Ozonized Water, Microwaves and Freezing Effects on Viability of Encysted Metacercariae in Fish Muscle

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Abstract

The objective of this study is to evaluate the impact of ozonized water, microwaves cooking and freezing to inactivate the encysted metacercariae (EMC) and evaluate the prevalence of it in both Tilapia and Mugil species fish from Port-Said, Egypt. A total of 275 fishes (150, from Tilapia species and 125, Mugil species) was collected from June 2016 to May 2017, the incidence of EMC was 71.63%,with the highest prevalence in Summer 89.55% followed by Autumn 86.25%, the Spring 60% and the lowest was in Winter 46.03%. The prevalence was 70.66% and 72.8% in Tilapia and Mugil fish respectively. From the biological experimental infestation of chickens 4 species of trematodes were recovered (2 from Tilapia) Metagonimus Yokogawai & Haplorchis Yokogawai. and (2 from Mugil) Heterophyes heterophyes & Heterophyes aequalis. The treatments of infested fish muscle revealed that Microwave cooking at 500 Watt for 2 minutes be sufficient to destroy any EMC in it, also freezing the fish at -4° C to -5° C for 10 days are keeping the fish completely safe to consumer. The treatments of fish muscle by ozonized water, 25 ppm/ 8 minutes and 30 ppm / 10 minutes not completely safe enough to consumer to eat raw fish but the result give that; as the ozone concentration increased and prolonged time the affectivity increased.

Keywords: *Fish, Encystedmetacercare, ozone, Microwave, Freezing.*

1. Introduction

Parasites are common in fish but do not present a health concern in thoroughly cooked fish. It's become a concern when consumers eat raw or lightly preserved fish, **[1].**More than 50 million people are infected with food borne treamatodes all over the world, the most infections due to eating raw or undercooked infected seafood products**, [2],** while **[3]**reported a 29 food borne parasitic illness outbreaks with consumption of raw or improper cooked seafood from 1972 to 2016.Fishes act as definite host as well as intermediate host for larval phases of parasitic encysted metacercariae **[4].** In addition to the economic loss, many of the trematodes, are also of human health importance. Eating raw or improperly cooked or processed fish is the cause of these infections for humans, and this has been reported all over the world **[5].**Ozone is 3 atoms of oxygen bound together formed by the action of ultraviolet light or atmospheric electrical discharges on oxygen, **[6].** Its can control parasites by destroying the wall of the cyst and cytoplasm by interfering with its chemical structure **[7],** it is used to sanitize food, drinking water, and surfaces, **[8],[9]**, Ozone improved quality and safety of all food products without forming hazardous residues in it**[10]-[12],**also with no alteration in color or taste **[13] .**Microwave ovens cook food "from the inside out" meaning from the center of the entire mass of food outwards depending on water content, the depth of initial heat deposition may be several centimeters or more **[14]**. The microbial destruction by microwave mentioned in 2 theory the 1st is that: the microwave inactivate microorganism directly by heat**[15].** 2nd suggestion water, fat, and other elements in the food absorb energy from the microwaves in a process called dielectric heating, circling molecules hit other molecules and put them into motion, thus diffusing energy, this energy, distributed as molecular spins, vibrations and/or translations raises the temperature of the food**, [16]**.Most parasites are fairly easy to destroy by freezing temperatures for a definite period of time; The Fish and Fishery Products Hazards and Controls Guide recommends a temperature below (-20°C) for 7 days or (-35°C) (internal) for 15 hours to kill the parasites of concern **[17].** Although the **[18],** indicated that freezing fish at-10°C for 5 days would kill all trematodes of worry**[19]**showed that the metacercariae of *Heterophyes* are very resistant to freezing; since they survived 30 h of storage at -10° C. The wants for destroy depend on the type of parasite, temperature applied, length of time needed to reach the final temperature in all parts of fish tissue, the time fish is held at the set temperature, the species, and the fat content of the fish**.[1],[2]**.

The present study was assumed to reveal the frequency prevalence of encysted metacercariae of trematodes in some fishes in port-Said Province and the effect of some treatments as ozonized water, microwave cooking and freezing as management ways of those fishes as a source of human infections with these trematodes.

