

Effect of Some Processing Methods on the Viability of Metacercariae Infecting Wild and Cultured Fish in El-Menia Governorate

Shimaa-Ahmed, M.¹, Hefnawy, Y. A.², Arafa, M. I.³ & Abd-El-Malek, A. M.^{2*}

¹Food Hygiene Department, Animal Health Research Institute, El-Minia Lab, Agriculture Research Center, Egypt

²Food Hygiene Department, Fac. of Vet. Med. Assiut Univ., Egypt

³Parasitology Department, Animal Health Research Institute, Assiut Lab, Agriculture Research Center, Egypt

***Correspondence to:** Dr. Abd-El-Malek, A. M., Food Hygiene Department, Fac. of Vet. Med. Assiut Univ., Egypt.

Copyright

© 2022 Dr. Abd-El-Malek, A. M., *et al.* This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Received: 24 August 2022

Published: 19 October 2022

Keywords: *Fish; Encysted Metacercariae; Microwave Cooking; Sodium Chloride; Potassium Lactate; Electrolyzed Water*

Abstract

The present work aimed to investigate the prevalence of encysted metacercariae (EMC) in wild and cultured fresh water fish in El-Menia Governorate, Egypt. In addition, the effects of some processing methods on their viability were investigated. A total of 200 fish samples (100 wild and 100 cultured) included 50 for each; Tilapia nilotica (*Oreochromis niloticus*), catfish (*Clarias gariepinus*), bajad (*Bagrushajad*) and carp fish (*Cyprinus carpio*) were randomly collected from markets and some fish farms from El-Menia city. Examination of encysted metacercariae (EMC) in the fish is commonly done by macroscopic examination and muscle compression technique.

The total prevalence of EMC among examined fish was 89.5%, it was 88% and 85% in wild fish and cultured fish, respectively. Microscopic EMC was detected in 83% and 73% of wild fish and cultured fish, respectively, while macroscopic EMC was detected in 5% and 12% of cultured fish and wild fish, respectively. *Tilapia nilotica* has the highest infection rate for both macroscopic and microscopic EMC in different examined fish species.

Microscopic E.M.C. was identified as: *Cynodiplostomum* E.M.C and *Prohemistomum* E.M.C while, macroscopic E.M.C identified as *Clinostomum phalacrocoracis*.

Effect of microwave cooking, acidic electrolyzed water (AEW) and mixture of sodium chloride with two concentrations of potassium lactate (2 and 4%) on infectivity EMC of *Tilapia nilotica* were studied through mice bioassay. The obtained results showed that infectivity of EMC was lost at high temperature of microwave cooking (400°C) for 2 and 5 min. While other control measures observed significant reduction in infectivity of EMC but not completely lost. The data were statistically analyzed. The current study revealed a high prevalence of zoonotic EMCs in different examined fish, which represents a potential risk to public health if consumed raw or improperly cooked. In conclusion, thorough microwave cooking was effective in reducing risk of encysted metacercariae in fish muscles.

Introduction

Worldwide, about 40 million people suffer from trematode infections associated with the consumption of raw or undercooked fish products [1,2]. Many research described fish as a source of serious parasitic disease which may be harmful for human or fish eating mammals if it consumed raw or improper cooked [3,4].

Muscles of infected fish with encysted metacercariae had also some alterations on their chemical composition. [5] Ghada et al. mentioned that heavy infection of fish muscles with EMC induced a significant decrease in protein, oil and ash contents with a significant. Also they noticed increase in the total volatile nitrogen, thiobarbituric acid number, peroxide value and free fatty acids in muscles of infected fish.

Heat inactivation of parasites proved effective for eliminating the risk of parasitosis but trematodes possessed a higher heat resistance [6]. The microwave has microbial destruction effect as it inactivate microorganism directly by heat [7]. Food ingredient absorb energy from the microwave (dielectric heating process) as circling molecules hit other molecules make motion which diffuse energy distributes as vibration which raise the food temperature [8].

The effects of different concentrations of salt, vinegar and acetic acid for different exposure times on viability of EMC were studied previously. [9] Ammar and Arafarecorded that dry salting of *O. niloticus* could kill the EMC within one hour of saltcontact, while marination in 5% acetic acid for one minute resulted in eradication of EMC after 2 hours of treatment.

