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MOLECULAR DETECTION OF *TOXOPLASMA GONDII* IN SOME TYPES OF FISHES CAUGHT FROM THE RIVER NILE CANALS

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Abstract

Toxoplasma parasite is one of the most successful creatures on earth being resistant to the dangers of nature and powerfully spread among a wide range of land and aquatic animals. However, the extent of aquatic spread is still not well studied. This study discovered extent of *Toxoplasma* parasite's spread in fresh water fishes commonly eaten by Egyptians living in some rural areas.

Quantitative real-time PCR was performed for molecular detection of *Toxoplasma* genomic materials within 4 types of fishes; *Mugil*, *Atherina*, *Tilapia* and *Anguilla* species. Five sites were selected within these types to be studied; intestine, gills, skin/muscle complex, brain and eye.

The results showed that *Toxoplasma* was molecularly detected in the four types of fish with the highest percentage of infection estimated to be within gills, followed by skin/muscles complex, then intestine and eye with 0% brain infection.

Keywords: *Toxoplasma*-aquatic -waterborne–fishes-qPCR

Introduction

Toxoplasma gondii that causes toxoplasmosis infects up to a third of the worldwide, mainly acquired by ingestion of food or water contaminated with oocysts shed by cats or by eating undercooked or raw meat containing cysts. Primary infection is usually subclinical, but in some patients cervical lymphadenopathy or ocular disease can be present. Infection acquired during pregnancy may cause congenital infection with severe damage to fetus. In immunocompromised patients, reactivation of latent disease causes life-risky encephalitis (Montoya and Liesenfeld, 2004). This unique parasite is considered one of the most successful creatures on earth, in terms of its attainment in live with powerful zoological spreading and infecting capability (Holland, 2003).

Toxoplasma stages were molecularly detected in Ancient Egyptian mummies (Khairat *et al,* 2013). The Ancient Egyptians were known to consider cats in the Pharaohs times as Goddess Best (Azab, 2018). However, the first human toxoplasmosis case was reported by Ruge (1952). Rifaat *et al.* (1962) in Tahrir Province reported toxplasmin skin positivity among introduced workers and Rifaat *et al.* (1965) in 12 governorates reported human positivity by Sabin-Feldman dye and agglutination tests. Toxoplasmosis was reported among infants with fever & neonatal jaundice (Wishahy *et al,* 1971a, b), with neurological pictures (Wishahy *et al,* 1972), and Abdel Salam *et al.* (1990) isolated *Toxoplasma* parasites from human placenta of aborted females. Saleh *et al.* (2014) detected toxoplasmosis among childbearing age females and (2016) documented *T. gondii* as occupational, nosocomial or hospital infection. Ibrahim *et al.* (2009) reported that sero-positive to specific anti-*Toxoplasma* IgG antibodies was 57.9%, 58.1%, & 44.7% in randomly collected samples, full-term pregnant women, & aborted ones, but the IgM was 10.5%, 6.5%, & 23.7% respectively. Elsheikha *et al.* (2009) detected *T. gondii* antibodies among asymptomatic blood donors. Taman and Alhusseiny (2020) found that in females *T. gondii* sero-prevalence ranged from 6.66% in Minia Governorate (Upper Egypt) to 97.7% in Sharkia Governorate (Lower Egypt).

Apart from man, Rifaat *et al.* (1967) in Giza isolated *Toxoplasma* RH strain from wild mongoose and Rifaat *et al.* (1971) detected marked serological toxoplasmosis pattern in stray dogs & cats. Al-Kappany *et al.* (2010) reported antibodies to *T. gondii* in 97.4% by modified agglutination test. Viable *T. gondii* was isolated from brain, heart, & tongue of 115/137 cats by mice bioassay. They added that of 112 sero-positive their tissues were bioassayed individually, *T. gondii* was isolated from hearts of 83 (74.1%), tongues of 53 (47.3%), & brains of 36 (32.1%). Abbas *et al.* (2020) reported that a large rate of stray cats, up to 95% were *T. gondii* infected, specifically in rural and suburban areas.

