Pakistan J. Zool., pp 1-6, 2023. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20220802150800

Monitoring and Molecular Detection of *Toxoplasma gondii* in Food: Vegetables, Fruits, and Fish as Neglected Vehicles for Toxoplasmosis in the Nile Delta of Egypt

Walid Elmonir[1]*, Ahmed Abdel-Fattah Tayel[2], Suzan Abdelbaky Kotb[1] and Wael Fawzy El-Tras[2]

[1]*Department of Hygiene and Preventive Medicine, (Zoonoses), Faculty of Veterinary Medicine, Kafrelsheikh University, Kafrelsheikh, Egypt.*
[2]*Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University, Kafrelsheikh, Egypt*

Article Information
Received 02 August 2022
Revised 26 September 2022
Accepted 23 October 2022
Available online 25 May 2023
(early access)

Authors' Contribution
WE, AAT, and WFT designed the study. SAK collected the samples. SAK, WE and AAT conducted the experimental work. WE analyzed the data. All authors wrote, reviewed and agreed to publish the manuscript.

Key words
***Toxoplasma gondii*, Molecular detection, Vegetables and fruits, Fish, Egypt**

ABSTRACT

This study was conducted in the Nile Delta, Egypt, to investigate the role of vegetables, fruits, and fish as a source of *Toxoplasma gondii* and to determine effective decontamination approaches. A total of 200 agricultural produce samples (50 per each of carrots, radishes, lettuces, and strawberries) and 315 fish samples (200 Mullet and 115 Tilapia) were collected. The *T. gondii* were identified in these samples by microscopy and molecular analysis. In total, 9% (18/200) of agricultural produce samples had *Toxoplasma* oocysts. *T. gondii* was detected in 14% of carrots, 12% of radishes, 4% of lettuces, and 6% of strawberries. There was no significant difference in prevalence of *T. gondii* between different fresh produce type (*P* value= 0.1 – 0.6). *T. gondii* was not detected in any fish samples. Two agriculture produce (1 carrot and 1 strawberry) samples were misdiagnosed by microscopic analysis, which emphasized the importance of molecular detection of *Toxoplasma* oocysts in environmental samples. To our knowledge, this is the first molecular detection of *T. gondii* oocysts in vegetables, and fruits in Egypt. Awareness of public and health professionals about these neglected vehicles of toxoplasmosis is urgently required.

INTRODUCTION

Toxoplasmosis is a common zoonotic disease caused by *Toxoplasma gondii*, a protozoan that is widely spread among animals and humans thoughout the world (Dumètre and Dardé, 2003; WHO, 2015), including Egypt (Al-Kappany *et al*., 2010; Elmonir *et al*., 2017). Toxoplasmosis is generally asymptomatic, but severe outcomes may occur in pregnant women after congenital transmission (e.g., abortions, fetal handicapping) and immunocompromised patients (e.g., those with CNS diseases) (WHO, 2015).

The ingestion of sporulated oocysts comprises an important mode of *Toxoplasma* transmission to humans (Shapiro *et al*., 2019). Recently, there has been increasing evidence indicating that people may pick up the oocysts by eating untreated vegetables and fruits (Ekman *et al*., 2012; Shapiro *et al*., 2019). *Toxoplasma* oocysts were recovered from these food vehicles at rates ranging from 0.3% to 9.7% (Lass *et al*., 2012, 2019; Lalonde and Gajadhar, 2016). The vegetables, and fruits may be contaminated with *T. gondii* oocysts by cat feces either directly or indirectly though contaminated soil in agriculture fields (Shapiro *et al*., 2019). Sporulated oocysts can survive for up to 18 months in soil under variable ambient temperature (Dumètre and Dardé, 2003; Dubey, 2004). This robustness of *Toxoplasma* oocysts combined with their hydrophilic and low adhesive nature facilitate their survival and easy dispersion between fresh produce vehicles and also humans or animals that may consume or contact these vehicles.