In recent years electrolyzed water (EW) is used as an alternative of traditional heat treatments for controlling pathogens. It has a wide range of food applications, including dipping and spraying [10]. It is safe for skin and mucous membranes, easy to handle and of low production cost [11].

Therefore the present study aimed to:

- Investigate the prevalence of the different encysted metacercariae in wild or culture fresh water fish at El-Menia Governorate.
- Study the effect of microwave, acidic electrolyzed water (AEW) and mixture of sodium chloride and two concentrations potassium lactate on the infectivity of EMC through mice bioassay.

Materials and Methods

1- Collection of Samples:

From March 2021 to February 2022, a total of 200 fish samples (100 wild and 100 cultured) were collected randomly from different localities and from some fish farms at El-Menia Governorate. The obtained samples were included tilapia (*Oreochromis niloticus*), catfish (*Clarias gariepinus*), bajeed (*Bagrus bajad*) and carp (*Cyprinus carpio*) fish (50 of each). Fish samples kept in ice box and examined directly at El-Menia regional Lab Animal health research institute.

2- Parasitological Examination:

Examination of Fish for Encysted Metacercaria

-Macroscopic Examination

The specimens under investigation were carefully examined by the naked eye for the detection of macroscopic metacercaria in musculature and gills [12].

-Microscopic Examination for Detection of Encysted Metacercariae

From each fish, snips (about one gram each) were taken from different muscles of the fish, especially head, near dorsal fins, tail regions. Muscle snips were compressed between two glass microscopic slides and examined under binocular dissecting microscope to detect EMC and to determine viability by observing their characteristic rotator movement [13]. The recovered encysted metacercariae in the fish flesh were prepared for further experimental study

3- Effect of Processing Methods on the Infectivity of EMC

Study the Effect of Acidic Electrolyzed Water (AEW) on the Infectivity of EMC

AEW (pH of 3.4 and free available chlorine ACC of 30ppm) was produced by electrolysis of tap water brined with sodium chloride (3%) through the electrolysis chamber [14]. The pH level of formed acidic

electrolyzed water was estimated using a digital pH meter (FSSAI, 2015). Also, ACC was estimated by chlorine test kit, according to [15].

-Pieces of heavily infected fish muscles (100 gm) were dipped in, PH (3.4) of EW for 15 minutes then thieved and cooled at refrigerator (4°C) for 8h.

Study the Effect of Microwave Cooking on the Viability of EMC

Pieces of heavily infected fish muscles (100gm) cooking in a microwave oven (700 W) at high temperature (400°) for 2 and 5 minutes in accordance with the recipe provided by Fresh factories [16].

Study the Effect of Mixture of Sodium Chloride and Potassium Lactate on the Viability of EMC

To detect the most applicable salts concentration, the experiment design into two treatment groups. The first one is composed mixture of sodium chloride 2% and potassium lactate 2%. The second treatment is composed mixture of sodium chloride 2% and potassium lactate 4%.

Pieces of heavily infected fish muscles (100gm) were soaked in corresponding treatments for 15 minutes and later cooled at 4°C for 8 hours before experimental work [17].

-After each treatment was done, the digestion for fish muscles samples was applied as described by [18] Garcia to obtain the suitable number of EMC that used in mice bioassay

Experimental Infection of Laboratory Animals

Bioassay testing was designed to inoculate mice with suitable number of EMC orally according [19].

A total of 28 albino mice weighing 200-300 gm reared one week and their feces were examined daily for any parasitic infestation to insure it is free from parasites then divided to 7 groups:

- The first group was infected with 250 EMC as a control positive group.
- The second group was infected with 250 EMC after treated with microwave at high temperature for 2m.
- The third group was infected with 250 EMC after treated with microwave at high temperature for 5m.
- The fourth group was infected with 250 EMC treated with potassium lactate (2%).
- The fifth group was infected of which 250 EMC treated with potassium lactate (4%).
- The six one was infected with infected of 250 EMC treated with AEW (pH 3. 4).
- The last group mice were left uninfected as negative control groups.

Mice were infected experimentally with (250) encysted metacercariae, in accordance with local Institutional Animal Care and Ethics Committee (Assiut University, Egypt. Recovery of adult worms from the

experimentally infected mice was done after egg detection in fecal examination. Positive infected mice were sacrificed within 10 days from the day of infection to collect and counted under a microscope, as in previous studies [20].