This study aimed to discover the extent of *Toxoplasma* parasite's spread in fishes that are commonly eaten by the low income Egyptians living in some rural areas.

Materials and Methods

Study area and samples: Tributaries and channels of the Nile River within three governortes: Giza, Beni-Suef and Al-Fayoum. Collection of fishes was carried out in summer time, August to September 2020 and was transferred to the laboratory for preservation until processed and prepared for DNA extraction. Fishes were caught by fishermen from the same governorate to ensure the origin of samples. The fishermen were emphasized to catch the most types of fishes usually consumed by the inhabitants of these areas. Total number of collected fishes was 80 of four types (20/each) as follows; *Atherina*, *Tilapia*, *Mugil* and *Anguilla* species, and total number of samples were 400 (5 sites for each fish species; 5×80=400). From each type, different tissues were collected from gills, intestine, skin/muscles complex, eye and brain.

DNA extraction and molecular analysis: Single tissue types of each group were pooled into 4 units for each type for a total 100 samples to be transported into sterile stomacher bags, and gently mixed with a mortar. The homogenate was diluted with an equivalent volume of TE buffer (Tris-EDTA) and then remixed within the stomacher. DNA extraction was performed on 200µl of homogenate using Charge-Switch™ gDNA Mini Tissue Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Magnetic bead based technology was used to isolate genomic materials without using dangerous chemical compounds, centrifugation, or vacuum manifolds (Marino *et al,* 2019). According to the manufacturer's instructions, the quality and quantity of the extracted DNA was analyzed by Nano-Drop® ND-1000 spectrophotometer. Samples related to positive pools were exposed individually to DNA extraction and subsequent molecular analysis.

The real-time PCR amplification was performed in which 529-bp repeated fragment of *T. gondii* genome was targeted (GenBank accession no. AF146527). Technique was completed using Applied Bio-systems kits and supplies within a net volume of 20µl including 10µl of master mix 2× with nuclease-free water of 4µl & 40ng of DNA genomic template. Primers were added, 10pmol, each; AF1 (CACAGAAGGGACAGAAGT) and primer AF2 (TCGCCTTCATCTACAG TC), 5pmol of the labeled TaqMan probe, AF 529 (6FAM-CTCTCCTCCAAGACGG CTGGBHQ), 2µl of 10×Exo IPC-VIC, 0.5µl of 50× Exo IPC DNA. Molecular amplification procedure was performed after 2min incubation at 50°C with 10min denaturation & then 40 full cycles amplification; 95°C for 15s, annealing & extension at 60°C for 60s.

Molecular grade water was used as negative control and positive control (*T. gondii* genomic DNA (ATCC 50174D™ from Rh-88 *T. gondii*; LGC Standards) was exposed to serial dilutions from 10^3 to 10^7 copies/ml to be quantitative molecularly analyzed and calculating parasitic load of fish samples by a standard reference curve. Validation was done following OIE & Codex Alimentarius Guidelines (Marino *et al,* 2017).

Results

Toxoplasma gondii genomic DNA was molecularly detected within all 4 fish species with overall 140 positive samples that gene-

rated fluorescence signals out of 400 samples (35%). Largest number of positive samples was found in *Tilapia* (40, or 28.5%), followed by *Mugil* (36, or 25.7%), *Anguilla* (34, or 24.2%) and the lowest one was in *Atherina* spp. (30, or 21.4%), but without significant difference (P>0.05). Quantitative molecular estimation of positive samples was between 1.3×10^1 to 7.6×10^8genomic equivalents. Crossing points showed values from 6.23 to 30.2 reflected different quantities of *Toxoplasma* genomic materials within the samples. Infection site was significantly higher in gills (48, or 34.29%), followed by skin/muscle (38, or 27.14%), then intestine (33, or 23.57%) and least one in brain (21, or 15%) with significant differences (P<0.05).

