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Serological and Molecular Identification of *Toxoplasma gondii* of One-humped Camels (*Camelus dromedarius*) in Al- Diwaniyah Province

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Abstract

The aim of this study conducted in Al- Diwaniyah province, in Iraq during the period from September 2023 to March 2024 was to determine seroprevalence of Toxoplasma gondii infection in camels with study the effect of some epidemiological factors such as sex, age and months on the rate of infection using enzyme-linked immunosorbent assay (ELISA) to determine the IgG antibodies against Toxoplasma gondii infection and molecular identification of these parasites.One hundred and forty 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. There was a statistically significant difference ($P<0.05$) in the prevalence between age groups of camels that showed that the positive rate in >5-10 age group was highest (61.53%). Statistical analysis of the data showed that there was no significant difference ($P>0.05$) in overall prevalence of camel's infection between sex. In the present study, significant difference ($P<0.05$) was recorded between infection rate during the months of the study and higher prevalence of infection rate was observed in February (90%) and January (75%), while lowest infection rate was observed in October (15% (While Molecular result was 39.28 % out of 140 camels tissue samples.

Keywords: Toxoplasma gondii, camels, ELISA, PCR, prevalence

Introduction

Toxoplasmosis is one of the most prevalent and important foodborne and waterborne zoonotic infectious parasitic diseases of medical and veterinary importance worldwide (1). It is caused by an obligate apicomplexan intracellular protozoan parasite Toxoplasma gondii that can be life threatening when opportunistic or congenital in both humans and animals. Therefore, its diagnosis requires accurate and rapid techniques that rely mostly on serology and molecular methods (2).

The parasite which is common in both humans and animals (sheep, goats, cattle, camels, horses, birds, mice, rats, and flies, in contrast to humans) act as intermediate hosts, except for feline species, which acts as a definitive and intermediate host, (3). Camels are infected with many different dis¬eases, including toxoplasmosis, and camels ac¬quire Toxoplasma infection by swallowing the sporulated oocysts that wild felids or cats shed in the environment, the prevalence of Toxoplasma infection in camels in the world depends mainly on the location (4).

The apicomplexan zoonotic Toxoplasma gondii has three infective stages: sporozoites in sporulated oocysts, which are shed in unsporulated form into the environment by infected felids; tissue cysts containing bradyzoites, and fast replicating tachyzoites that are responsible for acute toxoplasmosis , toxoplasmosis in animals, including humans, is transmitted by tachyzoites to the fetus through the placenta or by consuming drinks and foods contaminated with tachyzoites and brachyzoites, such as unpasteurized milk, unwashed fruits and vegetables, or undercooked meat (5,6). Camelus dromedaries is a multi-purpose animal bred for meat, milk, and transportation, camel meat is frequently consumed and is highly susceptible to the exposure of toxoplasmosis which may become the possible source of infection for the customers (7).

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Prevalence of T. gondii in camels is significant because of the continuous contamination of pastures by T. gondii oocysts which makes this parasite a common infectious agent among these animals (8).

Toxoplasmosis infection could lead to different impacts such as blindness, mental retardation in infected fetus, and abortions of pregnant, and even death (9). Toxoplasma has a wide variety of hosts, and an apparently global distribution, it is estimated that one third of the world's population is chronically infected with Toxoplasma (10). The seroprevalence differs considerably between countries, with incidence rates ranging between 0% and 90%, increasing with age, sanitary habits, consumption of undercooked meat, socioeconomic, weakened immunity, animal contacts, climatic factors, and others (11).

There are different ways to diagnose toxoplasmosis, such as microscopic, serological detection (latex agglutination test and enzyme-linked immunosorbent assay [ELISA]), bioassays, cell line culture, and molecular techniques (12). ELISA test which can define as a simple, rapid and accurate method for demonstration of IgM or IgG (13,14). Few previous traditional studies were carried out for diagnosis of T.gondii in camels, accordingly this study focused on serological and molecular of this detection these parasites by IgG ELISA test.

