Archives of Razi Institute, Vol. 78, No. 3 (2023) 1029-1034

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DOI: 10.22092/ARI.2022.360398.2579

Original Article

New Recording of *Toxoplasma gondii* in Wild Tortoise *Testudo graeca* Using Nested PCR Method

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Received 25 October 2022; Accepted 24 November 2022
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Abstract

Toxoplasmosis is one of the most widespread zoonotic diseases, especially in warm and humid areas, and affects all mammals, including humans and many herbivores and carnivores. The present study investigated the *Toxoplasma gondii* (*T. gondii*) parasite in tortoises for the first time in Iraq using PCR technology. A total of 28 tortoises/*Testudo graeca* (*T. graeca*) were collected between October 2018 and March 2019 from the study stations and then sent to the Animal House, which belongs to the Department of Biology, Faculty of Education, University of AL-Qadisiyah, Iraq, to perform the dissection. The body cavity was opened, and all organs were removed. The tortoises' liver, heart, and brain were removed and kept at -20ºC until use. Afterward, the samples were subjected to DNA extraction. The Nested-PCR technique was implemented using two pairs of primers, and then the PCR products were analyzed using 1.5% agarose gel electrophoresis. The amplification of the gene during the first cycle indicated that 10 samples gave positive results with a total percentage of (11.9%), including five liver samples, three heart samples, and two brain samples (17.85%, 10.71%, and 7.14%, respectively). On the other hand, during the second cycle of the reaction, the amplification of the gene was obtained in seven samples (8.33%). The highest percentage of the presence of the gene was recorded in the tortoises' liver (14.28%) and the lowest in their brain (3.57%). This study is among the first to investigate the molecular detection of *T. gondii* in wild tortoises (*T. graeca*) in Iraq. The findings imply that tortoises have a role in transmitting *T. gondii* and are believed to acquire infection by feeding on small invertebrate animals or plants contaminated with the oocysts of the parasite.

Keywords: Iraq, Nested-PCR, *Testudo graeca*, Tortoise

1. Introduction

One of the most prominent obligate intercellular and most common opportunistic parasites is *Toxoplasma gondii* (*T. gondii*). It can cause infection in all warm-blooded animals, leading to Toxoplasmosis, one of the most prevalent diseases recorded globally in humans and animals, such as sheep, goats, horses, birds, wolves, whales, dolphins, sea dogs, and snakes (1-3).

The parasite's life cycle is summarized in three different forms: 1) tachyzoite, in which it invades the macrophage and is found during an acute infection, 2) bradyzoite, which occurs during a chronic infection within the tissue infection, and 3) sporozoite, which exists in the oocysts (4). Felidae is the parasite's final host, while the rest are its intermediate hosts (5).

Testudo graeca (*T. graeca*) is a tortoise found in the Arab World, Middle East, as well as South and Eastern Europe, which lives in diverse natural environments, such as forests and deserts. It feeds on plants, leaves, seeds, fruits, and some small invertebrate animals,

including insects and arthropods. It is a cold-blooded animal whose body adapts to the heat of the environment it exists in, and its skin covers horny scales (6-9).

Because of previous studies on *T. gondii* in many hosts, this study was conducted to investigate this parasite in tortoises in Iraq for the first time.

2. Materials and Methods

2.1. Sampling

A total of 28 tortoises/*T. graeca* were collected between October 2018 and March 2019 from the study stations and then sent to the Animal Houses belonging to the Department of Biology, Faculty of Education, University of AL-Qadisiyah, Iraq, to perform the dissection. The body cavity was opened, and all organs were removed. Tortoises' liver, heart, and brain were removed and kept at -20ºC until use.

2.2. DNA Extraction from Tissues

The extraction of the DNA from the tortoises' tissues, which included the liver, heart, and brain, followed the method attached to the test kit (Anatolia Company, Turkey). A total of 10 mg of each tissue was taken and crushed by a Micro Pestle and then transferred to the Eppendorf tubes (1.5 ml). Afterward, 400 µl of lysis buffer and 20 µl of proteinase K were added and mixed by a vortex. The mixture was then put in an incubator for 60 min at 56ºC. A total of 400 µl of binding buffer was then added and mixed by the vortex for 15 sec, and the mixture was centrifuged at 12.500 rpm for 1 min. The mixture was then transferred to the spin column and centrifuged at 12.500 rpm for 2 min. Afterward, 500 µl of buffer W3 was added to the spin column and centrifuged at 12.500 rpm for 2 min, and the filtered liquid was eliminated. Subsequently, 500 µl of buffer W3 was added to the spin column and centrifuged at 12.500 rpm for 2 min, and then the spin was placed in a column in an Eppendorf tube (1.5 ml). After that, 50 µl of buffer EL4 was added to the spin column, and the tubes were left fixed for 5 min to ensure the filter absorbed the solution.