Significantly higher infection rate was only in skin/muscle complex and gills of *Mugil* spp. & *Anguilla* spp. respectively (P<0.05), with significant difference in skin/muscle complex and gills in same groups (P≤0.05)

Details were given in tables (1 & 2) and figures (1, 2, 3, 4, & 5).

Table 1: Positive samples within different sites in each studied groups

Fishes' type 20/type	Intestine (n=33)	Gills (n=48)	Skin/ Muscle (n=38)	Eye (n=21)	Test value	P- value
	No. Percentage	No. Percentage	No. Percentage	No. Percentage		
Mugil spp.	11 (33.3%)	6 (12.5%)	17 (44.7%)	2 (9.5%)	15.471	0.001
Tilapia spp.	7 (21.2%)	15 (31.3%)	11 (28.9%)	7 (33.3%)	1.280	0.734
Anguilla spp.	9 (27.3%)	14 (29.2%)	2 (5.3%)	9 (42.9%)	12.199	0.005
Atherina spp.	6 (18.2%)	13 (27.1%)	8 (21.1%)	3 (14.3%)	1.758	0.624

Table 2: Dominance of gills and skin/muscle complex as most affected sites within groups.

Fishes' type 20/type	Intestine (n=33)	Gills (n=48)	Skin/ Muscle (n=38)	Eye (n=21)
	No. Percentage	No. Percentage	No. Percentage	No. Percentage
Mugil spp. (n=36)	11(30.6%)	6 (16.7%)	17 (47.2%)	2 (5.6%)
Tilapia spp. (n=40)	7 (17.5%)	15 (37.5%)	11 (27.5%)	7 (17.5%)
Anguilla spp. (n=34)	9 (26.5%)	14 (41.2%)	2 (5.9%)	9 (26.5%)
Atherina spp. (n=30)	6 (20.0%)	13 (43.3%)	8 (26.7%)	3 (10.0%)
Test value	2.164	6.950	15.117	6.811
P- value	0.539	0.054	0.002	0.078

Discussion

In the present study, *Toxoplasma* genomic materials were molecularly detected in the 4 consumable fish. The highest *Toxoplasma* infection rate was only in skin/muscle complex and gills of *Mugil* spp. & *Anguilla* spp. respectively (P<0.05), with significant difference in skin/muscle complex and gills within the same fish group (P≤0.05). Also, *Toxoplasma* was detected by real time PCR in all fish types with the highest infection rate in the gills, followed by skin/muscles complex, then intestine and eye, but none in the brain. This may indicate that the source of infection is external through contaminated water to attach to the external body or invade the eye of fishes, or the parasite was unable to reach through the internal system. Intestine was molecularly examined to ascertain the capacity of fishes to retain *Toxoplasma* oocysts. Gills could trap the oocysts, as water filters.

Abroad, *T. gondii* oocysts were molecularly detected in well water and drinking water in Belgium (Mikaelian *et al,* 2000), France (Villena *et al,* 2004), Poland (Sroka *et al,* 2006), USA (Shapiro *et al,* 2019a) and Columbia (Campo-Portacio *et al,* 2021). Moreover, *T. gondii* oocysts were detected in the Coastal California (Miller *et al,* 2008), and retained infectivity in the seawater (Shapiro *et al,* 2019b). Besides, Dubey (1998) reported that preserved aquatic *Toxoplasma* oocysts after purification at 4°C were infective to mice for 54 months, and Lindsay and Dubey (2009) used the same experimental study, but with the artificial seawater found that oocysts remained infective to mice for up to 18 months.

Waterborne outbreaks of *Toxoplasma* infection were documented in Canada (Bowie *et al,* 1997), in Brazil (de Moura *et al,* 2006), and in India (Palanisamy *et al,* 2006). Jones and Dubey (2010) in USA approved water-

borne toxoplasmosis, and that a large human outbreak was linked to contamination of the municipal water reservoir in Canada by wild felids and the USA widespread infection of marine mammals. Also, Baldursson and Karanis, 2011) in Germany reported that about 199 outbreaks of human waterborne parasitic protozoa occurred in the period from 2004 to 2010, as 46.7% in the Australian continent, 30.6% in North America & 16.5% in Europe, *T. gondii* caused four outbreaks (2%).