T. gondii has long been considered a global parasite infecting substantially all warm-blooded animals. Sporadically, cold-blooded animals such as fish may be infected with this parasite (Nayeri *et al*., 2021). *In vitro* studies on tissue cells of cold-blooded animals showed that they could be infected by *T. gondii* at 37°C (Omata *et al*., 2005). Additionally, *in vivo* injection of

* Corresponding author: walid.elmonir@gmail.com
0030-9923/2023/0001-0001 $ 9.00/0

T. gondii oocysts intra-peritoneally in zebrafish resulted in the multiplication of tachyzoites in various tissues including heart, liver, spleen, brain, and blood vessel, with histological and pathological changes similar to that found in acute toxoplasmosis in mammals (Sanders *et al*., 2015). *T. gondii* parasite was detected molecularly at various rates (0.08% - 21.7%) in naturally exposed edible freshwater fish (Zhang *et al*., 2014; Aakool and Abidali, 2016) and marine fish (Marino *et al*., 2019) worldwide. In positive fish samples, *T. gondi* DNA was detected in GIT content, gills, and skin-muscle complex (Zhang *et al*., 2014; Aakool and Abidali, 2016; Marino *et al*., 2019). Moreover, Massie *et al*. (2010) confirmed the viability and infectivity of *T. gondii* oocysts in fish GIT content for up to 8 h by bioassay. All these aforementioned studies did not clarify whether fish can be infected naturally by *T. gondii* or they just act as mechanical carriers. In both cases, fish might be an under-reported source of human infection.

In Egypt, the high infection rate of *T. gondii* in cats (Al-Kappany *et al*., 2010; Elmonir *et al*., 2017) and the insufficient control of stray cats that can easily access agriculture fields may contribute to the high environmental contamination level of *T. gondii*. Furthermore, most of the physicians and researchers in Egypt focus on direct contact with cats and consumption of undercooked meat of infected animals as the possible routes of *T. gondii* infection. Hence, the role of vegetables, fruits, and fish in transmitting this disease still remains neglected in Egypt. Therefore, this study aimed to assess the role of vegetables, fruits, and fish as neglected vehicles of *T. gondii* transmission in Egypt, and to compare the efficiency of the identification methods of *T. gondii* oocysts in these vehicles.

MATERIALS AND METHODS

Vegetables and fruits

A total of 200 vegetable and fruit samples (50 strawberries, 50 carrots, 50 radishes, and 50 lettuces) were collected from farms and gardens located in 13 villages distributed in the following thee governorates in the Nile Delta of Egypt: Menoufia (30.52°N 30.99°E; 6 villages), Kafrelsheikh (31.3°N 30.93°E; 4 villages), and Gharbia (30.87°N 31.03°E; 3 villages) (Fig. 1). Stray cats (definite host for *T. gondii*) had free access to all the sampled fields and gardens as noted by the authors. The amount of each sample per type of vegetable or fruit was as follows: 0.5 kg strawberries, 0.5 kg carrots, 15–20 radishes, and 3–5 lettuces. All samples were transported in plastic bags to the laboratory.

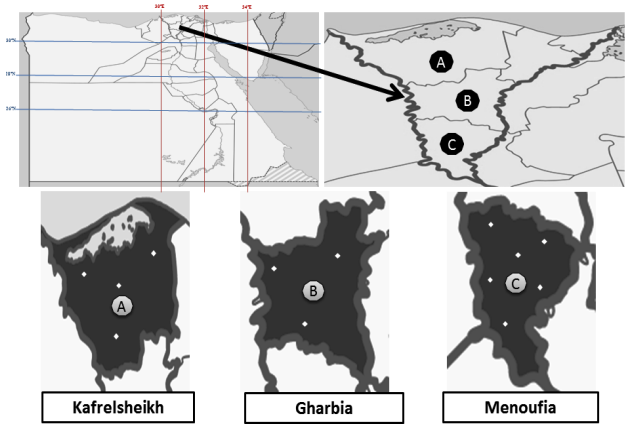

Fig. 1. Map of Egypt showing the sampling locations in the thee studied governorates of Nile Delta in Egypt. Kafrelsheikh governorate (Elhadadi , Bela, Sakha and Kafrmoger), Gharbia Governorate (Alhyatem, Kransho and, Fesha Sleem), and Menoufia Governorate (Tala-Babel, Berket Elsaba, Sheben Elkom, Menof-Tamalai, Elbagour-Kafr Shobra, and Ashmon -Sentres). White dots show sampling sites per each governorate.