Statistical Analysis

Statistical analysis was performed using Graph Pad Prism 8.0 software (Graph Pad Software Inc., San Diego, CA, USA). The data were analyzed using the chi-square test or Fisher's exact test to assess the association between prevalence and risk factors. While "P" value of <0.05 was considered statistically to be significant [21].

Results

The total infected rate of encysted EMC in examined fish was 89.5% (173/200), where microscopic EMC was detected in 78% (156/200) and macroscopic EMC were infected in 8.5% (17/200) of examined fish. Infection rate in wild fish and cultured fish was 88% & 85% respectively. Infection rate of cultured fish with microscopic metacercariae was 73% (73/100) and in wild fish was 83% (83/100). Macroscopic metacercaria was detected in 12% (12/100) of cultured samples, and 5 % (5/100) of wild fish samples (Table 1).

Table 1: Prevalence of EMC in examined Cultured and wild Nile fish

Parasite		Examined fish				Total (200)	
		Cultured fish (100)		wild Nile fish (100)			
		No. of inf.	%	No. of inf.	%	No. of inf.	%
EMC	Microscopic	73	73	83	83	156	78
	Macroscopic (Clinostoum sp.)	12	12	5	5	17	8.5
Total EMC		85	85	88	88	173	89.5

Microscopic EMC found in between the muscle fibers of infected fish; they were heavily distributed at posterior part of the body. The highest prevalence rate of microscopic EMC showed in 98 % (49/50) of examined Tilapia followed by Carb spp. 96% (48/50), in Bajad samples was 78% (39 /50), while lowest infection rate of microscopic EMC 40% (20/50) was found in examined Cat fish (Table 2).

Table 2: Prevalence of EMC in examined Cultured and wild Nile fish

Parasite		50 Sample for Each								Total	
		Carb		Catfish		Tilapia		Bajad			
		No. of inf.	%	No. of inf.	%	No. of inf.	%	No. of inf.	%	No. of inf.	%
EMC	Microscopic	48	96	20	40	49	98	39	78	156	78
	Macroscopic (Clinostoum sp.)	–	–	–	–	17	34	–	–	17	8.5

Morphologically microscopic EMC differentiated into:

Prohemistomum E.M.C: The cyst was small in size and spherical in shape about 300-350µm (325); it was yellowish brown in colour. The metacercaria is folded inside double layered transparent cyst wall containing fluid with dark granules. It is surrounded at each pole with adipose tissue (fig 1).

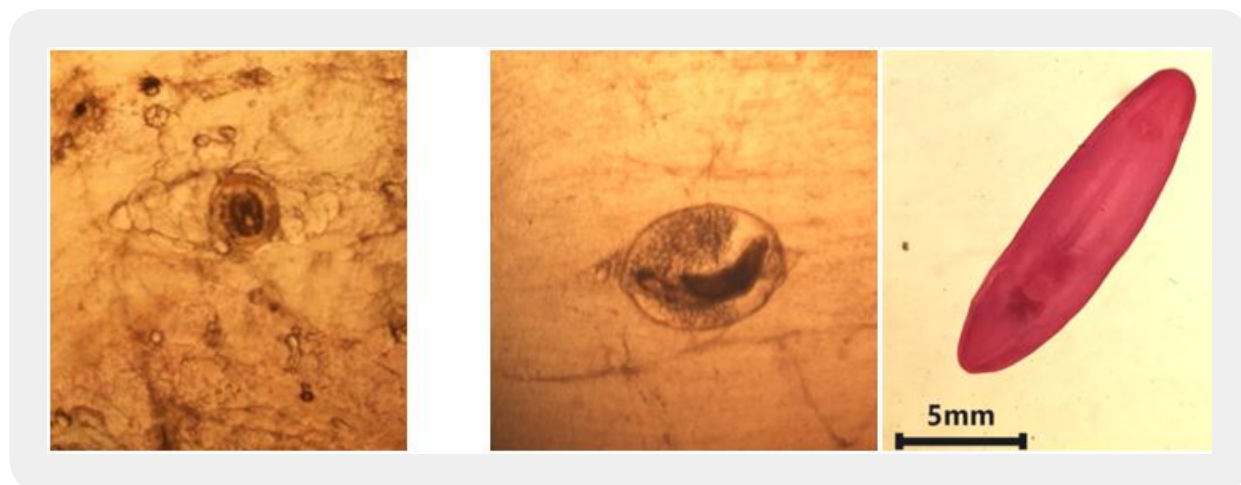


Plate1: showing different EMC detected in examined fish 1-*Prohemistomum E.M.C*x40. 2-*Cynodiplostomum E.M.C*.x40. 3-*Cl. Phalacrocoracismetacercariae*.

