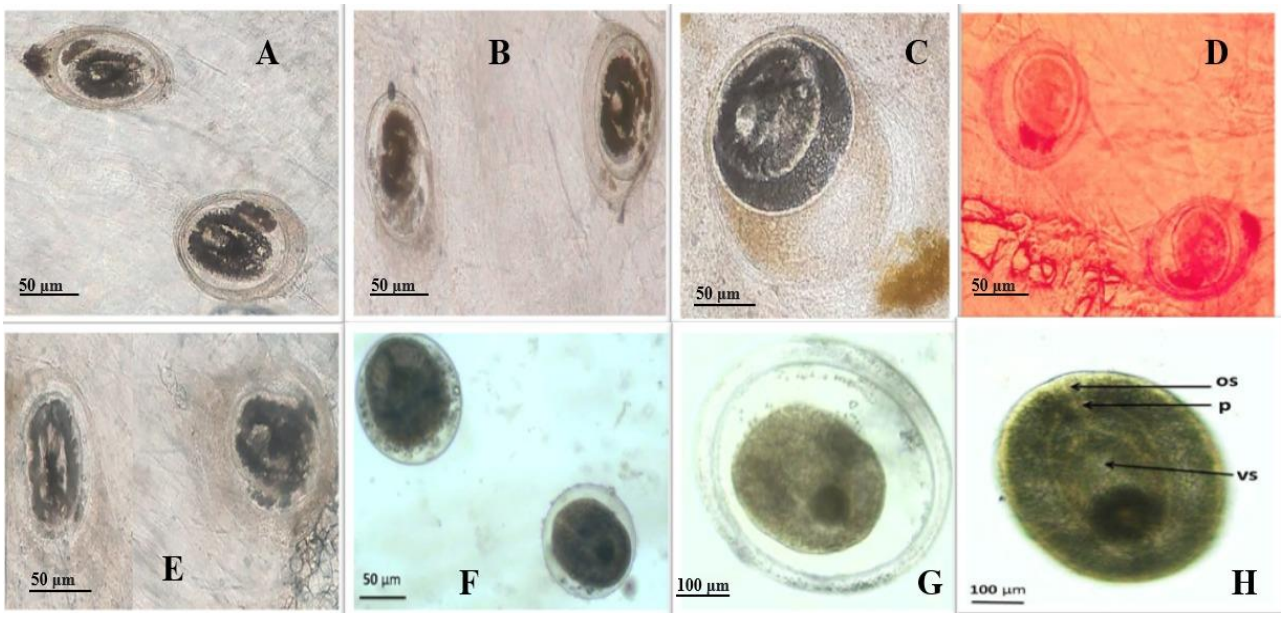

Fig. 2. Cyathocotylidae EMC in muscles of *O. niloticus*, **(A & B)** Unstained, **(D)** Stained and unstained Cyathocotylidae EMC in **(C)** kidney, **(E, F & G)** muscles of *Clarias gariepinus* by compression method, and **(H)** Cyathocotylidae excysted metacercariae from muscles of *Clarias garipinienus* by digestion method. (os = Oral sucker, vs = Ventral sucker and p = Pharynx)

4. Molecular identification

Fig. (3) shows the amplification of a 300bp product of the 28S rDNA region which was done by the morphologically identified Cyathocotylidae encysted metacercarial samples. The identification was based on the product size which revealed that samples follow family Cyathocotylidae.

Fig. 3. Analysis of 28S rDNA PCR products of encysted metacercariae by agarose gel-electrophoresis. The left lane constitutes 100bp (base pair) DNA ladder plus marker. Lanes from 1- 2 constitute PCR product for DNA the samples with the product an approximately 300 bp and Lane 3 is negative control

5. Experimental infection

The results of experimentally infected Wistar albino rats with muscles containing Cyathocotylidae metacercariae after 7 days post-infection are shown in Table (8). The recovered trematodes were *Prohemistomum vivax* (Fig. 4A), *Mesostephanus appendiculatus* (Fig. 4B), and *Mesostephanus fajardensis* (Fig. 4C). They were isolated from the small intestine of the infected rats.