II. Materials and methods

A. Fish samples:

During the period extended from June 2016 to May 2017, 275 fish specimens 125 *Mugil* spp., and150 of *Tilapia spp.*, were collected from Port-Said Province markets and fisheries. Fish was examined by naked eye for any external abnormality to detect the encysted metacercariae in skin, fins and musculature. Then kept in ice box and examined directly at Port-Said regional Lab Animal health research institute.

B. Parasitological examination:

Macroscopic examination: Fish was carried out for the detection of any cyst or nodules in different parts of fish body by a-naked eyes according to **[20].**

Microscopic examination of fish tissues: Fish was carried out using compression technique, fish body was skinned, eviscerated, fillet and divided to very small pieces of muscle mixed with few drops of saline solution and compressed between two glass slides (repeat 2-3times) to increase the detection rate and examined under microscope. The muscle pieces taken from different regions and depth for detection of encysted metacercariae, as described by **[21],**

C. Experimental infection:

A total of 30 chickens (21 days old) reared for7 days and their feces were examined daily for any parasitic infestation to insure it is free from parasites then divided to 6 groups 5 each in separate cages. The specimens of fish were skinned and eviscerated and detect the presence and density of *EMC* by compressed technique and microscope, every group fed the same weight of fish muscle and nearly the same number of *EMC*., the density of infection with *EMC* was recorded by examining one gram of muscle tissue in 10% of fish by using compression technique, the *EMC* were counted and recorded in all samples **[22].**

Group 1-The chickens fed infested tissue of Tilapia spp. fish

Group2-The chickens fed infested tissue of Mugil spp. fish.

Groups 3&4- The chickens fed infested fish which treated with ozonized water at 25 and 30 ppm to 8 and 10 minutes respectively. The ozonized water was generated from cold plasma ozone generator (MA5001 model), Germany, using oxygen with 220 volts obtained from (Egyptian center for treatment by ozone) Heliopolis**,** Egypt. The ozonized water kept in dark glass bottles in an ice box and used immediately. The infested fish muscle collected in petri dishes and soaked in ozonized water 25ppm for 8minutes (group 3) and 30ppm for 10 minutes (group 4) respectively then directly fed to chickens**.**

Group 5: The chickens fed infested fish which cooked by microwave 500 W/2 minutes.in a commercial home microwave oven (Clatronic, typeMWG-753B, with a rotating glass plate, frequency at 2,450 MHz, and power of1500 watt).

Group6: The chickens which fed the fish musculature infested by *EMC* and frozen to 10 days at (-4C°to -5C°) then thawed before feeding to chickens. **[23]**.

Daily examined feces of chickens until detection of trematodes eggs, and then the infected chickens sacrificed after 15 days post infection. The content of small intestine was, scrapped and collected in jars containing normal saline. Washing with normal saline was carried out several times to remove the coarse particles of the intestinal contents and mucus that may be attached to the parasite. The sediments were examined using dissecting microscope. The trematodes were collected in normal saline then picked up in a small bottles containing 5% neutral buffered formalin for preservation staining with alum carmine, adult trematodes counted and described according to **[24],[25]**.

III. Results

A. Parasitological examination (table 1): Revealed that from total of 275 fish samples examined 197were infested by encysted metacercariae one or more with prevalence of 71.63%.while if we look at table one can tell us that Tilapia spp.106 (70.66%) out of 150 examined fish were infested by metacercariae with the highest rate at the Summer season 89.1% followed by Autumn 84.2%and then followed by Spring 62.5% and finally the lowest season is Winter with record rate 45.7%.while the Mugil spp., Fish give 91sample from 125 was infested by metacercariae (72.8%) the lowest season also at Winter by rate of (46.4%) while the highest one was in Summer by infested rate 90% followed by Autumn 88.09% and Spring 56%.

Heterophyid metacercariae: detected in the musculature of Mugil spp., and Tilapia spp., the cyst was globular to elliptical, double walled, measured 0.22- 0.19 mm. The outer layer was thin and transparent while the inner one was homogeneous with bright color.

B. Experimental studies:

Fourspecies of Trematodes recovered from the experimentally infected chicken with encysted metacercariae, (Table 2).