Material and Methods

Ethical of approval

This research complies with the ethics rules of the University of Al-Qadisiyah, Iraq's Faculty of Veterinary Medicine (ethics permission number: 1890 in 28/8/2023).

Collection of blood samples

A total of 140 blood samples were collected randomly from camel's jugular vein. 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

For the purpose of the molecular test, a total of 140 tissue samples were taken from the same camels. A total of 250 grams of each organ—the diaphragm, the heart, and the uterus—were extracted from each and every camel that was slaughtered. Until the DNA extraction process was completed, the samples were kept at freezing temperatures and stored in separate plastic containers, each of which was designated with the appropriate label.

Serum preparation

The sera were separated by centrifugation the blood samples at 3000 round per minute for 10 minutes, the serum aspirated carefully by pipette into dry, sterile and labeled test tubes, and stored at -20ºC until used for serological test.

Enzyme linked immunsorbant assy (ELISA) Kit

ELISA kit was purchased from Sunlong Company in China country.

Principle of Camel toxoplasma antibody IgG (TOXO-IgG) ELISA.

This ELISA kit used Sandwich-ELISA as the method. The Microelisa stripplate provided in this kit has been pre-coated with an antigen specific to TOXO-IgG. Standards or samples are added to the appropriate Microelisa stripplate wells and combined to the specific antigen. Then a Horseradish Peroxidase (HRP)-conjugated antigen specific for TOXO is added to each Microelisa stripplate well and incubated. Free components are washed away. The TMB substrate solution is added to each well. Only those wells that contain TOXO-IgG and HRP conjugated TOXO antigen will appear blue in color and then turn yellow after the addition of the stop solution. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm. The presence of TOXO-IgG is determined by comparing with the CUTOFF value.

Procedure of Camel toxoplasma antibody IgG (TOXO-IgG) ELISA

1.In the Microelisa stripplate, leave two wells as negative control, two wells as positive control and one well empty as blank control. Number: the sequential number, corresponding sample of the microporous hole 2 per board should set negative control and positive control 2 holes, ck 1 hole (ck hole without samples and HRP-Conjugate reagent, the rest of the same step operation).

2.Adding samples: Negative and positive control in a volume of 50µl are added to the negative and positive control wells respectively. In sample wells, 40µl Sample dilution buffer and 10µl sample are added. Samples should be loaded onto the bottom without 5 touching the well wall. Mix well with gentle shaking.

3.Incubation: incubate 30 min at 37°C after sealed with Closure plate membrane.

4.Dilution: dilute the concentrated washing buffer with distilled water (30 times for 96T).

5.Washing: carefully peel off Closure plate membrane, aspirate and refill with the wash solution. Discard the wash solution after resting for 30 seconds. Repeat the washing procedure for 5 times.

6.Add 50 µl HRP-Conjugate reagent to each well except the blank control well.

7.Incubation as described in Step 3.

8.Washing as described in Step 5.

9.Coloring: Add 50 µl Chromogen Solution A and 50 µl Chromogen Solution B to each well, mix with gently

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shaking and incubate at 37°C for 15 minutes. Please avoid light during coloring.
10.Termination: add 50 µl stop solution to each well to terminate the reaction. The color in the well should change from blue to yellow.
11.Read absorbance O.D. at 450nm using a Microtiter Plate Reader. The OD value of the blank control well is set as zero. Assay should be carried out within 15 minutes after adding stop solution.

Determine the result

Test effectiveness: the average value of positive control ≥1.00; the average value of negative control ≤0.10.
The critical value (CUT OFF) calculation: critical value = the average value of negative control + 0.15
Negative judgement: if the OD value< CUT OFF, the sample is Camel TOXO-IgM negative.
Positive judgement: if the OD value ≥CUT OFF, the sample is Camel TOXO-IgM positive.

Molecular study
(Vitale (15)) designed a specific primer for the Sag3 gene to be used in the DNA detection of Toxoplasma gondii. This primer was provided by Scientific Researcher Company Ltd. in Iraq, table (1). The gSYNCTM DNA Extraction Kit was used to harvest genomic DNA from camel tissue samples, following the instructions provided by the company. The DNA that was acquired was assessed using a Nanodrop spectrophotometer (THERMO), a device produced in the United States.

