Genotyping and Phylogenic tree of Toxoplasma gondii in one-humped camels (Camelus dromedarius) in Al- Diwaniyah province in Iraq

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KEYWORDS
Genotyping and Phylogenic tree, one-humped camels

ABSTRACT
The current study is carry out in Al- Diwaniyah province; in Iraq between (September 2023 - March 2024) for determine the seroprevalence of Toxoplasma gondii in camels and molecular identification with phylogenetic analysis. (140) blood and tissue samples were collected from camels from the slaughter of the Al-Diwaniyah province. The results of total serological prevalence showed that 72 (51.42%) of the camels were infected. Genomic DNA was extracted from 140 camel's tissues samples and SAG3 gene was amplified by PCR. PCR results showed that camels tissues samples 55/140 (39.28 %) have SAG3 gene. Ten PCR positive products of local Toxoplasma gondii isolates were DNA sequencing and compared with other isolates in GenBank. After that, the phylogenetic relationship was analyzed for this parasite using phylogenetic UPGMA tree (MEGA X version). Sequencing and phylogenetic results of Toxoplasma gondii isolate (No.1, 3, 5, 6, and 10) were showed closed genetic related into Toxoplasma gondii genotype I. The camels T. gondii isolates (No.7) were showed closed genetic related into Toxoplasma gondii genotype II. Whereas, the camels T. gondii (No.2, 4, 8, and 9) were showed closed genetic related into Toxoplasma gondii genotype III. At total genetic change (0.01). The homology sequence identity between local The Toxoplasma gondii isolates and NCBI-BLAST related genotype of T. gondii were reveals that genetic homology sequence identity was ranged (99.32% - 100%).

1. Introduction

One of the most major zoonotic illnesses, toxoplasmosis is caused by the coccidian parasite *T. gondii* and is a highly contagious disease that affects almost all animals,human and birds worldwide (1).

Toxoplasma is a common global infection that is a member of the phylum Apicomplexa, which was identified by (2) (3, 4). Animals, including humans, may get toxoplasmosis via tachyzoites that enter the fetus via the placenta or by ingesting contaminated foods and liquids, such as raw milk, undercooked meat, and cheese (1).

Many diseases, including toxoplasmosis, may afflict camels. Toxoplasma infection is obtained by ingestion of sporulated oocysts shed by wild felids or cats in the surrounding area. Due to its frequent consumption, camel meat is very sensitive to toxo-plasmosis exposure, which might potentially infect consumers. Due to the ongoing pasture contamination by oocysts of T. gondii, the parasite are spread among the farm (4,1). Geographical factors mostly determine the global frequency of infection with Toxoplasma in camels (5).

According to reports, toxoplasmosis is very prevalent in camels in Iraq (6, 7, 8), with varying infection rates between 1998, 2006, and 2012 (6.04%, 16.35%, and 20.34%, respectively).

Toxoplasmosis can cause abortion or neonatal mortality in animal species, such as sheep, goats, horses, camels and deer (9).

Since the clinical indicators are not precise enough to diagnose the illness, further measures must be used to show antibodies or the organism (10). Toxoplasmosis was detect by many old techniques such as cell culture, genetic approaches, bioassays, and serological detection of IgG and IgM using the enzyme-linked immunosorbent assay (ELISA) and latex agglutination test (11). IgG antibodies indicate the history of prior infection when they are found in the blood of ill camels. IgM in the acute phase. Toxoplasma may be detected using one of the most basic methods, which is serological testing. Traditional *T. gondii* diagnostic techniques include the dye test, indirect immunofluorescence antibody test, and direct and indirect haemagglutination test; however, these approaches are not able to identify current infections. The IELISA test was created to estimate IgM antibody levels in relation to IgG antibody levels in order to identify recent infections (12).

The PCR method allows for quicker and more accurate parasite identification. (13).This research focused on identifying alternative techniques to enhance the identification of these parasites since there were few prior conventional studies conducted for the diagnosis of *T. gondii* in camels. These include molecular-based methods and immunoassays for early parasite identification in the subclinical stage.