Table 8. Results of experimental infection

Infectivity / Group	Source of EMC	A	B	Infective dose	(%)	Time of egg shedding	Isolated adult trematodes
Group1 (control group)	Tilapia (healthy muscles)	5	Nil	—	0.0	No eggs in feces	Nil
Group2 (control group)	catfish (healthy muscles)	5		—	0.0		
Group3	Tilapia (infected muscles)	5	5	6200	100.0	7 days	*P. vivax* *M. appendiculatus* *M. fajardensis*
Group4	catfish (infected muscles)	5	5	9065	100.0		*P. vivax* *M. appendiculatus* *M. fajardensis*

- **A:** No. of experimentally infected rats.
- **B:** No. of rats which took infection.
- **EMC:** Encysted metacercaria.

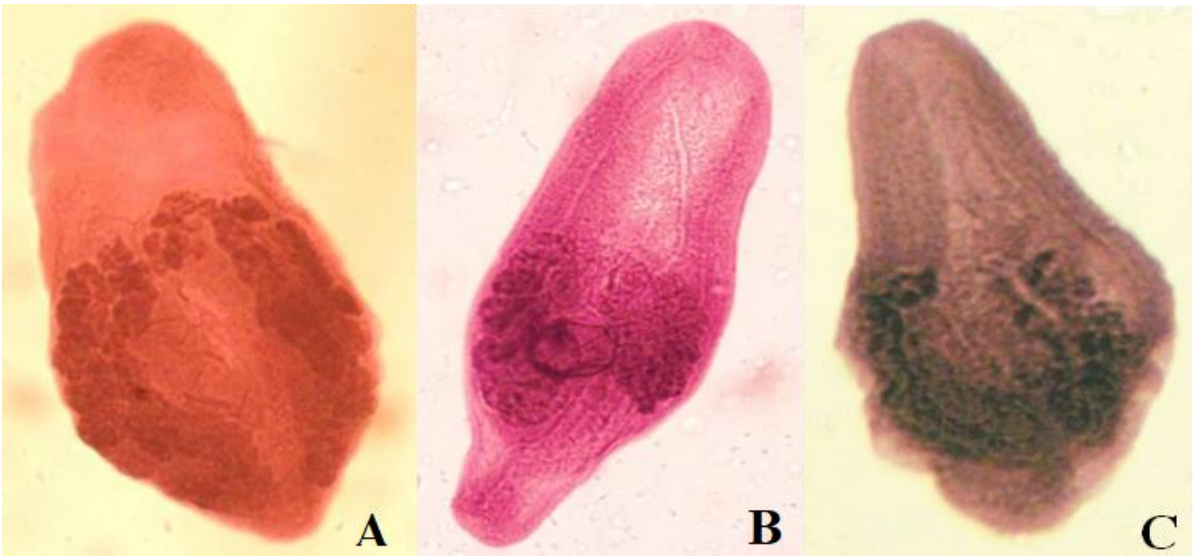

Fig. 4. Stained adult Cyathocotylid trematodes recovered from the intestine of experimentally infected white albino rats showing**: (a)** *Prohemistomum vivax*, **(b)** *Mesostephanus appendiculatus*, and **(c)** *Mesostephanus fajardensis*

6. Results of the effect of different food processing methods on the infectivity of Cyathocotylid encysted metacercariae that infect both *Oreochromis niloticus* and *Clarias gariepinus* muscles

The two positive control groups of white albino rats which were fed on infected muscles of *Oreochromis niloticus* and *Clarias gariepinus* containing Cyathocotylidae encysted metacercariae (EMC) had become infected with the isolation of 3 adult trematodes (*Prohemistomum vivax, Mesostephanus appendiculatus* and *Mesostephanus fajardensis)* from their small intestines with 100.0% infection ratio, as shown in Table (9). Results of the effect of electric oven temperature and freezing on the infectivity of the viable EMC affecting muscles of the 2 fish species were also recorded in Table (9).