These instruments measure the absorbance at a wavelength of 260/280 nm, which provides information on the concentration and purity of DNA. As can be seen in table (2), the approach was utilized in a manner that was consistent with the parameters that were provided by the company. Following that, agarose gel electrophoresis was utilized in order to investigate the outcomes of the PCR.
Table(1): Gene, primer, sequence and PCR product size.

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

Table(2): PCR Thermocycler Constructs reaction Conditions.

PCR step	Temp.	Time	repeat
Initial Denaturation	95C	5min	1
Denaturation	95C	30sec.	
Annealing	55C	30sec	35 cycle
Extension	72C	2min	
Final extension	72C	5min	1
Hold	4C	Forever	-

Statistical Analysis

The results of current study were examined by SPSS program (version-18), software (2010), using Chi-square test and a P value of ($p \leq 0.05$) were considered to report statistical (16).

Results

Total serological prevalence of toxoplasmosis in camels. For the first time in Al-Diwaniyah province, the present study detection 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.
Rate of toxoplasmosis in camels by ELISA according to ages.

Results of the present study showed that the positive rate in >5-10 age group was highest (61.53%). Statistically significant ($p<0.05$) difference was observed between age groups. (Table3).

Table (3): Rates of toxoplasmosis by ELISA according to age.

Age	No. of examined	No. of positive	Percentage (%)	X^2	P value
<2 year	**48**	**18**	37.5	**6.060**	**0.048**
>2-5	40	22	55		
>5-10	52	32	61.53		
Total	140	72	51.42		

*P<0.05

Rate of toxoplasmosis in camels by ELISA according to sex.

The result of the study recorded that the differences between males and females' infection rate not statistically (P >0.05) significant. (Table 4).

Table (4): Rates of toxoplasmosis by ELISA according to sex.

Sex	No. of examined	No. of positive	Percentage (%)	X^2	P value
Male	72	**38**	52.77	**0.108**	**0.742**
Female	68	**34**	50		
Total	140	72	51.42		

*P>0.05

Rate of toxoplasmosis according to year months

Monthly examination of 140 samples from September 2023 to March 2024 was shown in table (5).

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The highest infection rate was observed in February (90%) and January (75%), while lowest infection rate was observed in October (15%) with significant (P<0.05) differences was found between prevalence of infections according to months.

Table (5): Infection rate of toxoplasmosis according to months.

Month	No.of examined	No. of positive	Percentage (%)	X^2	P value
September	20	3	15%	56.732	0.000
October	20	4	20		
November	20	9	45		
December	20	13	65		
January	20	15	75		
February	20	18	90		
March	20	10	50		
Total	140	72			

*P<0.05

Results obtained by PCR technique

The polymerase chain reaction (PCR) was performed on the DNA samples that were described with high concentrations ranging from 400 to 710 ng/µl and purity levels ranging from 1.6 to 1.8. The overall findings of the PCR technique revealed that out of a total of 140 camel tissue samples, 55 (39.28 percent) were found to be positive for the SAG3 gene. (Figure 1).

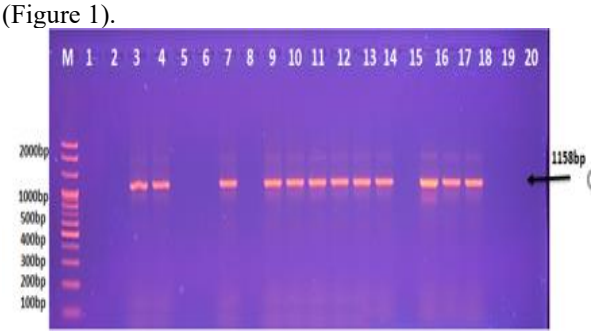

Figure (1) Agarose gel electrophoresis image that showed the PCR product analysis of SAG3 gene in Toxoplasma gondii from extracted DNA of camel's isolates. Where M: marker (2000-100bp) shown some positive Toxoplasma gondii isolates at (1158bp) PCR product.