***Table (1)**: Seasonal prevalence of the encysted metacercariae in the examined fish muscle*.

Season	Tilapia spp.			Mugil spp.			Total		
	No. ex.	+v		No. ex	+ v		No. ex	+v	
		No.inf.	%		No. inf.	%		No. inf.	%
Summer	37	33	89.1	30	27	90	67	60	89.5
Autumn	38	32	84.2	42	37	88.o.9	80	46.03	86.25
Winter	35	16	45.7	28	13	46.4	63	29	46.03
Spring	40	25	62.5	25	14	56	65	39	60
Total	150	106	70.6	125	91	72.8	275	197	71.63

No. ex. = number of fish examined+ v = fish positive to *EMC*. No.inf. = number of fish infested.

Table (2):- Prevalence of trematodes adult parasites from experimentally infected chickens with *EMC* infested fish muscle

Helminthes, spp.,	Fish ,spp.	No. chickens fed	No. infested	%
Heterophyes hetrophys	Mugil	5	3	60
Heterophyes aequalis	Mugil	5	2	40
Haplorichis yokogawai	Tilapia	5	1	20
Metagonimus yokogawi	Tilapia	5	4	80

No. =number % = percent

Table (3): Effect of microwave, freezing and ozonized water on infectivity of *EMC* in fish fed to experimental chickens.

Treatments	No. fed	No. infected	Infectivity %	Adult trematodes
Microwave 500W/2minutes.	5	0	0. 0	----------
Freezing-4 -5°C/10 days	5	0	0. 0	----------
Ozone water 25ppm/8 minutes.	5	2	40	*H. heterophyes*
Ozone water30 ppm/10 minutes.	5	1	20	*H. heterophyes*

No. fed =number of chickens fed infested fish with *EMC*.
No. infected= number of chickens recovered adult trematodes.
H. heterophyes = *Heterophyes heterophyes*