Table 9. Effect of different food processing methods on the infectivity of cyathocotylidae encysted metacercariae that infect both *Oreochromis niloticus* and *Clarias gariepinus*

Group	Source of infection	Infective dose (No. of EMC /rat)	Time of exposure for electric oven temperature (250°C)	Time of exposure for freezing (-15- -17°C)	No. of experimentally infected rats	No. of rats that took the infection	Infectivity (%)	Time of egg shedding
Group 5 (Control positive)	Tilapia (Infected muscles)	2100	……	……	5/ each group	5	100.0	After 7 days
Group 6 (Control positive)	Catfish (Infected muscles)	3050	……	……		5	100.0	After 7 days
Group7	Tilapia (Infected muscles)	1100	/15 min	……		0	0.0	None until 7 days
Group 8	catfish (Infected muscles)	1950	/15 min	…….		5	100.0	After 7 days
Group 9	Tilapia (Infected muscles)	2100	/20 min	……..		0	0.0	None until 7 days
Group 10	Catfish (Infected muscles)	3050	/20 min	…….		5	100.0	After 7 days
Group 11	Tilapia (Infected muscles)	1300	…….	/3 days		0	0.0	None after 7 days
Group 12	Catfish (Infected muscles)	2250	…….	/3 days		0	0.0	None after 7 days
Group13	Tilapia (Infected muscles)	2100	……	/7 days		0	0.0	None after 7 days
Group 14	Catfish (Infected muscles)	3050	……	/7 days		0	0.0	None after 7 days

- EMC: Encysted metacercariae. No: Number.

DISCUSSION

Fish can harbor numerous pathogens, such as the metacercarial infection which are pathogenic to fish and can also be pathogenic or potentially pathogenic to man **(Aly *et al.*, 2005)**. The current study investigated encysted metacercarial infections in freshwater fish from various perspectives, including their prevalence, seasonal prevalence, distribution, and prevalence in different regions and organs of fish bodies. Their developing adult worms and how to control zoonotic digeneans among human beings through different processing methods were also recorded.

The results revealed no severe clinical signs among infected fish. Some cases of *Oreochromis niloticus* showed inflammations all over the body surface, with hemorrhages on pectoral fins, scale loss, skin erosions and excessive mucus secretion. In *Clarias gariepinus,* skin erosions and ulcerations were detected all over the body surface while infected *Mugil capito* showed black spots on the skin of the posterior part and reddening of the fins. These results comparatively agree with the result documented by **Aly *et al.* (2005)** who stated that skin darkening, excessive mucus secretion, detached scales, variable-sized erosions, and necrosis of the skin were detected in *O. niloticus* infected with encysted metacercariae; **Abd rabo *et al.* (2017)** who showed black coloration and ulcer on the abdomen and dorsal aspect of the body of *C. gariepinus;* and **Derwa *et al.* (2019)** who reported that naturally infected *O. niloticus* with encysted metacercariae suffer from hemorrhages and darkening of the skin and frayed fins. While in *C. gariepinus* there was erosion all over the body, hemorrhages and rubbing fins. These signs can be caused due to the encystation while playing a role as a stress factor causing a decrease in fish defiance and an increase in their susceptibility to other diseases **(Skinner, 1982)**.

In the current study, the total prevalence of EMC in all examined fish from both governorates was 80.0%; it represented 84.15, 85.0, and 69.15% among *Oreochromis niloticus*, *Clarias garipienus* and *Mugil capito*, respectively. These results are approximately similar to those recorded by **Derwa *et al.* (2019)** in Ismailia (70%) with a prevalence of 62 and 80% for *O. niloticus* and *C. gariepinus* and by **Elghayaty and Tadros (2020)** in Port-Said (71.63%) with a prevalence of 70.66 and 72.8% in the tilapia and *Mugil* fish, respectively. In contrast, **Hefnawy *et al.* (2019)** recorded 60% in El Minia City with a prevalence of 70.0% *in C. lazera* and 50.0% in *T. nilotica* and **Saad *et al.* (2019)** recorded 64% in Giza Governorate, with a prevalence of 82.8% and 35.8% in *O. niloticus* and *C. gariepinus*, respectively. The prevalence of encysted metacercariae was different from one study to another due to several factors, including the study area, the location where fish were obtained, and water pollution level with human, animal, and bird waste.