Moreover, the centrifuge was at 12.500 rpm for 2 min. The DNA was kept at -20ºC. The purity and concentration of the DNA were measured using a Nanodrop spectrophotometer. The PCR output was analyzed using agarose gel electrophoresis (1.5%). A mock control (DNA extracted from the brain of the inbreed BALB/c mice, which serologically confirmed that they were harmful to Toxoplasmosis) was isolated simultaneously with the samples as the negative control for the confirmation of the procedure accuracy. The sensitivity and specificity of the Nested-PCR (N-PCR) test were calculated based on the obtained results as the percentage of positive and negative samples, according to Al-Khalidy (10).

2.3. Nested-PCR

Two pairs of primers were designed based on Khan, Su (11), as shown in table 1. The N-PCR interaction included two cycles. In the first round, an interaction occurred to amplify the 340 bp segment of the *GRA6* gene of *T. gondii*. The size of the reaction mixture in this cycle was 20 µl, and the components indicated in table 2 were mixed by a vortex and placed in the PCR thermal cycler, as shown in table 3.

In the second round, the primer 310 pb of the *GRA6* gene was amplified, in which 1 µl of positive sample output for the first reaction was taken as the template for the second-round reaction. The components and the size of the reaction mixture in the second cycle are shown in table 2. In this step, the inner primers were used, and the samples were placed in the thermal cycler.

Table 1. Nucleotide sequence of nitrogenous bases for Primers and N-PCR product size

Primers		DNA Sequence	Product size (bp)
Outer	F	5-ATTTGTGTTTCCGAGCAGGT-3	340
	R	5-GCACCTTCGCTTGTGGTT-3	
Inner	F	5-TTTCCGAGCAGGTGACCT-3	310
	R	5-TCGCCGAAGAGTTGACATAG-3	

Table 2. The components reaction mixture of N-PCR (1st and 2nd cycle)

N-PCR master mix(1st round reaction)			N-PCR master mix(2st round reaction)		
DNA(template)		5µl	DNA (template)		1µl
master mixes		10µl	master mixes		10µl
Nested-PCR	F. Primer	1µl	Nested-PCR	F. Primer	1µl
	R. Primer	1µl		R. Primer	1µl
	PCR water	3µl		PCR water	7µl
Total volume	20µl		Total volume	20µl	

Table 3. N-PCR program to amplify the GRA6 gene using a thermal cycler (1st and 2nd cycle)

Steps	Temperature (°c)	Time (sec.)	Number of cycles
Initial denaturation	94	300	1
Denaturation	94	45	35
Annealing	58	45	
Extension	72	60	
Final extension	72	600	1

2.4. Statistical Analysis

The data were analyzed using the SPSS software (version 10.5), and the Chi-Squared test was used to determine the significant differences under the significance level of $P \leq 0.05$.

3. Results

To amplify the *GRA6* gene of the *T. gondii*, 84 samples were selected from the distributed tissue according to the organ type using the N-PCR.

The results of the first round showed 10 samples (11.9%) in which the *GRA6* (340 bp) gene was amplified (Figure 1), including five liver samples (17.85%), three heart samples (10.71%), and two brain samples (7.14%). In the second round, there was an amplification of the *GRA6* (310 bp) gene in seven samples (8.33%) (Figure 2), which included four samples of the liver (14.28%), two samples of the heart (7.14%), and one sample of the brain (3.57%). The sensitivity and specificity of the N-PCR were also confirmed (70 and 96%), respectively.

The results showed significant differences in the amplification rate of the *GRA6* gene among the organs ($P \leq 0.05$), as shown in table 4.

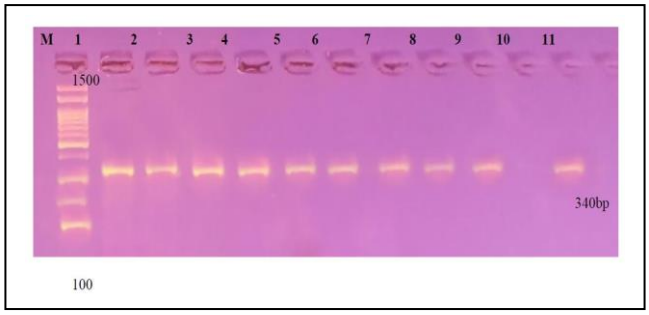

Figure 1. Amplified GRA6 gene electrophoresis in N-PCR (1st cycle), 1-5 represent liver samples, 6-8 represent heart samples, 9 & 11 represent brain samples, and column M represents a Ladder bearing molecular weight 100-1500