Cynodiplostomum E.M.C: it was ovoid in shape surrounded by two transparent cyst walls. As result of metacercariae body movement in the cyst their shape constantly changed (fig 2). It measured 620 -750µm long by 380- 500µm wide (700 x 450µm).

The macroscopical EMC were found in the branchial and pharyngeal regions of *Tilapia* spp. only and their infection rate was 34% (17/50). Morphologically macroscopic EMC differentiated into:

Clinostomum phalacrocoracis E.M.C: The cysts were visible, oval or spherical in shape, covered with a thin yellowish cyst wall and measured 1.5-3.6mm. The living metacercariae characteristic by its elongated body with slightly wider at the middle and were actively moving. Its length about 1.2 cm and about 4 mm thickness. Their oral sucker is smaller than the ventral one and intestinal ceca were run to the posterior part of the body until genital pore. Both testis and ovary located at the posterior third of body (fig 3).

The Effect of Some Processing Methods on the on the Viability of EMC through Mice Bioassay:

1-Microwave cooking of fish (700/W) at high temperature for 5 minutes completely destroys the encysted metacercariae used in experimentally infection, where mice were free from any adult trematodes. Also, microwave cooking at 700/W at high temperature for 2 minutes destruct most EMC inoculated in experimentally infected mice, and where the infectivity rate was 0.40% (Table 3).

Table 3: Effect of different treatments on the viability of EMC through mice bioassay

Experimental group	M. of adult worm / M. of EMC.	Infectivity rate	Chi-square	P. value
Control group	62/250	24.9%		
Treatment with Microwave 1- high temperature (400° c) for 2 m***	0/250	0.0%	140.7	P> 0.0001
2- high temperature (400° c) for 5 m***	1/250	0.40%	146.6	P> 0.0001
Treatment with mixture of 1- Potassium Lactate Conc. (2%) sodium chloride (2%)**	45.33/250	18.13%	10.26	P> 0.05
3-Potassium Lactate Conc. (4%) sodium chloride (2%)***	15.33/250	6.13%	101.0	P> 0.0001
Treatment with Electrolyzed water Treated with Electrolyzed water PH (3.4)***	9.33/250	3.73%	140.7	P> 0.0001

2- Regarding the effects of acidic electrolyzed water (EW) on the viability of the encysted metacercariae, mice inoculated on EMC treated with acidic EW (PH 3.4) revealed that EW was not sufficient to completely destroy EMC in examined fish sample. The infectivity rate was 3.73% in experimentally infected mice (Table 3).

3-Different concentrations of mixture of sodium chloride and potassium lactate were not sufficient to completely destroy EMC in examined samples. The affectivity on viability of EMC was increased by the concentration of potassium lactate. The infectivity rate was as follows: sample treated with mixture of sodium chloride (2%) and potassium lactate (2%) was 18.13%, while sample treated with mixture of potassium lactate (4%) and sodium chloride (2%) was 6.13% (Table 3).

Discussion

Parasitic infection of fish in tropical and subtropical countries represents a serious problem for aquaculture as result of their huge economic losses [22]. They affect growth rate especially for young fish as well as they lose their market value [23]. The presence of metacercariae is very common especially in freshwater fish and they may cause public health problems [24].

In this study the overall infection rate of EMC in examined fish was 89.5%. This wide variety of EMC in different species of examined fish reflected the presence of intermediate and definite hosts in our study region. The present results was relatively similar that obtained by previous researchers as [25] in Assiut Governorate and [26] in Giza Governorate, who reported infection rate with different EMC was 84.75%, 84.8% and 82.8% respectively. On other hand, the present results considered higher than those reported by [27,28] they recorded that the prevalence of EMC in fish was 44.6%, 23.2%, and 56.3% respectively. Such variations in the frequency EMC may be attributed to various factors including the locality from which fish were caught and the degree of water pollution with human, animal and birds excreta and feeding habit of fish. [29] Skinner reported any environmental stress or chronic exposure to pollutants leads to release of corticosteroids which are immunosuppressant so fish become susceptible to any pathogenic organism.