The result of the seasonal prevalence of encysted metacercariae in the examined fish detected that the highest prevalence of EMC in *Oreochromis niloticus* was in winter (100.0%) and the lowest was in spring (71.65%), while in *Clarias gariepinus,* there was

no significant difference ($P > 0.05$) in the prevalence of all seasons, which was 86.65, 85.0, 85.0, and 83.3[a] in autumn, summer, winter and spring, respectively, with the highest prevalence was in autumn and the lowest was in spring. In *Mugil capito*, the highest prevalence was in summer (88.3%) and the lowest was in spring (50.0%). These results are in agreement with the results of **Hassan *et al.* (2012)** and **Derwa *et al.* (2019)** considering the highest prevalence of *Oreochromis niloticus* in winter, while it disagrees with the results of **ElKamel *et al.* (2014)**, **Hefnawy *et al.* (2019)** and **Youssef *et al.* (2020)** with respect to the highest prevalence of *Clarias lazera* in summer. Moreover, it contradicts with the results of **Abd rabo *et al.* (2017)**, **El-Shahawy (2017)**, **Derwa *et al.* (2019)** and **Yassen *et al.* (2023)** for the highest prevalence of EMC in African catfish in winter. However, our results contradict those of **Satour *et al.* (2019)**, **El-Seify (2021)** and **Yassen *et al.* (2023)**, who reported the highest prevalence of encysted metacercariae (EMC) in *O. niloticus* during summer. Additionally, our findings differ from those of **Kotb *et al.* (2014)** and **El Assal and Mohamed (2018)**, who found the highest prevalence of EMC in *Mugil capito* during spring and winter, respectively. This variation in seasonal prevalence is influenced by numerous reasons, such as fish feeding habits, and host immune response at different temperatures **(EL-Shahawy *et al.*, 2017)**.

Regarding the results of the distribution of the recovered EMC in different body parts and organs of examined fish, it was revealed that, there was no significant difference ($P > 0.05$) between the prevalence in gills and muscles of *Oreochromis niloticus*, and the highest prevalence was 87.44 and 82.16%, respectively, while the prevalence in muscles of *Clarias gariepinus* and *Mugil capito* was significantly higher with 100.0 and 90.82[a], respectively, than other infected tissues. These results match with those of **Nouh *et al.* (2010)** and **El-Gayar and Aly (2013)** regarding the highest distribution of EMC in the gills of *O. niloticus.* Additionally, they align with the results of **Nouh *et al.* (2010)**, **Saad *et al.* (2019)** and **Yassen *et al.* (2023)** for the highest distribution of EMC in muscles of *C. gariepinus.* Similarly, **Ghorbaland and Merwad (2018)** reported the highest distribution of EMC in muscles of *M. cephalus.* This variation in EMC tissue distribution could be attributed to the sample size, the procedures of sampling, fish species, and other epidemiological and statistical factors which can be responsible for the differences in-between different results of the studies **(Oidtmann *et al.*, 2013)**.