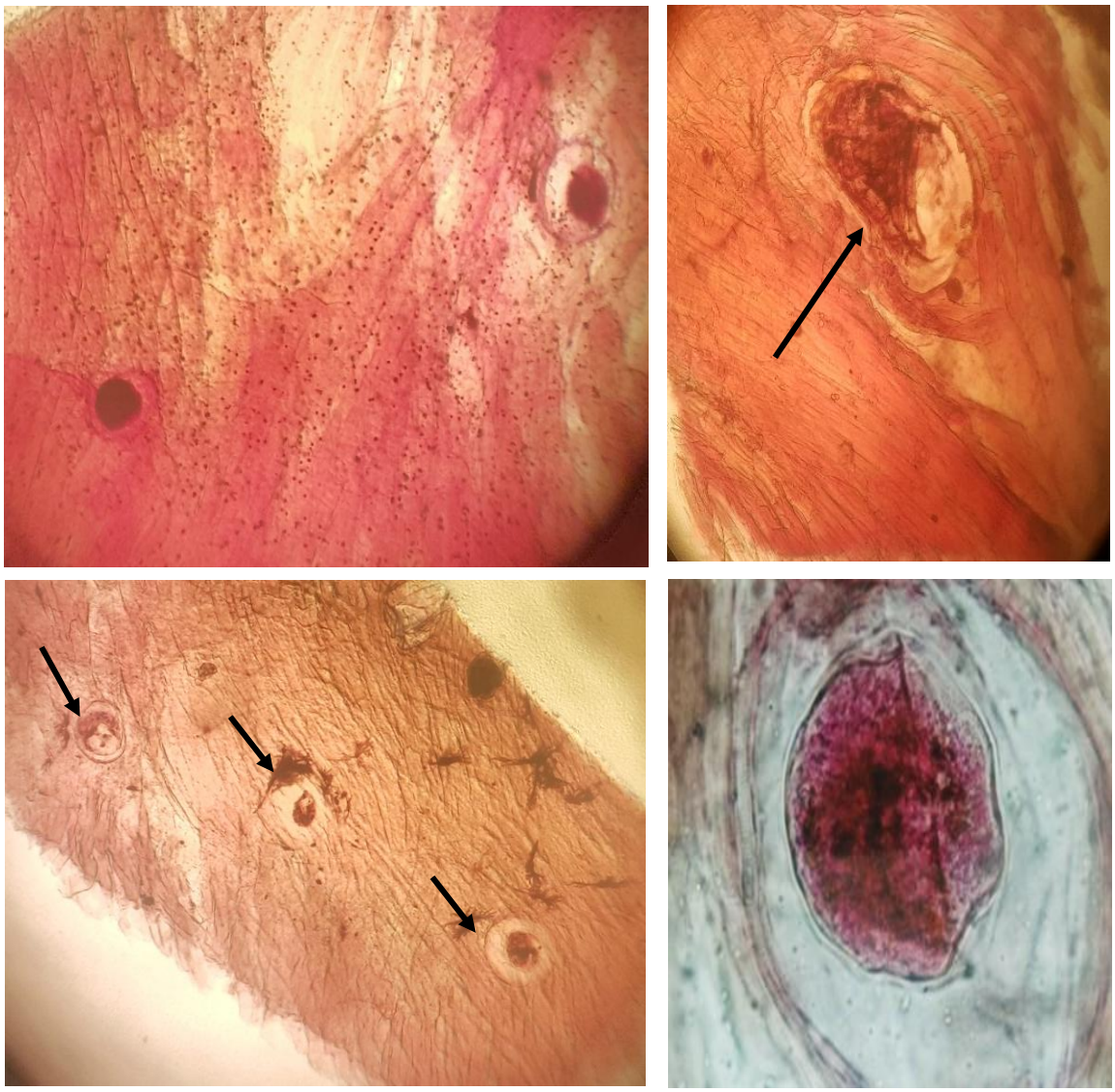

Fig. 1: Muscle of fish infected with unidentified *EMC* (showing several cysts) stained (acidified alum carmine)

(A- B)*: EMC* in Tilapia muscle (x10).

C- Heavy infested Mugil muscle with *EMC* (x10).

D-*EMC* in Mugil muscle (x40).