2. Methodology

Collection of samples

Collection of blood samples

(5 ml) of 140 blood samples were taken from jugular vein of the camel. Each blood sample was put in gel tube with information include date, age and sex, then transferred in cold box to the laboratory for preparation of the sera.

Collection of tissue samples

140 tissue samples were taken from the same camels; approximately, 250 gm of each organ (diaphragm, heart and uterus) were collected from each slaughtered camel, and put individual plastic labeled containers for molecular test that kept frozen until DNA extraction.

Serum preparation and ELISA test:

The blood samples were separated by centrifuge at 3000 rpm for ten minutes to produce the sera. The serum was then carefully pipetted into dry, sterile, and labelled test tubes, and it was kept at -20ºC until it was needed for the serological test. To identify anti-*T. gondii* IgG antibodies in serum samples, an indirect ELISA kit was bought from MyBioSource Company in the United States.

Molecular study:

For polymerase chain reaction, *Sag3* gene primer that used in DNA sequencing method for genotyping of *Toxoplasma gondii* were designed according to (14). Providing of the primers are done by (Scientific Researcher. Co. Ltd. Iraq) table (1). Genomic DNA was extracted from Camel tissue samples by using gSYNCTM DNA Extraction Kit and done according to company instruction. The DNA that was extracted was examined using a device known as a Nanodrop spectrophotometer (THERMO, which was manufactured in the United States). By analysing the absorbance at 260 and 280 nanometers, this instrument determines the concentration of DNA as well as its purity status. As shown in table (2), this method was carried out in accordance with the instructions provided by the firm. Afterwards, agarose gel electrophoresis was used in order to examine the PCR results.

Table (1): Gene, primer, sequence and PCR product size.

Primers	**Sequence (5'-3')**		**Amplicon**
SAG3 gene	F	ATGCAGCTGTGGCGGCGCAG	1158bp
	R	TTAGGCAGCCACATGCACAAG	

Table(2): PCR conditions (temp, time and cycle number)

PCR step	**Temp.**	**Time/min**	**cycle**
Initial Denaturation	**95**	**5**	**1**
Denaturation	**95**	**Half min**	**35**

Annealing	**55**	**Half min**	
Extension	**72**	**2**	
Final extension	**72**	**5**	**1**
Hold	**4C**	**Foreve**	**-**

DNA sequence method:

The discovery of genetic variation between Camel *Toxoplasma gondii* isolates and Standard NCBI reported *SAG3 Toxoplasma gondii* isolates was studied using DNA sequencing. The PCR *SAG3* gene-positive results were sent in an ice bag by DHL to the Macrogen Company in Korea, where an AB DNA sequencing machine was used to do the DNA sequencing. Molecular Evolutionary Genetics Analysis (Mega 6.0) was used to conduct the DNA sequencing analysis. The evolutionary distances were determined by using the Maximum Composite Likelihood approach by phylogenetic tree UPGMA technique, in addition to performing multiple sequence alignment analysis of the incomplete *SAG3* gene based on ClustalW alignment analysis.

3. Results and discussion

Total serological prevalence of toxoplasmosis in camels:

For the first time in Al-Diwaniyah province, our work detects the infection rate of *T. gondii* in camels from the slaughter of the Al-Diwaniyah province using the indirect IgG- ELISA test. One hundred and forty random blood samples were collected and analysis for detection toxoplasmosis using ELISA test. The results revealed that 72 (51.42%) of the camels had positive result.

Results obtained by PCR technique:

A PCR was performed on DNA samples that had high concentrations (4–710 ng/µl) and purity levels (1.6–1.8). According to the PCR technique's overall findings, 55 (or 37.28%) of the 140 camel tissue samples tested positive for the *SAG3* gene (figure 1).