Otherwise, the distribution of encysted metacercaria (EMC) in different muscle regions. It was detected that the prevalence was significantly higher in tail region (95.9[a]) of *Oreochromis niloticus*, and the lowest prevalence was recorded in the head region (54.85[b]), while there was no significant difference ($P > 0.05$) between trunk and head regions of *Clarias gariepinus*, with a prevalence of 99.0[a] and 89.65[ab], respectively, but the lowest prevalence (82.3[b]) was recorded in tail region. In *Mugil capito*, the highest prevalence (93.9%) was recorded in the trunk region followed by 66.95 and 57.75% in the head and tail regions, respectively. Our results concur with the results obtained by

Youssef (2015) and **Hefnawy *et al.* (2019)** considering the highest prevalence of *O. niloticus* since it was firstly in the posterior part (tail region) then middle part (trunk region) and the anterior part (head region). Morover, **Abdallah *et al.* (2009)** and **Saad *et al.* (2019)** results agree with our results for the highest prevalence of *C. garipienus* as it was in the trunk and head regions. On the other hand, the present results disagree with those of **Youssef (2015)**, **El-Shahawy *et al.* (2017)** and **Youssef *et al.* (2020)** who reported the highest infestation of *C. lazera* in the posterior region, followed by the middle region, and the lowest infection was in the anterior region.

The results revealed that the highest prevalence of EMC in *Oreochromis niloticus* was in weights ≤50g (100.0%) while in *Clarias garipienus*, there was no significant difference (*P*> 0.05) in lengths of >35- 40, >40- 45, >25- 30, and >30- 35cm with a significant difference of prevalence was 93.8, 87.1, 83.65, and 82.5^{a}, respectively, and there was a significant difference (*P*< 0.05) between them and lengths >45- 50cm, as they showed the lowest significant (55.0^{b}). In contrast, in *Mugil capito*, the highest prevalence was in weights >50- 100g (100.0%). Similarly, the results reported by **Saad *et al.* (2019)** mentioned that the highest prevalence of *O. niloticus* was in weights less than 50g (95.1%), while the highest prevalence (48.0%) was in weights 250- 300g for *C. garipeinus.* Meanwhile, **Aly *et al.* (2005)** added that, the lowest prevalence of EMC was in weights less than 50 and over 300g for *O. niloticus* and *C. lazera*, respectively. Moreover, **Awosolu *et al.* (2018)** reported that the lowest prevalence was 24.24% in weights <50g for *O. niloticus.*

Regarding lengths, the results revealed that there was no significant difference (*P*> 0.05) between the prevalence of encysted metacercariae in lengths >15- 20 and ≤15cm in *Oreochromis niloticus* with a prevalence of 87.15 and 84.75%, respectively. In *Clarias gariepinus*, there was no significant difference (*P*> 0.05) in lengths of >35- 40, >40- 45, >25- 30, and >30- 35cm with the prevalence of 93.8, 87.1, 83.65, and 82.5%, respectively. Moreover, there was no significant difference between prevalence in lengths >20- 25 and >25- 30cm of *Mugil capito* with a prevalence of 79.3 and 63.3%, respectively.

In the current study, morphological and genetic characteristics were used to identify the encysted metacercariae. The genetic characteristics of the recovered EMC were done based on utilizing the genomic PCR reactions on the extracted DNA from metacercariae using 28S rDNA gene. A 300bp amplicon from the DNA of metacercariae was obtained. These results showed that metacercariae isolated from infected *Oreochromus niloticus* and *Clarias gariepinus* belonged to the family Cyathocotylidae. Cyathocotylidae metacercariae recovered from muscles, kidney and liver of *Oreochromus niloticus* and *Clarias gariepinus.* These results relatively agree with those obtained by **Saad *et al.* (2019)** who detected Cyathocotylid EMC from the gills and muscles of *O. niloticus* and C. *gariepinus* and **Elaswad *et al.* (2021)** who found that the encysted

metacercariae which infect *Clarias gariepinus* belonged to the family Cyathocotylidae based on the morphological identification.