Aramini *et al.* (1999) in Canada reported that world's first documented toxoplasmosis outbreak associated with a municipal water supply was recognized in 1995 in Victoria, British Columbia, Canada, which hypothesized that domestic cat (*Felis catus*) or cougar (*F. concolor*) feces contaminated a surface water reservoir with *Toxoplasma gondii* oocysts. They concluded that contamination of Victoria's water supply with oocysts potentially occurred during the study period and future waterborne toxoplasmosis outbreaks in this and other communities are possible. Dabritz and Conrad (2010) in Morro Bay, California found that 3/326 (0.9%) cats were actively shedding oocysts, with approximately 244-253 million oocysts per cat.

Moreover, Massie *et al.* (2010) in USA reported that from bottlenose dolphins, to walruses, to sea otters, the parasitic protozoan *T. gondii* infects marine mammals worldwide. They added that the oocysts persisted in the fish for at least 8hr post-exposure and remained infectious inside the fish's alimentary canals and that northern anchovies and Pacific sardines filtered *T. gondii* oocysts from seawater under experimental conditions.

Zhang *et al.* (2014) in China reported eight aquatic animals infected with *T. gondii.* Van de Velde *et al.* (2016) in Belgium examined samples from 589 marine mammal species and 34 European otters (*Lutra lutra*), stranded on the coasts of Scotland, Belgium, France, The Netherlands and Germany for *T. gondii.* Brain samples were analyzed by PCR for parasite DNA. Blood and muscle fluid samples were tested for specific antibodies using a modified agglutination test (MAT), a commercial multi-species ELISA and IFA. They found that out of 193 animals tested by PCR, only two harbored porpoise (*Phocoena phocoena*) cerebrum samples from the German coast were positive. Serological results showed with a cut-off value of 1/40 dilution in MAT, 141/292 animals (41%) were positive, by IFA, 30/244 samples (12%) were positive at a 1/50 dilution. ELISA yielded 7% positives with a cut-off at S/P ratio$\geq$50; and 12% with cut-off at S/P ratio$\geq$20. The high positivity in MAT may be an overestimation due to high degree of samples haemolysis, but ELISA results could be an underestimation due to using a multispecies conjugate.

Marino *et al.* (2019) in Italy examined a total of 1,293 fishes from 17 species obtained from wholesale and local fish markets for *T. gondii* DNA by Real-time PCR from separately pooling intestines, gills & skin/muscles collected from each fish species. They found that 32/147 pooled samples from 12 different fish species contaminated with *T. gondii* DNA 16 of skin/muscle and 11 of the intestine and gills. The quantitative analysis confirmed that positive fish samples were contaminated with the *Toxoplasma* genomic DNA to an extent of 6.10×10^{-2} to 2.77×10^{4} copies/ml (qPCR), and of 1 to 5.7×10^{4}copies /ml (dPCR). They concluded that fishes are not competent biological hosts for *T. gondii*; but, they can be contaminated with *T. gondii* oocysts flowing via freshwater run-offs (untreated sewage discharges, soil flooding) into the marine environment, thus acting as mechanical carriers. They suggested that fish species sold for human consumption can be accidentally involved in route transmission of parasite in marine environment and that foodborne transmission of toxoplasmosis risk to fish consumers should be in mind.

Martins *et al.* (2021) in Portugal reported *T. gondii* infection in pinnipeds 169 (8.9%) and added that some of them with fatal consequences. They found that sera from were tested for antibodies to *T. gondii* by the modified agglutination test with a cut-off titer

of 25. An overall sero-prevalence of (95% confidence interval: 5.1-14.2) was observed. The antibody titers of 25, 50, 100, 1600 & ≥3200 were in five (33.3%), two (13.3%), five (33.3%), one (6.7%) and two (13.3%) animals, respectively.