In the current study, infection rate of microscopic encysted metacercaria was relatively higher in wild fish than cultured fish; it was 83% & 73% respectively. This result agree with [9] who mentioned that the prevalence rate of microscopic EMC was 88.3 % and 26.7% in wild fish and cultured fish respectively in Assiut Governorate. Difference of percentage between wild and farmed fish may be attributed to fish raised in rivers, canals, streams and lakes may be more exposed to infect snails/cercariae, and reservoir hosts, compared to fish raised on farms in farms, where the pond environment may not favor the presence of snails [30].

Contrary to this result was recorded by [31] Eissa *et al.* who reported that the infection rate in farmed and wild fish in Sharkia Governorate was 67.33% and 42.33% respectively. Also This may attributed to overcrowding in some fish farms, which affects the immune status of the fish as a result of increase environmental contaminants in these farms [32].

In the current study, prevalence of microscopic EMC was varied according fish species. The highest infection rate of microscopic EMC was detected in Tilapia "*O. niloticus*" (98%) and the lowest infection rate with EMC was detected in cat fish "*C. garpienus*" (40%). This result cleared that tilapia "*O. niloticus*" thought to be the main second intermediate host for trematodes EMC in Egypt. This result is nearly similar to those given by [25].

Difference of infection rate between fish species may be attributed to the difference in the habitat, food supply and abundance of both aquatic snails and the aquatic birds which play the main role to complete the life cycle of some trematodes and climatic variations of the fish sampling areas [25].

Microscopic metacercariae recovered in the present work were identified as *Prohemistomatide* and *Cyn. odiplostomatide*, their morphologically coincided with that previously reported by [12,26,33].

In other hand, macroscopic encysted metacercaria was identified as *Clinostomum phalacrocorais*. It was detected only in *Tilapia niloticus* and their total infection rate was 34%, it *Cl. Phalacrocoracis* metacercaria was recorded as a parasite only for Nile tilapia and known as Yellow grub [34]. It was found previously in Egypt by; [9] Ammar and Arafa they detected it in 19.2% in examined wild *O. niloticus* and they not detected it in farmed Tilapia. [35] Manal *et al.* described it as yellow cyst in 39.16% of *O. niloticus* from the River Nile at El-Minia District. Also, [36] Mai *et al.* recorded that the infection rate of *Cl. Phalacrocoracis* EMC among the investigated *O. niloticus* in Egyptian water was 60.93%. Regarding to the morphological characters, description of *Cl. phalacrocoracis* detected in the present work agree with [36,37].

The Effect of Some Traditional Processing Methods on EMC

As shown in table (3) microwave cooking by 700/W at high temperature for 5 & 2 minutes were sufficient to destroy all EMC in muscle of infested fish, where intestine of mice fed on microwave cooked fish were free from any adult trematodes. Our result agrees with many authors; [38] determined that microwave was very effective on larval stage of *Opisthorchis metacercariae* and it destroyed it completely. [39] Panupan *et al.* found that a heating process, by microwaving at 400 or 800 W for at least 5 min, could kill metacercariae in all sizes of fish. Also [40] revealed that using microwave 500 W/2 minutes was enough to destroy EMC. Heating process of microwave ovens depending on water content, the depth of initial heat deposition may be several centimeters or more and it cook food “from the inside out” [41]. The microbial destruction by microwave done by; 1- inactivates and destruct microorganism directly by heat [7]. 2- dielectric heating which means water, fat, and other elements in the food absorb energy from the microwaves, 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 [8]. [42] Ali and Al-Mahmoud recorded that the effectiveness of heating depends on several factors, such as, species of parasite, fat content and fillet thickness of fish.

Dry salting was the most effective processing mean to kill the EMC in *O. niloticus* muscles within one hour [9]. The current study investigated effect of two concentrations of potassium lactate in mixture with sodium chloride for 15 minutes on infectivity of EMC. Data summarized in Table 3 verify that both of concentrations used had significant values on the viability of EMC compared to control, but not sufficient to destroy all EMC in examined fish muscle. Previous studies of anti-parasitic effect of mixture (sodium chloride and potassium lactate) indicated that mixture of (2% sodium chloride and 1.4% potassium lactate) was able to prevent transmission of *T. gondii* experimentally either in cats or mice [17,43].