Fish

A total of 315 fresh fish: 115 *Oreochomis niloticus* (Tilapia) and 200 *Mugil cephalus* (Mullet) were collected from fishermen at Lake Burullus, at Kafrelsheikh governorate (30°22′–31°35′ N; 30°33′–31°08′ E). Fish samples were transferred in ice box for same-day analysis at the laboratory.

Microscopic detection of T. gondii *oocyst*

Each of the vegetable and fruit samples (carrot root, strawberries fruits, or leaves of radishes and lettuce) was added to fill a space of 500 ml of a glass vessel. Then, the sample was added to 1l of 1% Tween 80 in a 3-l glass vessel and mixed by shaking for 2 h at 100 rpm. The vegetables or fruits were removed, and the oocysts were recovered using the calcium carbonate flocculation method as described by Lass *et al*. (2012). The concentrated oocyst pellet was purified by the sucrose flotation (Villena *et al*., 2004). Briefly, the oocyst pellet was mixed at a 1:3 ratio in a sucrose suspension (1.15 specific gravity), and the suspension was centrifuged at 4°C for 10 min (1250 ×*g*). Then, 2 ml of the supernatant was transferred to 8 ml of distilled water, and centrifugation was repeated. The resulting supernatant was discarded, and the final purified oocyst pellet was separated into two parts; one part was used for microscopic examination, and the other was used for genomic DNA extraction.

For fish samples, a smear of intestinal content of each sample was examined microscopically at 100× and 400×

magnifications, and *T. gondii*-like oocysts (9–12 µm in size) were recorded.

DNA extraction from T. gondii

For vegetables and fruits, *T. gondii* oocyst pellet (as mentioned in method section 2.2) was lysed by five freezing (−80°C) thawing (65°C) cycles, followed by proteinase K digestion at 60°C for 1 h. DNA extraction was conducted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

For fish, genomic DNA was extracted from fish muscles using the DNeasy Blood and Tissue Kit and from the intestinal content using the QIAamp DNA Stool Mini Kit (Qiagen). The extraction methods followed the manufacturers' protocol with exception of five freezing-thawing cycles, and *proteinase* K digestion for 1 h. at 60°C, as for vegetables and fruits.

Real-time PCR identification of T. gondii *DNA*

Real-time PCR identification of *T. gondii* was conducted using Toxo-F/Toxo-R primers (Table I) targeting the 98-bp fragment of the B1 gene of *T. gondii* RH strain (Lin *et al*., 2000). The amplification reaction mixture consisted of 10 µl SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Foster City, California), 1 µl of each primer (500 nM), 5 µl of DNA template (50–100 ng) in a final volume of 20-µl. Amplification was started by polymerase activation at 95°C for 10 min, succeeded by 40 cycles of (95°C for 15 s and 60°C) for 1 min in the iQ5 thermocycler (Bio-Rad, Foster City, California). Both positive control (*T. gondii* RH strain genomic DNA) and negative control (sterile distilled water) were used to confirm the efficiency of the reaction and the absence of contamination, respectively. The cycle theshold (Ct) value was determined, and samples that showed Ct values <40 were considered to be positive. All negative samples were reexamined for possible PCR inhibitors by a mixture of 2 µl of sample DNA and 1 µl of *T. gondii* control DNA as a template for the reaction (Lass *et al*., 2012). If inhibitors were present, the PCR was repeated with the addition of 8 µg of bovine serum albumin (Boehinger, Mannheim, Germany) to the amplification mixture (Villena *et al*., 2004).

Standard PCR detection of T. gondii *DNA*

PCR identification of *T. gondii* was conducted using the primers pairs TOXO B22/ TOXO B23 (Table I) that target a 115-bp of the B1 gene of *T. gondii* RH strain (Basso *et al*., 2007). The 25 µL PCR mixture consisted of 5 µL of DNA template (~50 ng), 12.5 µL of Emerald Amp MAX PCR Master Mix (Takara Bio, Kusatsu, Japan), 1 µL of each primer (20 pmol), and 5.5 µL sterile distilled water. The PCR cycling begins with 94°C for 7 min, succeeded by 35 cycles of (94°C for 1 min, 60°C for 1 min, and 72°C for 1 min), and a final extension for 10 min at 72°C. The PCR cycling was conducted in an Applied Biosystem thermal cycler (Applied Biosystems, Foster City, USA). The distilled water and DNA of *T. gondii* (RH strain) were used as negative control and positive control, respectively.