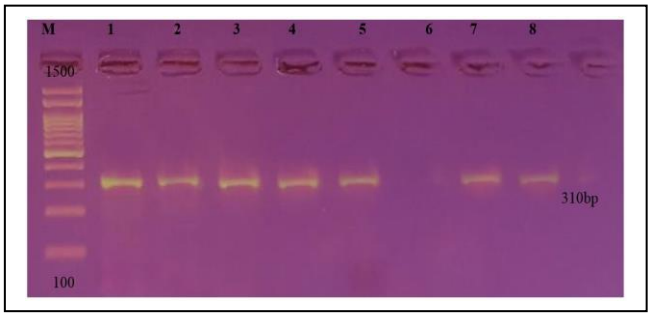

Figure 2. Amplified GRA6 gene electrophoresis in N-PCR (2nd cycle), 1-4 represent liver samples, 5 & 7 represent heart, 8 represent brain sample, and column M represents a Ladder bearing molecular weight 100-1500

Table 4. Numbers and percentages of GRA6 gene amplification using N-PCR (1st and 2nd cycle)

Name of the infected organ	Samples examined number	Positive tissue samples number of N-PCR (first cycle)	Positive tissue samples number of N-PCR (second cycle)
liver	28	5 (17.85)	4 (14.28)
heart	28	3 (10.71)	2 (7.14)
brain	28	2 (7.14)	1 (3.57)
total	84	10 (11.9)	7 (8.33)
Sensitivity		%70	
specificity		%96	

4. Discussion

The N-PCR is used when diagnosing *T. gondii* is challenging by other diagnostic methods. This method is also employed to investigate *T. gondii* in tissue samples containing a few DNA copies of the target, distinguish them from the host DNA, and identify protozoa, such as *T. gondii* (12).

The PCR technique targets several genes specific to the *T. gondii*, including *B1*, *Sag1*, *fo1*, *b10*, *tub2*, *tub1*, *hsp70*, *gra4*, and *sag3* (13).

The molecular diagnosis has a high sensitivity of up to 97.4% and a specificity of 100% (14). This technology can detect *T. gondii* in the body. It can also detect less than 10 tachyzoites as it detects living and dead parasites (15). This technique is an alternative to serological tests. It reveals the DNA of the parasite in the serum and various body fluids, such as blood, amniotic fluid, cerebrospinal fluid, vesicular cystic fluid, urine, as well as aqueous and vitreous fluid in children with congenital infection (16). The results of the N-PCR for the amplification of the *GRA6* (340bp) gene in tortoises' tissue samples showed that in 10 out of the 84 samples, the gene was amplified (11.9%) during the first round of the reaction, which included five liver samples, three heart samples, and two samples of the brain. In the second reaction cycle, the *GRA6* (310 bp) gene was amplified in seven samples (8.33%). It was noted that the lowest percentage of amplification occurred in the brain samples (3.57%), compared to the liver and heart samples. This study is consistent with the findings of previous studies (17), indicating that seven species of reptiles, including tortoises, were infected with *T. gondii*.

The amplification rate of the *GRA6* gene in the liver, heart, and brain in this study was lower than the amplification rate (66%) recorded by (18-21) in red-footed tortoises (*Chelonoidis carbonaria*) and that (80.88%) recorded by Nasiri, Teymurzadeh (3) in snakes using the same gene, as well as its proximity to the amplification ratio (16.66%) recorded by Anah and Al-Mayali (1) in wild and aquatic snakes. The difference in ratios can be due to the difference in the hosts used in the study, the nature of nutrition, the number of samples and organs used, in addition to the diagnostic methods used. Another reason is that *T. graeca* does not prey on vertebrates in the bradyzoite phase, but it does consume small invertebrate animals, such as insects and arthropods, or plants, such as leaves, seeds, and fruits, which may be contaminated with the oocysts of *T. gondii*. The reason for the lack of amplification of the *GRA6* gene in some samples during the second cycle of the interaction can be interpreted on the basis that these samples may contain other parasitic organisms similar to *T. gondii*. Therefore, it gave a positive result during the first cycle of the interaction but not in the second one. The outer pair of primers link to the location of the template tape. This site may share a group of organisms that may belong to the same family or the same genus, whereas the inner pair of primers is associated with the location of the template that is of high specificity to species or subspecies, which is consistent with what was pointed out by (22, 23).

Tortoises are linked to the transmission of *T. gondii* and acquire the infection by feeding on small

invertebrates or plants contaminated with *T. gondii* oocysts.

Authors' Contribution

A. A. Saad contributed to collecting the samples and statistically analyzing the data. A. A. Sadiya contributed to the implementation of the N-PCR. All authors discussed the results, commented on the manuscript, and gave the final approval for the final version to be submitted.

Ethics

All procedures were conducted in accordance with the ethical standards of the institution at which the studies were conducted. The current study was approved by the Ethics Committee of the Department of Biology, Faculty of Education, University of AL-Qadisiyah, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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