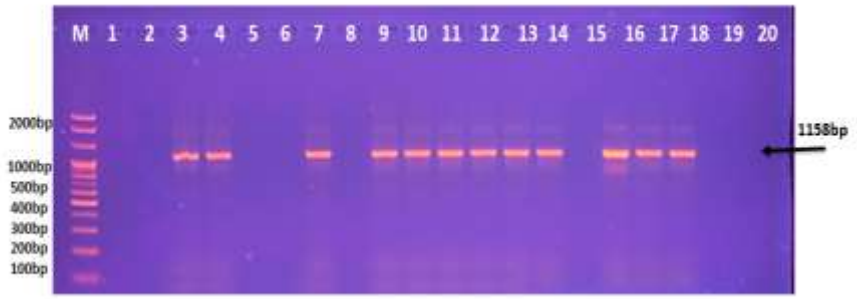

Figure (1): electrophoresis of the PCR product of *SAG3* in *T. gondii* of camels isolates. M: marker (2000-100bp) the bands mean positive *T. gondii* at (1158bp)

Result of DNA sequencing and phylogenetic tree construction.

The DNA sequencing method was carried out for genotyping of Camels *Toxoplasma gondii* based on surface protein *SAG3* gene. The local sequence isolates was alignment with NCBI-Blast related *Toxoplasma gondii* genotypes. The multiple sequence alignment analysis of surface antigen (*SAG3*) gene complete sequence was NCBI-Genbank *T. gondii* genotypes based ClustalW alignment analysis by (MEGA X). The nucleotide sequences of the surface antigen gene revealed substitution mutations and nucleotide similarities according to multiple alignment analysis. The Maximum Composite Likelihood technique via phylogenetic UPGMA tree (MEGA X version) was used to calculate the evolutionary distances. *Toxoplasma gondii* isolates (No.1, 3, 5, 6, and 10) were showed closed genetic related into *Toxoplasma gondii* genotype I. The camels *T. gondii* isolates (No.7) were showed closed genetic related into *Toxoplasma gondii* genotype II. Whereas, the camels *T. gondii* (No.2, 4, 8, and 9) were reveals closed genetic related into *Toxoplasma gondii* genotype III. At total

genetic change (0.01). The similarity sequence correspondence between local The NCBI-BLAST connection and *T. gondii* isolates Table (3) and Figures (2, 3) demonstrate the genetic homology sequence identity range of 99.32% - 100% for the *T. gondii* genotype.