Discussion

The ELISA test was used in the current study since it is a more specific approach for seroepidemiological examinations of T. gondii infection in animals (17). Despite the economic importance of camels in Iraq, there is a general dearth of awareness regarding camel illnesses, particularly toxoplasmosis. To the best of our knowledge, this is the first study to examine the risk factors for T. gondii infection in Al-Diwaniyah Province.

In the present study, prevalence of Toxoplasma gondii in 140 blood 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 of (120) serum sample (18). Nearly similar results were recorded by (19) in Saudi Arabia (45.44%) and (20) in Ethiopia (40.49%) out (451). This finding was higher than that obtained by (21) in Egypt, (22). (10%, 8% respectively). Also, this study disagrees (23) revealed that 27 out of 180 (15%) camels had the serologic evidence of T. gondii exposure. The obtained result disagreed with the result of (24) who found a higher infection rate (96.9%) in Sudan. Study in AL-Najaf province in Iraq by (25) revealed that 16 (22.8%) serum samples were positive for toxoplasmosis.

The present study disagrees with study of (26) that show T. gondii antibodies were found in serum samples at the rates of (36.58%) out (108) camels, in different governorates in Egypt. (Saad (27)) mentioned that the camels feed mainly on the dry matter that is present in the desert, and this feed fairway is contaminated by cats' feces, which is the main source of Toxoplasma oocytes. These differences may be resulting from geographical conditions, management systems and may be attributed to that the stray cats were widely spread in camels' farms leading to contamination of the environment with the oocysts that were shed in their feces. Also, the difference of Toxoplasma seroprevalence in our study and previous studies was ascribed to the difference of employed serological tests used in the diagnosis of the parasite, the initial serum dilution, climatic variation from region to another, age of the examined camels and frequency of felines on the farms.

Rates of toxoplasmosis by ELISA according to age in present study showed that the positive rate in >5-10 age group was highest (61.53%) . Statistically significant (p<0.05) difference was observed between age groups .

The present study agree with (25) which showed that the percentage of ELISA positivity increased with increasing age of animals and there was significant differences (P<0.05) between the infection rate in adult and young. The finding compatible with the finding of (28) and (29) . It may explained that the animal

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exposure to T. gondii and acquisition of infection increased when the longer animal lives. The previous results indicated that the younger camels had stronger immunity than the older aged camels ((18) . The present findings were consistent with those reported by (30) which indicated that the seropreva¬lence of T. gondii infection was higher in older camels (70.6%) than that of youngers (18.5%). The current high seroprevalence rate of T. gondii in older camels is due to the camels' movement to agriculture fields and feeding in the field areas, where they are more exposed to T. gondii com¬pared with younger camels (31).

Our result was compatible with result reported by (28) which mentioned the titers of antibodies were highly significant (P<0.01) between young and adult infection and the percentage of positivity increased with increasing animals age. Also this study agree with study by (20) that explained the T. gondii infection was significantly higher in camels of ≥ 8 years old (56.52%) than camels of ≤ 4 years old (34.26%) . (Ahmed (18)) pointed to the relationship between age of the examined camels and the infection rate, the study showed that there was a difference in the prevalence of infection among the different age groups, ELISA test showed that the camels of ages ranged from 6-10 years had higher infection rates than the camels of ages ranged from 1-5 years. The present study also were similar to that observed by (32) in Sudan, (33) in Iraq, (34) in Iran, (20) in Ethiopia. Moreover, the prevalence rate of seroreactivity increased by increasing age of the camels. The obtained results disagreed with the results of (35) in Sudan, (36) in Sudan , (37) in China and (23) in Iran , they noted that there was no significant difference in the prevalence among camels of different age group.

In studies conducted in Sudan, by (38) and by (37) in China recorded no significant association with T. gondii according to age groups , while(27) mentioned that younger camels had the highest infection rate .