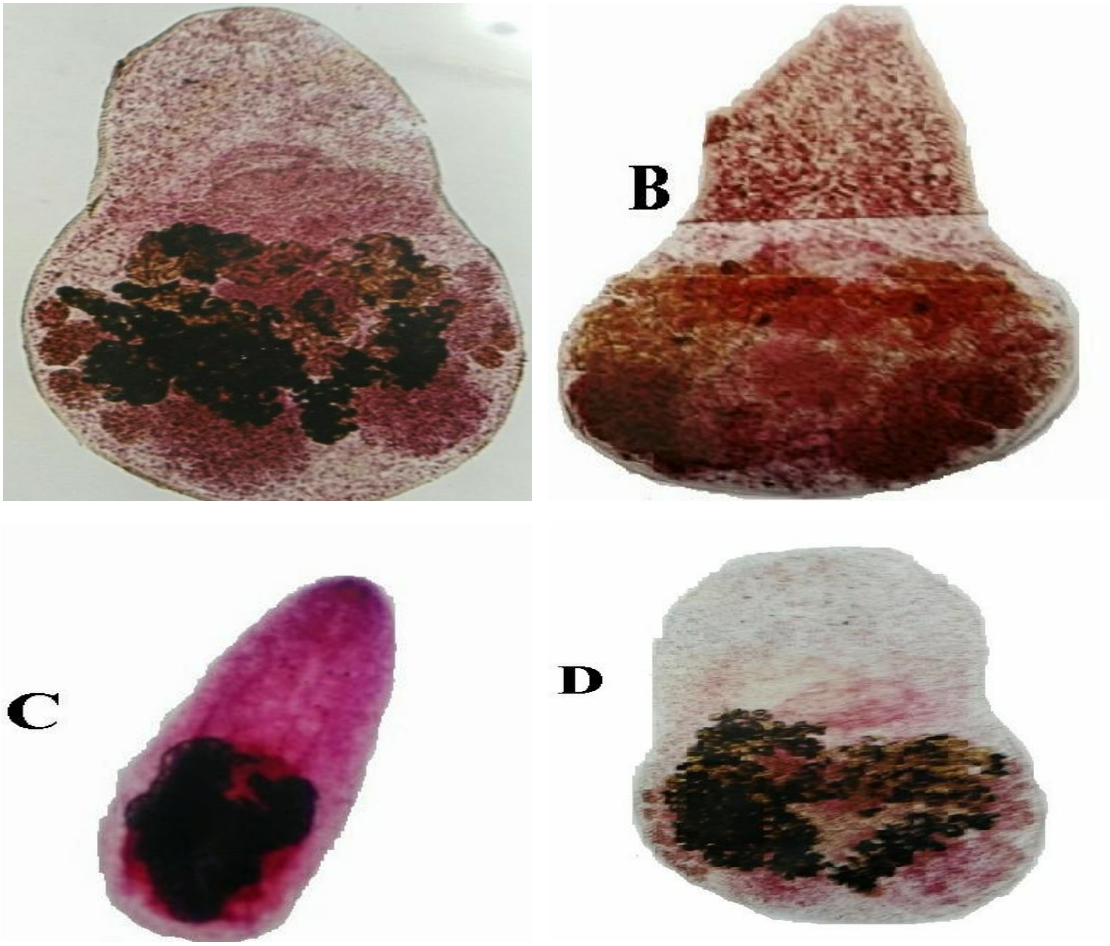

Fig (2): Adult *Heterophyid* trematodes recovered from experimentally infected chickens.Stained (acidified alum carmine)

(A): *Heterophys heterophyes*. (x10).

(B): *Heterophys aequalis*. (x40).

(C): *Haplorchis yokogawai*. (x10).

(D):*Metagonimus yokogawai*. (x10).