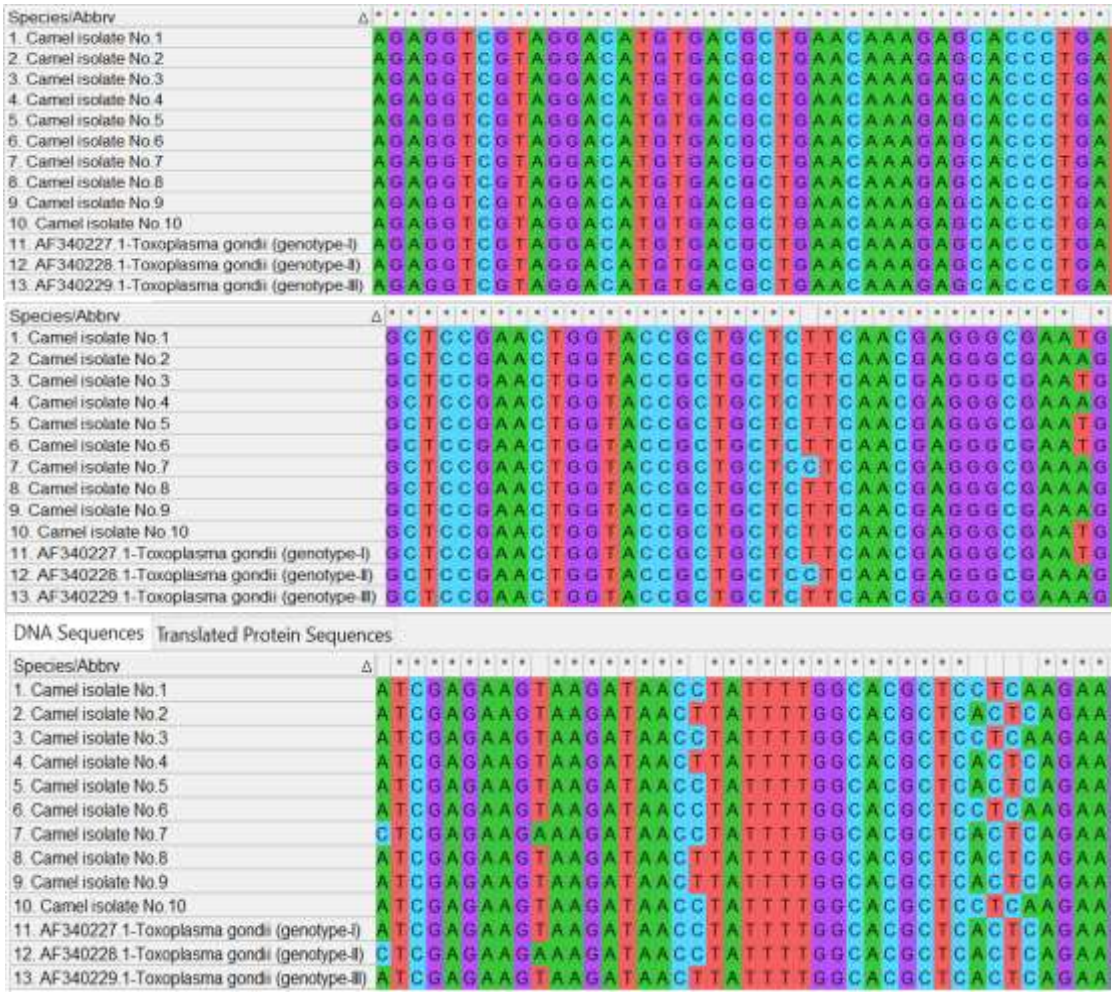

Figure (2): The surface antigen gene full sequence in camels *T. gondii* was subjected to multiple sequence alignment utilising ClustalW alignment analysis based on NCBI-Genbank *T. gondii* genotypes using MEGA X. The surface antigen (*SAG3*) gene sequences' similarity and substitution mutation were shown by the multiple alignment analysis.

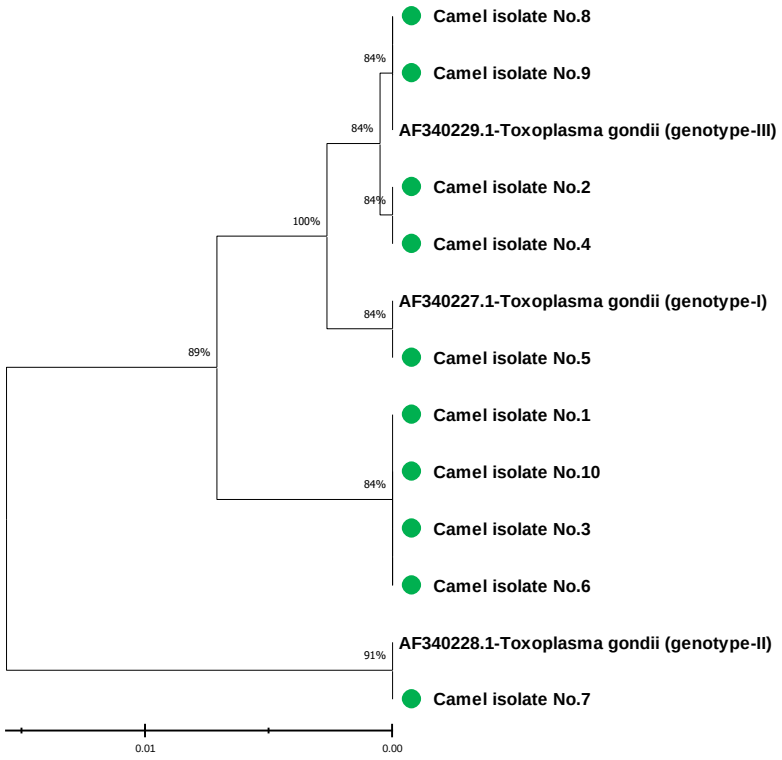

Figure (3): Phylogenetic tree based on *T. gondii* surface antigen sequence (*SAG3*) gene, which was utilised for the genotyping analysis of the camels. Using a phylogenetic UPGMA tree and the Maximum Composite Likelihood approach, the evolutionary distances were calculated (MEGA X). It was discovered that the camel *T. gondii* isolates (No. 1, 3, 5, 6, and 10) were closely related to *T. gondii* genotype I. *T. gondii* isolates from camels (No. 7) were shown to have tight genetic relationships with *T. gondii* genotype II. Conversely, *T. gondii* isolates from camels (No. 2, 4, 8, and 9) demonstrated closed genetic relationships with *T. gondii* genotype III. At the whole genetic shift (0.01).