Statistical analysis

The univariate logistic regression was conducted using SPSS statistics software version 21.0. (IBM SPSS Inc., Armonk, NY, USA).

RESULTS AND DISCUSSION

The overall prevalence of *T. gondii* oocysts in vegetables and fruits samples was 9% (Table II). This result was consistent with the prevalence of 9.7% reported in Poland (Lass *et al*., 2012), but it was higher than other reports in Canada (0.26%) and China (3.6%) by Lalonde and Gajadhar (2016) and Lass *et al*. (2019), respectively. Cats commonly access agricultural fields and bury their feces in the soil, which may easily spread *T. gondii* oocysts to agricultural produce. Afonso *et al*. (2007) recorded that *T. gondii* oocysts were concentrated in the soil near cat defecation sites, and hence a greater number of oocysts are deposited in the agriculture fields (e.g., vegetables and fruits) where cats can easily access.

Carrots were the most contaminated fresh produce (14%), whereas lettuces were the least contaminated (4%), however these differences were not significant (Table III;

Table I. The primer used for detection of *Toxoplasma* DNA in different samples.

Methods	Primers	Primer sequence (5' – 3')	Product size
Real time PCR	TOXO-F/ TOXO-R	TCCCCTCTGCTGGCGAAAAGT	98 bp
		AGCGTT CGTGGTCAACTATCGATTG	
Standard PCR	TOXO B22/ TOXO B23	AACGGGCGAGTAGCACCTGAGGAGA	115 bp
		TGGGTCTACGTCGATGGCATGACAAC	

*The primers were purchased from Metabion (Metabion, Steinkirchen, Germany).

Table II. Frequency distribution of *T. gondii* oocysts in vegetables, fruits, and fish samples in this study.

Location*	Category	Vegetables and fruits					Fish
		Carrot	Radishes	Lettuce	Strawberry	Subtotal	
Kafrelsheikh	Sample No.	16	15	15	12	58	315
	Positive (%)	3 (18.7)	1 (6.7)	0 (0)	1 (8.3)	5 (8.6)	0 (0)
Menoufia	Sample No.	18	19	22	38	97	-
	Positive (%)	1 (5.5)	3 (15.8)	1 (4.5)	2 (5.3)	7 (7.2)	-
Gharbia	Sample No.	16	16	13	-	45	-
	Positive (%)	3 (18.7)	2 (6.2)	1 (7.7)	-	6 (13.3)	-
Total	Sample No.	50	50	50	50	200	315
	Positive (%)	7 (14)	6 (12)	2 (4)	3 (6)	18 (9)	0 (0)

*There was no significant difference between prevalence of *T. gondii* in different governorates at *P* value <0.05.

P value > 0.05). This may highlight that the prevalence rate of *T. gondii* in vegetables and fruits is more associate with rate of soil contamination by oocysts rather than type of fresh produce. Higher odds for isolation of carrots (3.9 times compared to lettuce) may be attributed to the fact that carrots are buried under the ground with a higher probability of soil contamination or due to the nature of the carrot roots that have hair-like structures that have been reported to trap *Toxoplasma* oocysts (Kniel *et al*., 2002).

Table III. Univariate logistic regression for association between samples type and positive molecular status of *T. gondii* oocysts.

Sample type	Percent	Odds	P-value	CI 95%
Lettuce	4	-	-	-
Carrots	14	3.9	0.1	0.8 - 19.8
Radishes	12	3.3	0.2	0.6 - 17.1
Strawberries	6	2.1	0.6	0.2 - 9.6

*Significant difference at *P* < 0.05; CI: Confidence Interval; Brackets: Percent.

A previous study in Egypt showed that around 12% of residents do not wash vegetables or fruits before consumption (Hamed and Mohammed, 2019). Consuming untreated vegetables and fruits has been reported to cause outbreaks of *T. gondii* illnesses elsewhere (Ekman *et al*., 2012) and might also contribute to several cases of asymptomatic infections (Shapiro *et al*., 2019). Hence, our findings may emphasize the public health risk linked to the ingestion of untreated vegetables and fruits in Egypt.