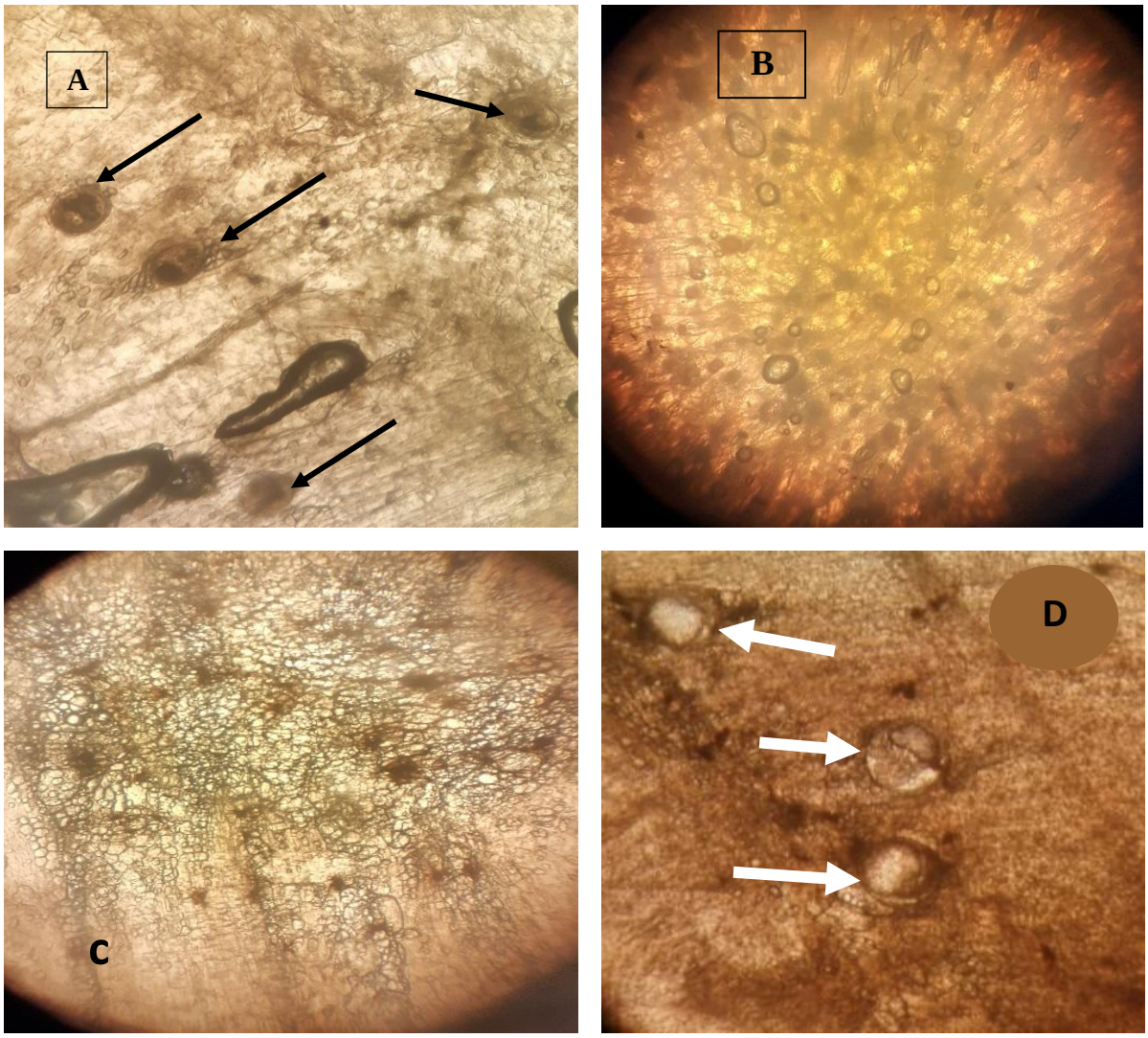

Fig. (3): A- & D: *EMC* (arrows) Heavy infested Tilapia and Mugil spp. muscle without staining (x40).

B-Fish muscle after cooking by microwave at 500 W for 2minutes without staining (x 40).

C- Fish muscle after exposure to soaked in ozonized water at 30 ppm for10 minutes without staining (x40).

C. Morphology of adult flukes

***-Heterophyes heterophyes:* fig.2 (A)**
The adult worms were recovered from the duodenum and jejunum of experimental infected chicken with Mugil fish containing metacercariae. Its small pear shape fluke, attenuated anteriorly and broad round posteriorly, covered with spines, most numerous anteriorly, measured 0.86 mm in length and 0.57 mm in width. The oral sucker was sub terminal and 0.08×0.08 µm in diameter, with small pharynx and muscular, somewhat long esophagus. The caeca reach to the terminal end posterior to testes, and ventral sucker was well-developed, thick wall, situated at end of anterior third of the body. The genital pore surrounded with well-developed genital sucker. The ovary was small, gall bladder measuring 94.5x81 µm, pre testicular. The two testes were oval, horizontal measuring 162-148.5 µm. The eggs were minute, embryonated light brown and operculated.

***-Heterophyes aequalis*, fig.2 (B)**
The adult worms were collected from the small intestine of experimentally infected chicken, fed metacercariae of *Mugil* spp. The prepatent period was 11 days. A minute pear shape distomal worm covered with scale-like spine, most numerous anteriorly. The body length was 0.49 mm and 0.24-0.26mm width, with round minute sub terminal oral sucker, 36.30µm in diameter. Pharynx was small and muscular, measured 36.30x33µm. and long esophagus 0.19mm. The intestinal caeca were short, terminate at the level of the ovary. There is well developed ventral sucker, situated in the middle of the body, measured 75.90x52.80µm. The genital sucker was small, present posterior lateral on the left side of the acetabulum and measured 42.90x29.70µm. Two testes were elongate, lied side by side in the caudal region, and small, sub globular ovary pretesticular measuring 52.82x52.82µm. pear-shaped vitelline glands, coiled uterus extended from the acetabulum to the testes with minute, embryonated yellowish brown, thick shell operculated and measured 20 x 12µm Eggs.

***Metagonimus yokogawai*: fig.2 (C)** The adult worm obtained from the small intestine of the experimentally infected chicken, fed metacercariae of Tilapia spp. The prepatent period 8 days. It is a small, flattened, oval, covered with minute spines numerous anteriorly measured 1.02-1.20 x 0.49 -0.58mm, body,with sub terminal small oral sucker, measured 81-1.08x81-1.08µm , sub globular muscular pharynx, moderately long esophagus, the intestinal caeca were simple and terminated at posterior extremity. The acetabulum was clear; it usually fused with the genital sucker forming acetabula-genital apparatus. Tests were Tests were ovoid, nearly horizontal at posterior end. Ovary was subglobular, right to medium line. Vitellaria appeared as small follicles laterally extending along ovario-testicular zone. The eggs were small, thick and oval.