Ahmadpour *et al.* (2022) in Iran reviewed and meta-analyzed the prevalence of *T. gondii* infection among sea animal species worldwide and highlight the existing gaps. They concluded that spread of *T. gondii* among marine animals can affect the health of humans and other animals; in addition, it is possible that marine mammals act as sentinels of environmental contamination, especially the parasites by consuming water or prey species.

In Egypt, as to edible meat or non-human toxoplasmosis, Rifaat *et al.* (1969) reported *T. gondii* in chickens, Rifaat *et al.* (1977) isolated *Toxoplasma* from farm animals, and Michael *et al.* (1977) reported high ELISA-antibodies among camels. El-Massry *et al.* (2000) detected anti-*Toxoplasma* antibodies by modified AT in 59.5% of domestic turkeys, 47.2% of chickens, and 50% of ducks.

In Egypt, apart from meat as source of zoonotic infection, Shaapan and Ghazy (2007) isolated *T. gondii* from meat of horses. Haridy *et al.* (2009) reported ELISA anti-*Toxoplasma* antibodies in draught horses in the Greater Cairo, and Haridy *et al.* (2010) reported high positive ELISA anti-*Toxoplasma* antibodies in donkeys and their milk. Mikhail *et al.* (2017) in Giza Governorate reported ELISA anti-*Toxoplasma* antibodies among the commensal rodent species *Rattus norvegicus* and *R. rattus*, trapped from different localities. Only, El Shazly *et al.* (2007) in Dakahlia Governorate detected *Toxoplasma* oocysts among other water polluted gastrointestinal protozoa infective stages.

Conclusion

No doubt, *T. gondii* is a zoonotic parasite that can cause morbidity and mortality in man, domestic animals, and terrestrial & aquatic wildlife. Risk of foodborne transmission of toxoplasmosis to fish consumers must be deeply explored with special attention in studying the infectivity of the identified disease. This must be in concern with the Public Health, Veterinary, and Environmental Policy Authorities

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Explanation of figures

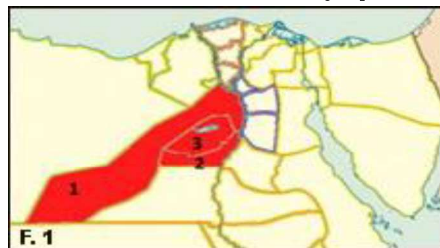
Fig. 1: Map showed governorates 1; Giza, 2; Beni-Suef and 3; Al-Fayoum governorates, with tributaries and channels of Nile River.

Fig. 2: Fishing at selected areas by nets (A&C) or by hook (B). Different types of fishes used in the study (D): *Mugil* (E): *Atherina* and (F): *Tilapia* (red arrows), and *Anguilla* species (yellow arrow).

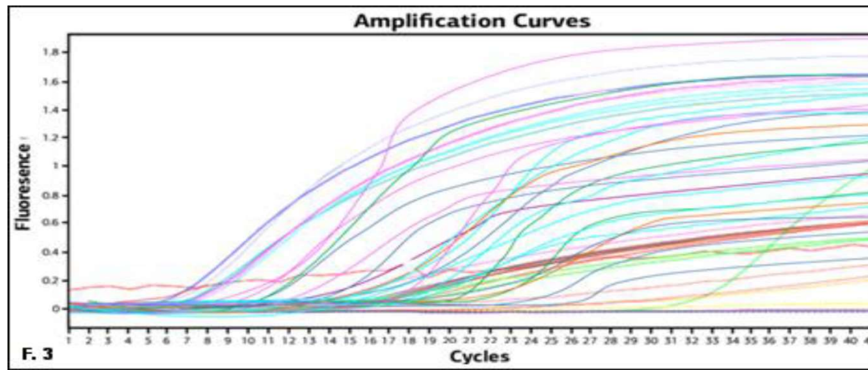
Fig. 3: Amplification curves of positive samples in a real-time PCR, crossing points ranged from 6.23 to 30.2 reflected different quantities of *Toxoplasma* genomic materials.