Electrolyzed water (EW) is mainly divided into two different types, acidic(AEW) and alkaline(BEW), AEW has a very powerful effect as a disinfectant. There are several factors affects antimicrobial activity of EW; the available chlorine concentration (ACC), the pH value and the oxidation reduction potential (ORP) in addition to storage time [44].

Regarding to the effect of AEW on the infectivity of EMC; in the present work, infective rate of EMC was significant decreased (3.73%) in mice group fed on fish muscle treated with acidic Electrolyzed water pH (3.4) as shown in Table 3. According to the available literatures, the present work is considered the

first study of anti-helmenthic effect of acidic electrolyzed water(AEW). While anti-protozoan effect of electrolyzed oxidizing water (EOW) was recorded by [45] who mentioned that administration electrolyzed oxidizing water (EOW)in mice experimentally infected with *Trypanosoma cruzi* showed a beneficial effect on parasitaemia, general physical condition, and mortality. On the other hand the effectiveness of acidic electrolyzed water (AEW) in reducing airborne microorganisms was investigated previously by [58] they mentioned that slightly acidic electrolyzed water spray can used as potential method for reducing microbial presence in layer houses.

Conclusion

Our results cleared high incidence of different encysted metacercariae in muscles of examined fresh water fishes in El-Minia Governorate. This needs to raise awareness in fish health management and application of suitable control measures. According to mice bioassay study; microwave cooking was the best way to kill metacercariae in fish muscle. Furthermore, AEW and mixture of sodium chloride and potassium lactate (different concentrations) gave significant values for inactivation the EMC in fish muscle.

Bibliography

1. Chai, J. Y., Murrell, K. D. & Lymbery, A. J. (2005). Fish. borne parasitic zoonoses: status and issues. *Int. J. Parasitol.*, 35(11-12), 1233-1254.
2. Pinlaor, S., Onsurathum, S., Boonmars, T., *et al.* (2013). Distribution and abundance of Opisthorchis viverrini metacercariae in cyprinid fish in northeastern Thailand. *Korean J. Parasitol.*, 51(6), 703-710.
3. EFSA (2010). European Food Safety Authority.
4. Phan, T. V., Ersbøll, K. A., Nguyen, V. K., Madsen, H. & Dalsgaard, A. (2010). Farm. level risk factors for fish. borne zoonotic trematode infection in integrated small. scale fish farms in Northern Vietnam. *PLoS Negl. Trop. Dis.*, 4(7), e742.
5. Ghada, A. K. K., Nagwa, M. E., Eman, K. B., Shimaa, S. Gh.S. & Khaled, M. (2018). Oreochromis niloticus and Clarias gariepinus Fish Infected with Digenean Metacercariae: Experimental Infection of Puppies and Some Chemical Alterations in Fish. *Asian J. Animal Vet. Advances.*, 13(4), 352-359.
6. Huss, H. H., Ababouch, L. & Gram, L. (2003). Assessment and management of seafood safety and quality. Food Agriculture Organization (FAO) fisheries technology.
7. Heddleson, R. A. & Doores, S. (1994). Factors affecting microwave heating of foods and destruction of food born pathogen. *A review J. Food Protection.*, 57(11), 1025-1037.
8. Zitzewitz, W.P. (2011). The Handy Physics Answer Book. Visible Ink, 2nd ed., Brownstown, MI, USA.