***Haplorchis yokogawai*fig.2 (D):** Collected from the ileum of chickens experimentally infected with the metacercariae in the muscles of Tilapia spp., it is oval or pear, tiny shaped (0.66×0.28 mm). With spinney, cuticle, oral sucker measures (0.032×0.043). The acetabulum is embedded in the parenchy-ma, a short oropharynx leads to an esophagus measuring 0.025-0.048 mm (0.036 mm). The testis is single, spherical lies near the posterior extremity Pretesticular ovary about 0.073×0.065 mm, and the uterus contains numerous eggs extends from behind the bifurcation of intestinal caeca to the posterior end. Few follicular small vitellaria, lies at the posterior part in the ovario-testicular zone.

***D.* The treated groups in Table (3):** showing that the chicken groups fed on fish muscle treated with ozonized water revealed that ozonized water 25ppm for 8/minutes and 30ppm for 10 minutes were not sufficient to destroy all EMC in examined fish muscle. This finding was proved by adult trematodes recovering from small intestine of experimentally infested chickens but with increased time and concentration of ozonized water increased affectivity of it on viability of EMC. We were observed that the chickens which fed on fish muscle treated by ozone water 25 ppm for 8 minutes, 2 chickens from 5 give adult trematodes after scarification with infestation rate (40%). While the chickens which fed infested fish muscle soaked in ozonized water 30 ppm for 10 minutes 1 chicken from 5 infected chickens was positively with parasite with infestation rate 20% Microwave cooking by 500/W for 2 minutes can destroy the encysted metacercariae in muscle of infested fish which chickens fed on these microwave cooked fish were free from adult trematodes. Also, cooking by microwave changes the feature and texture of muscle to .(cooked appearance (fig.3, B)
Freezing the infested fish muscle at – 4C°to – 5C° for 10 days could destroy the all EMC in fish muscle this finding achieved by no any recovered trematodes from the intestines of chickens fed on it.

(1):

V. Discussion

***A. prevalence of encysted metacercariae in the examined fish*:**
Was 71.63% which nearly similar to **[26]** 76.5% in fresh water and**, [27]** 71.9% in brackish water but higher than **[25],** 22%. The prevalence of EMC in Tilapia spp., was 70.6% which similar to**, [26]**, 70%, **[28]** 77.7% and **[29]** 74.8% but higher than **[30]** 19.31%; **[31]** 42.8% and **[32]** 45%. While the Prevalence of encysted

metacercariae in *mugil spp.,* was 72.8%, this result higher than **(30)**36.36 %, and**[25]**51.5%. *Heterophyid metacercariae* were detected from the two fish spp., similar to obtained by **[33, 34,35**]; It is usually very difficult to identify metacercariae for different species **[36**], so metacercariae collected from fish were feeding experimentally to chickens (3weeks old) to obtain and identify the adult trematodes. Two species identified from Mugil fish (*Heterophyes heterophyes*)and (*Heterophyes aequalis*) and two from Tilapia (*Haplorchis yokogawai*) and *Metagonimus yokogawai* which agreed with **[28]** who detected *Heterophyes heterophyes* and *Metagonimus yokogawai* in different species of fish in port-said province and **[29]** were detected *Heterophyes heterophye*s and *Metagonimus yokogawai* in different species of fish in Ismaillia province. Also, **[34]** detected*Heterophyes heterophye*s, *Heterophyes aequalis* and *Haplorchis yokogawai* from *Mugils pp*., and *Tilapia spp*., from brackish water in Dakahlia and with**[27]**who detected *Heterophyes hetrophyes* and *Hetrophyes spp*., from fresh and brackish water fish. the seasonal variation of infection cleared the highest incidence was in Summer (89.55%) followed by Autumn (86.25%), Spring (60%) and the lower one in Winter(46.03%).These results lower than, **[29]**in seasons Summer (94.7%), Spring, (84%) and Winter (47.6%) but higher from Autumn Season (84%).Also lower than **[28]** who detected(95.9%) in Summer followed by Spring (84%) and Winter (49.2%) but higher in Autumn (80.64%).The variation in incidence may related to the differences in the habitat, food supply, abundance of both aquatic snails and aquatic pisciverrous birds, which play the main role to complete the life cycle of some digenetic trematodes**, [35].**

