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

By
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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 toxoplasm 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 governorates: 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, Wal-tham, 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 (TCGCCTTCATCTACAGTC), 5pmol of the labeled TaqMan probe, AF 529 (6FAM-CTCTCTCCAAGACGGCTGGBHQ), 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 10⁵ to 10⁷ 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 \(1.3 \times 10^1\) to \(7.6 \times 10^8\) genomic 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 fish group (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

<table>
<thead>
<tr>
<th>Fishes’ type 20/type</th>
<th>Intestine (n=33)</th>
<th>Gills (n=48)</th>
<th>Skin/ Muscle (n=38)</th>
<th>Eye (n=21)</th>
<th>Test value</th>
<th>P- value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mugil</em> spp. (n=36)</td>
<td>11 (33.3%)</td>
<td>6 (12.5%)</td>
<td>17 (44.7%)</td>
<td>2 (9.5%)</td>
<td>15.471</td>
<td>0.001</td>
</tr>
<tr>
<td><em>Tilapia</em> spp. (n=40)</td>
<td>7 (21.2%)</td>
<td>15 (31.3%)</td>
<td>11 (28.9%)</td>
<td>7 (33.3%)</td>
<td>1.280</td>
<td>0.734</td>
</tr>
<tr>
<td><em>Anguilla</em> spp. (n=34)</td>
<td>9 (27.3%)</td>
<td>14 (29.2%)</td>
<td>2 (5.3%)</td>
<td>9 (42.9%)</td>
<td>12.199</td>
<td>0.005</td>
</tr>
<tr>
<td><em>Atherina</em> spp. (n=30)</td>
<td>6 (18.2%)</td>
<td>13 (27.1%)</td>
<td>8 (21.1%)</td>
<td>3 (14.3%)</td>
<td>1.758</td>
<td>0.624</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Fishes’ type 20/type</th>
<th>Intestine (n=33)</th>
<th>Gills (n=48)</th>
<th>Skin/ Muscle (n=38)</th>
<th>Eye (n=21)</th>
<th>Test value</th>
<th>P- value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mugil</em> spp. (n=36)</td>
<td>11 (30.6%)</td>
<td>15 (37.5%)</td>
<td>11 (27.5%)</td>
<td>7 (17.5%)</td>
<td>7 (17.5%)</td>
<td></td>
</tr>
<tr>
<td><em>Tilapia</em> spp. (n=40)</td>
<td>7 (17.5%)</td>
<td>15 (37.5%)</td>
<td>2 (5.9%)</td>
<td>9 (26.5%)</td>
<td>6.811</td>
<td>0.078</td>
</tr>
<tr>
<td><em>Anguilla</em> spp. (n=34)</td>
<td>9 (26.5%)</td>
<td>14 (41.2%)</td>
<td>2 (5.9%)</td>
<td>3 (10.0%)</td>
<td>6.811</td>
<td>0.078</td>
</tr>
<tr>
<td><em>Atherina</em> spp. (n=30)</td>
<td>6 (20.0%)</td>
<td>13 (43.3%)</td>
<td>8 (26.7%)</td>
<td>3 (10.0%)</td>
<td>6.811</td>
<td>0.078</td>
</tr>
</tbody>
</table>

### 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 Karranis, 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≥50; and 12% with cut-off at S/P ratio≥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 $6.10 \times 10^{-2}$ to $2.77 \times 10^{4}$ copies/ml (qPCR), and of 1 to $5.7 \times 10^{4}$ 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.


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.

Authors’ contributions: All authors equally contributed in the study.

Conflict of interest: Authors neither have conflict of interest nor received fund

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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* (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 4 fish groups.

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