Table (3): Homology sequence identity between *Toxoplasma gondii* camel and NCBI BLAST *T. gondii* genotypes isolates

Local *Toxoplasma gondii* No.	**accession number**	**Homology sequence identity**		
		Genotypes	**accession number**	**Identity %**
Camel isolate No.1	PP791834	Genotype: I	AF340227.1	100%
Camel isolate No.2	PP791835	Genotype: III	AF340229.1	99.32%
Camel isolate No.3	PP791836	Genotype: I	AF340227.1	99.33%
Camel isolate No.4	PP791837	Genotype: III	AF340229.1	100%
Camel isolate No.5	PP791838	Genotype: I	AF340227.1	99.32%
Camel isolate No.6	PP791839	Genotype: I	AF340227.1	100%
Camel isolate No.7	PP791840	Genotype: II	AF340228.1	100%
Camel isolate No.8	PP791841	Genotype: III	AF340229.1	99.33%
Camel isolate No.9	PP791842	Genotype: III	AF340229.1	100%
Camel isolate No.10	PP791843	Genotype: I	AF340227.1	99.33%

Discussion

Since the ELISA test offers a more focused method for seroepidemiological analysis of *T. gondii* in animals, it was used in the present investigation (15). Even though camels are very important economically in Iraq, people are often unaware of the diseases that may affect them, especially

toxoplasmosis. To the best of our knowledge, this is the first research in Al-Diwaniyah Province looking at the risk factors for infection of *T. gondii*.

In the present study, prevalence of *T. gondii* in 140 bloods revealed high infection rate (51.42%). The high prevalence of *Toxoplasma gondii* of camels in Al-Diwaniya province could be attributed to the degree of environmental contamination with oocysts due to the bad hygiene practices leading to the accumulation of feces, free-ranging behavior, limited veterinary services, carcasses and animal offal rids by local butchers who play an important role in the spread of parasites. Also, contaminated soil is an important source of infections by *Toxoplasma gondii* parasites. The prevalence of camels *Toxoplasma gondii* found in this study was close to that reported in a previous study conducted in the Egypt, with an overall prevalence of (52.5%) out 120 serum sample (16). Nearly similar results were recorded by (17) in Saudi Arabia (45.44%) and (18) in Ethiopia (40.49%) out (451), and that rates was higher than (19) in Egypt and (20) (10% and 8% respectively). Also this study disagree with (21) that showed that 27/180 (15%) camels had sero positive of *T. gondii*. Also, our results disagreed with (22) in Sudan who detect an infection rate (96.9%). Study in AL-Najaf province in Iraq by (23) revealed that 16 (22.8%) serum samples were positive for toxoplasmosis.

The previous research contradicts a study by (24) which indicated that serum *T. gondii* antibodies were present in camels in several Egyptian governorates at a rate of 36.58% out of 108. (25) mentioned that camels eat dry matter contaminated with *T.* oocytes from cat faeces, and that variations in the environment are caused by geographic conditions, management practices, and current stray cats that were introduced into camel farms and subsequently contaminated the environment with oocysts from cat faeces. The discrepancy in Toxoplasma seroprevalence and our investigation was also attributed to variations in the serological assays used for parasite detection, the initial serum dilution, regional variations in climate, the age of the camels under examination, and the incidence of cats on the farms.

Because of their high sensitivity, specificity, speed, usefulness, and ability to identify the parasite's DNA, methods based on PCR used as an accurate tools of *T. gondii* (26) since its inception.For the purpose of providing a more sensitive instrument that enables specific amplification of the *SAG3* gene, the polymerase chain reaction (PCR) methodology has been used to validate the outcomes of serological tests, which are represented by the Eliza test as a diagnostic procedure.