9. Ammar, M. A. M. & Arafa, M. I. (2013). Cryptosporidium and other zoonotic parasites in *Oreochromis niloticus* in Assuit governorate. *Assiut Vet. Med. J.*, 59(139), 142-151.
10. Athayde, D. R., Flores, D. R. M., Silva, J. S., Silva, M. S., Genro, A. L. G., Wagner, R., *et al.* (2018). Characteristics and use of electrolyzed water in food industries. *Int. Food Res. J.*, 25(1), 11-16.
11. Al. Haq, M. I., Sugiyama, J. & Isobe, S. (2005). Applications of electrolyzed water in agriculture and food industries. *Food Sci. Technol. Res.*, 11(2), 135-150.
12. Mahdy, O. A., Manal, A. A., Essa, A. A. & Easa, M. (1995). Parasitological and pathological studies on Heterophyid infection in *Tilapia* species from Manzala Lake, Egypt. *Vet. Med. J. Comp. Pathol. Clin. Pathol.*, 8(2), 131-145.
13. Huang, Y. R., Hung, Y. C., Hsu, S. Y., Huang, Y. W. & Hwang, D. F. (2008). Application of electrolyzed water in the food industry. *Food Control*, 19(4), 329-345.
14. Farah, R. I. & Ali, S. N. A. (2021). Electrolyzed water generated on site as a promising disinfectant in the dental office during the COVID-19 pandemic. *Frontiers Public Health*, 9, 629142.
15. El. Zawawy, L. A. (2008). Viability and infectivity of encysted metacercariae (EMC) of *Pygidiopsis genata* in fish. *J. Egypt. Soc. Parasitol.*, 38(3), 945-956.
16. Hill, D. E., Sreekumar, C., Gamble, H. R. & Dubey, J. P. (2004). Effect of commonly used enhancement solution on the viability of *Toxoplasma gondii* tissue cysts in pork loin. *J Food Protec.*, 67(10), 2230-2233.
17. Garcia, L. S. (2001): Diagnostic medical parasitology (3rd Edition). ASM Press, Washington, D.C.
18. Jennes, M., De Craeye, S., Devriendt, B., Dierick, K., Dorny, P. & Cox, E. (2017). Strain- and dose dependent reduction of *Toxoplasma gondii* burden in pigs is associated with interferon- γ production by CD8⁺ lymphocytes in a heterologous challenge model. *Frontiers Cellular Infection Microbiol.*, 7, 232.
19. Sriraj, P., Aukkanimart, R. & Boonmars, T. (2013). Does a combination of opisthorchiasis and ethyl alcohol consumption enhance early cholangiofibrosis, the risk of cholangiocarcinoma? *Parasitol. Res.*, 112(8), 2971-2981.
20. Dong, H., Lu, Y. Y., Su, R. J., Wang, Y. H., Wang, M. Y., Jiang, Y. B. & Yang, Y. R. (2018). Low prevalence of antibodies against *Toxoplasma gondii* in dairy cattle from China's central region. *BMC Veterinary Research*, 14(1), 315.
21. Borji, H., Naghibi, A., Nasiri, M. R. & Ahmadi, A. (2012). Identification of *Dactylogyrus* 233 spp. and other parasites 234 of common carp in northeast of Iran. *J. Parasitic Dis.*, 36(2), 234-238.

23. Rim, H. J., Sohn, W. M., Yong, T. S., Eom, K. S., Chai, J. Y., Min, D. Y., *et al.* (2008). Fishborne trematode metacercariae detected in freshwater fish from Vientiane Municipality and Savannakhet Province, Lao PDR. *Korean J. Parasitol.*, 46(4), 253-260.
24. Eissa, I. A. M., Mona, M. A., Hala, M. E. & Aly, S. M. (2001). Yellow grub disease among *Oreochromis niloticus* in relation to food quality and pathological alterations. *Sues Canal Vet. Med. J.*, IV(2), 179-194.
25. Taher, G. A. (2009). Some studies on metacercarial infection in *Oreochromis niloticus* in Assiut Governorate and their role in transmission of some trematodes to dogs. *Assiut Univ. Bull. Environ. Res.*, 12(1), 63-79.
26. Saad, M. S., Salem, A. M., Mahdy, O. A. & Ibrahim, E. S. (2019). Prevalence of metacercarial infection in some marketed fish in Giza governorate, Egypt. *J. Egypt. Soc. Parasitol.*, 49(1), 129-134.
27. Chi, T. D., Turnbull, J. F. & Pham, A. T. (2008). Prevalence of zoonotic trematodes in fish from Vietnamese fish farming community. *J. Parasitol.*, 94(2), 423-428.
28. El. Sheikh, H. M. & El. Shazly, A. M. (2008). Host-dependent variations in the seasonal prevalence and intensity of heterophyid encysted metacercariae (Digenea: Heterophyidae) in brackish water fish in Egypt. *Vet. Parasitol.*, 153(1-2), 65-72.
29. Skinner, R. H. (1982). The interrelation of water quality, gill parasites and gill pathology of some fish from South Biscayne Bay Florida. *Fishery Bull.*, 80, 269-280.
30. Nguyen, D. T., Anders, D., Ly, T. L. K. & Darwin, M. (2007). Survey for zoonotic liver and intestinal trematode metacercariae in cultured and wild fish in an Giang Province, Vietnam. *Korean J. Parasitol.*, 45(1), 45-54.
31. Eissa, I. A. M., Diab, A. S. & Badran, A. F. (1996). Studies on some internal parasitic diseases among wild and cultured *Oreochromis niloticus* fish. 7th Sci. Cong. 17-19. p 274-289.
32. Khaldi, E. L. & Aziza, T. F. (2010). Effect of different stress factors on some physiological parameters of Nile tilapia (*Oreochromis niloticus*). *Saudi J. of Biolog. Sci.*, 17(3), 241-246.
33. Mousa, W. M., Mahdy, O. A. & Kandil, O. M. (2000). Electrophoretic analysis to confirm the identification of some kinds of encysted metacercariae from *Oreochromis niloticus*. *Assiut Vet. Med. J.*, 43(85), 199-209.
34. Caffara, M., Locke, S. A., Halajian, A., *et al.* (2019). Molecular data show *Clinostomoides Dollfus*, 1950 is a junior synonym of *Clinostomum Leidy*, 1856, with redescription of metacercariae of *Clinostomum Brieni* n. comb. *Parasitol.*, 146(6), 805-813.