B-The results in (table 3) showed that the Effect of ozonized water on the infectivity of metacercariae revealed that ozonized water as 25 ppm to 8 minutes and 30 ppm to 10 minutes was not sufficient to destroy all encysted metacercariae in fish muscles. This finding was proved by recovering adult worms from small intestine of experimentally fed chickens. 2 chickens from 5 by infectivity rat 40% in group treated with ozonized water 25 ppm / 8 minutes and this percent decreased to 20%(one chicken from five reveal adult trematode) in group treated with ozonized water 30ppm / 10 minutes which agree with **[7**), which proved that low concentrations of ozone was not valid to kill cyst of parasites and partially agreed with **[37**], who studying the effect of ozone on viability of *Clonorchis sinensis* metacercariae in vitro and found that it was effective and the affectivity increased with time and concentration. Our result disagree with **[38];**who found ozone was very effective on (*Cyprinus Capria*) cyst with concentration of 20 ppm. It may be attributed to the fact that Ozone is not stable in hot climate and destroyed within 20-30 minutes**.** Our results revealed that using microwave 500 W/2 minutes is sufficient enough to destroy EMC from infested fish muscle and it's proved by complete absence of adult trematodes in chickens fed fish after microwave cooking, our result confirmed by many author studying as **[39]**; when used microwave 400- 800 W /1 minute a small number of metacercariae were observed with abnormal morphology but after 5 minutes it was completely destroyed. While **[40]**; used the microwave oven in compared to conventional one and proved that microwave need less time to kill *Anisakis simplex* larva isolated or impeded in muscle .reference**[41];** impact the microwave radiation on larval stage of *Opisthorchis* metacercariae and it was very effective to destroy it completely. Also, **(38);** treated the oocytes of (*Cyprinus Capria*) by microwave at 50W / 15 minutes and it was very effective to kill it totally. The effectiveness of heating depends on several factors, such as, species of parasite, fat content and fillet thickness offish, **[1].** The freezing at -4°Cto -5°Cfor 10 days is sufficient to inactivate the EMCin fish muscle which proved by there was no trematodes recovered from the chickens fed these fish which agree with **[42];**who found that freezing of *Tilapia* spp. fish muscles at -2° C for a period not less than 9 days may be considered enough to kill the contained metacercariae and also with **[23],** who found that freezing of *Tilapia* spp.,at -4°C for 10 and 12 days prevent the infection to experimental animals by metacercariae and **[47];** who proved that metacercariae in *Tilapia* lost their viability by freezing at-5°C /48 hours, and partially with **[44];**who found that freezing of *EMC* at -4 °C for 10 days was sufficient to abolish their infectivity, but some were remained viable. While our results disagree with **[45],** who reported that metacercariae of *Clonorchis sinesis* from fresh water fish remained viable after frozen storage at -12 °C for 10-12 days and -20°C for 3-7 days and,**[46];**who found freezing at -10°C /24 hours could not kill all EMC of *Echinostoma Capri*,**[33].,**found that freezing at -10°C /24-48 hours not enough to kill all metacercariae in fish muscle. Also, **[47];**revealed that freezing at -10°C for 24 hours was proved lethal to the metacercariae of both *Prohemistomum Vivax* and *Haplorchi spumilio*. Freezing may cause damage to parasites by both a dehydration caused by the formation of ice crystals in the cysts accompanied by an accumulation of external solutes, and through mechanical damage caused by ice crystals forming in the parasite tissues, **[48].**

V. Conclusion

1-All wild caught fish must be considered at risk of containing viable parasites of human health concern if these products are to be eaten raw or almost raw.

2-For that many treatments processes must be taken before consuming.

3-Ozone act as good anti-oxidant too many protozoa, viruses, microbes, and more but it is need more investigation as treatment to the *EMC* in fish muscle.

4-To minimize the risk of fish born trematodesIt's always best to cook thoroughly were cooking using microwave is effective.

5-Freezing raw fish remains the most effective way to ensure that viable parasites are not present.

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