Differentiation of *Toxoplasma gondii* has been accomplished by the use of a variety of molecular techniques that are based on polymerase chain reaction (PCR), including PCR, RFLP, and RT-PCR (15). For the purpose of this investigation, the usual polymerase chain reaction (PCR) method was used to amplify the *SAG3* gene by extracting DNA from camel tissue samples.

The total results of PCR reveals that camel's tissues samples 55/140 (39.28 %) were positive for *SAG3* gene. A previous study conducted in the in Al-Diwaniyah province by (27) was recorded 18/32 (56.25%) infection rate for toxoplasmosis in the tissue samples of camels. Also (28) recorded 13(26%) tissue samples of camels were positive for the *T. gondii* by Nested-PCR. Other study by reported a prevalence rate of Toxoplasmosis in the examined samples 13/65 (20%) using PCR. (24) recorded the rates of *T. gondii* DNA by PCR in camels were 14.63%. PCR have high specificity and sensitivity for toxoplasmosis diagnosis; furthermore, *SAG3* gene having higher copies in genome of *T. gondii* (29). (30) referred that among 122 camel's sample were 6.60% infected with *T. gondii*, It may be explained by the fact that IgG antibodies are created later in the infection and the parasite is localised in the organs and tissues rather than circulating in the blood, as well as the reduced frequency of *T. gondii* DNA found by PCR in contrast to *T. gondii* antibodies. Additionally, it's possible that the tiny sample sizes and the random distribution of cysts in the tissues—one cyst for every 50 to 100 grammes of tissue may be to blame (31).

In this study, The DNA sequencing method was carried out for determine the genotyping of *T. gondii isolates*, based on surface protein *SAG3* gene products. The local camels *Toxoplasma gondii* isolates (No.1, 3, 5, 6, and 10) were showed closed genetic related into *Toxoplasma gondii* genotype I. The

camels *T. gondii* isolates (No.7) were showed closed genetic related into *Toxoplasma gondii* genotype II. Whereas, the camels *T. gondii* (No.2, 4, 8, and 9) were demonstrated closed genetic related into *Toxoplasma gondii* genotype III.

The current study was consistent with those reported by (32) they recorded *T. gondii* type I, II, III in camels. Also (33) detected *T. gondii* type II, III in camels. (34) recorded *T. gondii* (type II, III) in camels, while (35) detected only *T. gondii* II type in camels.

In a study conducted by (36) that involved direct genotyping of *T. gondii* tested in fluid specimens that collected from 18 cases of ocular toxoplasmosis, three samples contained a type I strain, and five new recombinant genotypes were discovered. However, only one strain of type III and three strains of type II were found to be present in the samples. Four isolates from sheep in Iran were reported to include type II and III (37). Furthermore, type I was found in Iranian domestic animals according to previous investigations (38). Animal samples showed a high frequency of genotypes II and III (39).

Research conducted in the United States and Europe has shown that Type II strains are the most often found genotype in both people and animals (40, 41, 42). When (43) in the USA and (44) in Italy looked into *T. gondii* among goats, they discovered clonal Type III *T. gondii*. Type II was the predominant lineage in aborted instances and was responsible for spontaneous abortion, according to findings on the genotypes of parasites circulating in aborted foetuses by (45).While types II and III are comparatively avirulent, type I viruses are quite virulent (46, 47).

Goats from the Northeastern area of Brazil were exposed to two isolates that were introduced as recombinants of Types I/III (48). According to (49) the genotyping of the 33 isolates in sheep and goats showed that 29 (87.88%) were Type II, 3 (9.09%) were Type III, and 1 (3.03%) was unusual. Our findings did reveal, however, that the *T. gondii* genotypes that afflict camels are also responsible for the disease in people, sheep, goats, and cows.

It's likely that certain genotypes can only infect particular hosts, but further research is required to confirm this theory. There isn't much information available about camels. Clinical toxoplasmosis cannot infect these animals (50). Finding the genotypes of *T. gondii* may lead to the development of novel approaches for the diagnosis, treatment, immunisation, prevention, and the parasite control in both humans and animals (40).

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