Prohemistomum vivax, Mesostephanus appendiculatus and *Mesostephanus fajardensis* were isolated from the small intestine of experimental infestation of Wistar albino rats weighing 175g by cyathocotylid encysted metacercariae, which were infecting the muscles of *Oreochromus niloticus* and *Clarias garipienus* after 7 days post-infection. These results are nearly the same as that recorded by **Satour *et al.* (2019)**, who found *Prohemistomum vivax* in the small intestine of 100- 200g albino rats after 7- 14 days post-infection. Additionally, **Saad *et al.* (2019)**, who recovered *P. vivax* and *M. appendiculatus* from the small intestine of rats and **Youssef *et al.* (2020)**, who isolated *P. vivax* and *Mesostephanus* sp. from small intestine of rats after seven days post-infection. While these results differed from those obtained by **Taher (2009)** by obtaining *Prohemistomum vivax* from the small intestine of puppies, and **Nouh *et al.* (2010)** who detected *Prohemistomum vivax, Mesostephanus appendiculatus* and *Mesostephanus fajardensis* from the small intestine of puppies.

The variances in the detected adult worms from variable laboratory animals as stated above might be described by the change in the physiological condition and acidity of the stomach of all hosts in addition to the final host's differences and other anatomical factors, which are useful for the establishment of the parasites **(Shalaby *et al.*, 1989)**.

Based on this study, the effect of cooking on the infected muscles of *Oreochromis niloticus* and *Clarias gariepinus* with cyathocotylid EMC revealed that cooking the infected muscles of *Clarias gariepinus* with cyathocotylid EMC at 250°C for 15- 20min was not sufficient to destroy all EMC, whereas it was sufficient to destroy EMC in *Oreochromis niloticus.* The difference in this result may be traced back to the huge number of EMC in *Clarias gariepinus* compared to *Oreochromis niloticus* and the difference in the composition of the muscles of both fish. These results is different from the results obtained by **Abou Eisha *et al.* (2008)**, who mentioned that grilling the infected *O. niloticus* with EMC for 15- 20 minutes at 60- 80°C was sufficient to destroy the encysted metacercariae which infected the *Oreochromis niloticus* muscles; the current findings concur with those of **Elghayaty and Tadros (2020)** who reported that, cooking in a microwave at 500Watt/ 2min is appropriate to kill all encysted metacercariae in muscles of the tilapia and mugil spp. **Marcus *et al.* (2012)** and **Sripan *et al.* (2017)**, in their study added that the proper cooking of fish can reduce the viability of all metacercaria that infected fish.

In this study, the effect of freezing on the infected muscles of *Oreochromis niloticus* and *Clarias gariepinus* with Cyathocotylid EMC revealed that freezing at -17 to -15°C for 3 or 7 days was sufficient to destroy all EMC in the muscles of both fish. The previous studies revealed different results as follows: **Abou Eisha *et al.* (2008)** reported that freezing the infected fish with EMC at -10°C for 72- 96 hours was appropriate to kill all EMC, but freezing at -10°C for 24 and 48h was not adequate to kill all EMC in fish

muscles; **El-Sayad *et al.* (2014)** observed that freezing at −15°C for 2 weeks was adequate to destroy all EMC in *T. nilotica.* **Youssef *et al.* (2016)** proved that freezing at -10°C for 24h was appropriate to kill the metacercariae in *T. nilotica* and *C. lazera.* Additionally, **Satour *et al.* (2019)** recorded that freezing at −10°C for 14 days and deep freezing at -30°C for 24h was appropriate to destroy all metacercariae on *O. niloticus*. The best period for which the frozen metacercariae remains relies on the size and species of the EMC and from which species of fish were obtained **(Abdallah *et al.*, 2009)**. The WHO has suggested freezing as an appropriate method to decrease the danger of fish-born zoonosis **(WHO, 1979)**.

CONCLUSION

This study concluded that Cyathocotylid EMC parasitized *Oreochromis niloticus* and *Clarias garipienus* all over the year and varied according to climatic changes and can be transmitted to humans by eating them undercooked or raw. Their adult worms, *Prohemistomum vivax* and *Mesostephanus* spp. are of zoonotic importance. Freezing of the infected muscles of *O. niloticus* and *C. garipienus* with cyathocotylidae EMC at -17 to -15°C for 3 or 7 days was sufficient to destroy all EMC in these fish muscles and cooking at 250°C for 15- 20min was sufficient only to destroy EMC in *O. niloticus.*

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