None of the fish samples (intestinal content and muscle samples) showed evidence of *T. gondii* infection (Table II, Fig. 2) by all diagnostic tests used in this study. In contrast, other reports detected *T. gondii* DNA in fish at variable rates (0.08% - 21.7%) in China (Zhang *et al*., 2014), Iraq (Aakool and Abidali, 2016), and Italy (Marino *et al*., 2019). This finding highlights the minimum role of fish in the epidemiology of toxoplasmosis in this study region.

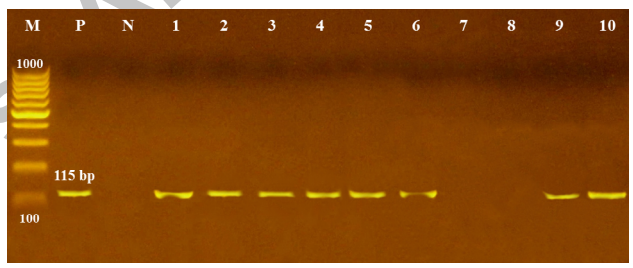

Fig. 2. Standard PCR detection of *T. gondii* DNA in various samples in this study. M, DNA marker. P, Positive control. N, Negative control. Lanes 1 and 2, Carrot samples. Lanes 3 and 4, Radishes samples. Lanes 5 and 6, lettuce samples. Lanes 7 and 8, fish samples (Tilapia and Mullet). Lanes 9 and 10, Strawberries samples.

Regarding the comparison of the microscopic and molecular detection methods in our study, the results revealed that two of vegetables/fruits samples were misdiagnosed and one sample was not detected by the microscopy examination (Table IV). This finding is in agreement with previous reports (Dumètre and Dardé, 2003; Shapiro *et al*., 2019) and highlights the importance of using molecular techniques in the identification of *T. gondii* oocysts in environmental samples.

CONCLUSION

In conclusion, this study highlighted that vegetables and fruits may play a role in transmitting toxoplasmosis in Egypt. To our knowledge, this is the first molecular identification of *T. gondii* oocysts in vegetables, and fruits in Egypt.

Table IV. Comparison between different diagnostic tests of *T. gondii* oocysts in vegetables, fruits, and fish samples in this study.

Type of sample	Positives/total samples (%)			Variance in results/ Method
	Microscopic detection	Standard PCR detection	Real-time PCR detection	
Vegetables/ Fruits				
Carrot	8/50 (16)	7/50 (14)	7/50 (14)	One NC-PCR
Radishes	5/50 (10)	6/50 (12)	6/50 (12)	One ND-Microscope
Lettuce	2/50 (4)	2/50 (4)	2/50 (4)	No difference
Strawberries	4/50 (8)	3/50 (6)	3/50 (6)	One NC-PCR
Subtotal	19/200 (9.5)	18/200 (9)	18/200 (9)	Two NC-PCR / One ND-Microscope
Fish				
Mullet	0/200	0/200	0/200	No difference
Tilapia	0/115	0/115	0/115	No difference
Subtotal	0/315	0/315	0/315	No difference
Total	19/515 (3.7)	18/515 (3.5)	18/515 (3.5)	Two NC-PCR / One ND-Microscope

*NC-PCR, not confirmed by PCR; ND-Microscope, not detected by microscope.

It is important to inform the public and health professionals about the newly discovered sources of infection and the possible new methods of *T. gondii* transmission to humans in Egypt.

ACKNOWLEDGEMENT

We would like to thank Dr. Nagwa Elhawary, Parasitology Department, Veterinary Medicine, Kafrelsheikh University, Egypt for technical support.

Funding

This research was financially funded by the Science and Technology Development Fund (STDF), Egypt, (Project no.: 5354).

IRB approval

The protocol of this study was approved by the institutional Animal welfare, Hygiene and Zoonoses committee at Kafrelsheikh University, Egypt (KFS-2018/6).

Ethical statement

The consents were obtained from the owners of vegetables farms and fishermen for the participation in this study and the publication of any relevant data.

Statement of conflict of interest

The authors have declared no conflict of interests.

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