According to sex , rate of toxoplasmosis by ELISA of the present study recorded that the differences between males and females infection rate not statistically significant (P >0.05) . The prevalence of infection among males was 52.77% out 72 while in the females was 50% out 68, similar to the study of (25) that recorded was no significant difference observed (P>0.05) between females and males infection. Our result was compatible with (28) , who showed total percent 16.4% in males and 18.6% in female. (Yektaseresht (23)) in their study recorded no significant relationship was detected between the seroprevalence of T. gondii infection with sex in camels , also agrees with the results of studies by (20) , (38), (39) and (40), (41) they recorded no significant relationship was observed between males and females. Our study disagree with study of(26) that show the rates of infection were higher in males than in lactating and non-lactating females. The present finding not compatible with the finding of (28) showed that, percent of infection in female animals(30.4%)out 141 were more than that in male animals(21.9%) out 291. (Shehzad (42)) recorded that the infection rate was higher in male camels (50.2%) than in female camels (16.5%).(Gebremedhin(31)) and(Selim (43)) recorded that the female camels had higher seroprevalence rate for T. gondii than male camels which could be owing to a reduced physiological or immunological status because of pregnancy and lactation stress. Contrary to these findings, (44) observed higher prevalence for toxoplasmosis in males than females .

Concerning the year months, significant differences in seroprevalence between the months , monthly examination of 140 samples from September 2023 to March 2024 was showed the highest infection rate was observed in February (90%) and January (75%) , while lowest infection rate was observed in October (15%) with significant differences was found between prevalence of infections according to months. Similar results previously reported by of (18) they recorded the infection rate was higher in the winter (76.7%), followed by summer (53.3%). (Al-Kabi (45)) noted that existence of cats is important in the epidemiology of toxoplasmosis based on environmental conditions, which can keep oocysts for months and years, and also IgG exists for long time in animals and can be found at different months or seasons of the year.

PCR based techniques, have been developed and used for accurate identification and diagnosis of Toxoplasma gondii because of their high sensitivity, specificity, rapidity, utility and detect the parasite's DNA (14). The PCR technique has been used to corroborate the results of serological tests represented by the Eliza test as a diagnostic method, being a more sensitive tool allowing specific amplification of SAG3 gene.

A number of PCR-based molecular assays (PCR, PCR-restriction fragment length polymorphism (RFLP), and real-time PCR) have been conducted for differentiation of Toxoplasma gondii (15). In the present study extraction of DNA from camel's tissue samples was used to amplification SAG3 gene using conventional PCR technique.

The total results of PCR technique showed that out of 140 camel's tissues samples 55 (39.28 %) were positive for SAG3 gene. A previous study conducted in the in Al-Diwaniyah province by (16) were recorded 18/32 (56.25%) infection rate for toxoplasmosis in the

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tissue samples of camels. Also (Khattab (17)) recorded 13(26%) tissue samples of camels were found 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. (Wallander (14)) recorded the rates of T. gondii DNA by PCR in camels were 14.63%. PCR method shows the high sensitivity and specificity for the detection of toxoplasmosis; furthermore, SAG3 gene having higher copies in genome of T. gondii (18). (Khamesipour (41)) referred that among 122 camel's sample were 6.60% infected with T. gondii. Which can be explained by the lower prevalence of T. gondii DNA detected by PCR in comparison to T. gondii antibodies may be due to the fact that IgG antibodies are produced late in the infection, and the parasite is localized in the organs and tissues rather than circulated in the blood. Also May due to the low concentration of cysts in the tissues and random distribution (one cyst per 50 – 100 g) of tissue and by the small size of samples (20).

Conclusions

Toxoplasma infection in camels is relatively high in Al-Diwaniyah
provinces.The prevalence of Toxoplasma infection according to ages of camels was highest in >5-10 age group than other ages groups.

The highest infection rate was observed in winter months (February January) and lowest infection rate was observed in October .There is no effect of sex on the prevalence of infection.

The PCR technique was more sensitive and specific than the ELIZA test for detection the of Toxoplasma. gondii.

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Conflict of Interest.

the authors declare that There is no conflict of interest.

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