35. Manal, A., Sahar, E. & Shaban H. A. (2018). Morphological and electrophoretic differentiation of two Clinostomatid Metacercariae infecting *Oreochromis niloticus* from the River Nile at El. Minia District, Egypt. *Egypt. Acad. J. Biolog. Sci.*, 10(1), 105-115.
36. Salem, M. A., Abdel-Maogood, S. Z., Mohamed, A. & Mahdy, O. A. (2021). Comparative morpho-molecular identification of *Clinostomum phalacrocoracis* and *Clinostomum complanatum* metacercariae co infecting Nile tilapia in Egypt. *Egyptian J. Aquatic Biolo. Fisheries*, 25(1), 461-475.
37. Thabit, H. (2004). Studies on some parasites of some Nile fishes in Assiut governorate. M.V.Sc. Thesis, Fac. of Vet. Med., Assiut. Univ.
38. Pel'gunov, A. N., Riabov, I. N. & Filippova, A. I. U. (2005). Use of microwave ovens in the prevention of opisthorchiasis. *Med. Parazitol. (Mosk.)*, Jul-Sep(3), 42-45.
39. Panupan, S., Thidarut, B., Jiraporn, S., Ratchadaw, A., Pranee, S., *et al.* (2017). Simplified techniques for killing the carcinogenic, *Opisthorchis Viverrini* Metacercariae in Cyprinid Fish. *Asian Pac. J. Cancer Prev.*, 18(6), 1507-1511.
40. Hanan, A. A., Elghayaty, I., Samar, W. & Tadros, A. (2020). Ozonized water, microwaves and freezing effects on viability of encysted metacercariae in fish muscle. *SSRG Int. J. Vet. Sci.*, 6(1), 6-15.
41. Sohn, W. M., Kim, J. A. & Song, H. J. (2005). Two species of Goby, *Boleophthalmus pectinirostris* and *Scartelaos* sp., as the new second intermediate hosts of Heterophyid fluke in Korea. *Korean J. Parasitol.*, 43(4), 161.
42. Ali, H. B. & Al. Mahmoud, S. S. (2009). Pathological effect of *Cryptosporidium mansonioocysts* isolated from fish and treated with infra. red, microwave and ozone in common carp. *Iraq J. Vet. Sci.*, 23(1), 187-192.
43. Nageib, B. R. & Mohamed M. H. (2021). Effect of mixture of sodium chloride and potassium lactate on the viability of *Toxoplasma gondii* in meat. *Assiut Vet. Med. J.*, 67(169), 37-53.
44. Chen, Bo. Kai & Wang, Chin. Kun (2022). Electrolyzed water and its pharmacological activities: A Mini. *Review. Molecules*, 27(4), 1222.
45. Olivia, R. M., Juan, J. C., Silvia, D. C., Rodolfo, A. G., *et al.* (2020). Electrolyzed Oxidizing Water Modulates the Immune Response in BALB/c Mice Experimentally Infected with *Trypanosoma cruzi*. *Pathogens*, 9(11), 974.