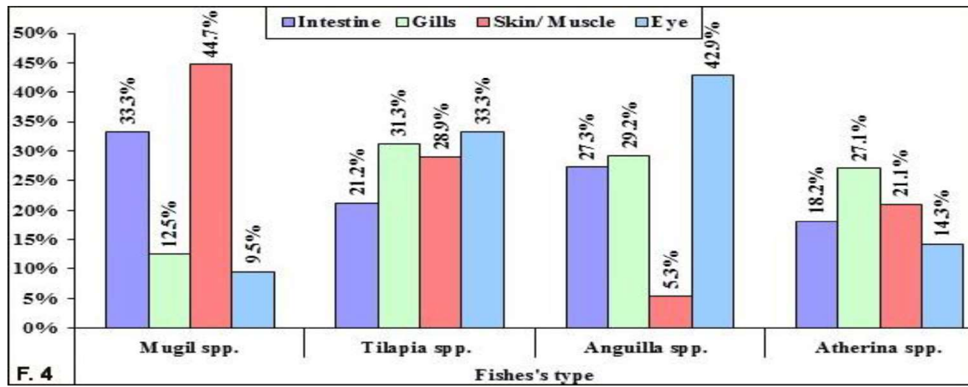
Fig. 4: Bar chart represents percentages of infection within different sites of t 4 fish groups.

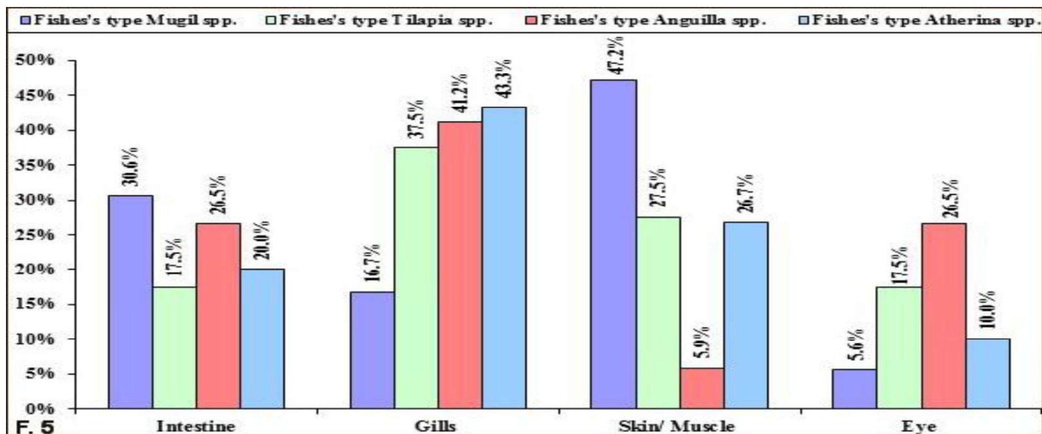
Fig. 5: Bar chart represents percentages of infection within different sites of 4 fish groups.



A
B
C
D
E
F
F. 2


Amplification Curves
Fluoresence
Cycles
F. 3


Intestine
Gills
Skin/ Muscle
Eye
33.3%
12.5%
44.7%
9.5%
21.2%
31.3%
28.9%
33.3%
27.3%
29.2%
5.3%
42.9%
18.2%
27.1%
21.1%
14.3%
Mugil spp.
Tilapia spp.
Anguilla spp.
Atherina spp.
Fishes's type
F. 4


Fishes's type Mugil spp.
Fishes's type Tilapia spp.
Fishes's type Anguilla spp.
Fishes's type Atherina spp.
30.6%
17.5%
26.5%
20.0%
16.7%
37.5%
41.2%
43.3%
47.2%
27.5%
5.9%
26.7%
5.6%
17.5%
26.5%
10.0%
Intestine
Gills
Skin/ Muscle
Eye
F. 5