SEROLOGICAL AND MOLECULAR DIAGNOSIS OF *TOXOPLASMA GONDII* AMONG EWES AND HORSES IN DUHOK PROVINCE-IRAQ

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ABSTRACT

This study was aimed to demonstrate the seroprevalence and molecular detection of *Toxoplasma gondii* among ewes and horses as well as to determine the risk factors for infection in Duhok province. Sera and blood samples from 700 ewes and 62 horses were collected. Sera were examined by indirect ELISA for detection of anti-*T. gondii* IgM antibodies and in molecular study, DNA was extracted, then by PCR, *B1* gene was amplified and the product visualized and sent off for sequencing. Serologically, the prevalence of toxoplasmosis was 17.7 (11/62) and 28.9 (202/700) in horses and ewes by ELISA respectively. Present of cats on the farm was significantly associated with *T. gondii* infected ewes in the farm. While, Age, number of abortion and history of abortion has no role in infection in ewes. On the other hand, age and sex have no role in prevalence of toxoplasmosis among the horses. Only 2 samples among 11 seropositive samples by ELISA were give positive results by PCR in rate 18.2% in horse, while in ewes 13 samples from 60 randomly selected seropositive by ELISA were found to be positive by PCR in rate 21.7%. Results of this study indicate that prevalence of *T. gondii* among ewes and horses was high and cats have a role in prevalence of infection among the ewes.

Keyword: Toxoplasmosis, ELISA and PCR

التشخيص المصلي والجزيئي لطفيلي مقوسات كوندي *Toxoplasma gondii* بين الأغنام والخيول في محافظة دهوك- العراق

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المستخلص

تهدف هذه الدراسة لتقييم الانتشار المصلي والكشف الجزيئي لطفيلي *Toxoplasma gondii* في الأغنام والخيول وكذلك عوامل خطورة الإصابة به في محافظة دهوك. عينات من الدم والامصال جُمعت من 700 من الأغنام و62 من الخيول. الامصال فحصت بواسطة اختبار الاليزا غير مباشر Indirect ELISA للكشف عن الاجسام المضادة IgM لمضاد *T. gondii* وكذلك تم استخلاص الحامض النووي الرايبوزي منقوص الاوكسجين (DNA)، ثم ومن خلال اجراء اختبار تفاعل البوليميريز المتسلسل (PCR)تم عزل وتضخيم مَورُوث *B1 gene*ومن ثم الناتج الظاهر تم ارساله لمعرفة التسلسل الجيني. اَظهرت نتائج اختبار الاليزا للأمصال معدل لانتشار داء المقوسات 17.7 (11/62) و28.2 (202/700) بين الخيول والأغنام على التوالي. كان لوجود القطط ارتباط معنوي بإصابة الأغنام بالطفيلي *T. gondii*، بينما لم يكن للعمر وعدد حالات وتاريخ الإجهاض أي دور في انتشار الطفيلي في الأغنام. ومن ناحية أخرى كان للعمر والجنس دور في انتشار الإصابة بداء المقوسات بين الخيول. فقط حالتين من ضمن 11 حالة موجبة لاختبار الاليزا كانت موجبة لاختبار (PCR) وبمعدل 18.2%في الخيول، بينما كان المعدل في الأغنام 21.7%من 13 حالة موجبة لاختبار تفاعل البوليميريز المتسلسل (PCR) من 60 حالة أُختيرت عشوائياً كانت موجبة مصلياً لاختبار الاليزا. اَظهرت نتائج الدراسة الحالية ان نسبة انتشار طفيلي ال *T. gondii* بين الأغنام كان مرتفعاً وكان للقطط دور في انتشار الإصابة بهذا الطفيلي بين الأغنام.

كلمات مفتاحية: داء القطط، اختبار الاليزا وتفاعل البوليميريز المتسلسل

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INTRODUCTION

Toxoplasmosis is an important zoonotic disease that infects all warm-blooded animals, including humans as intermediate hosts. Felids are the key species in the life cycle of this parasite because they are the only hosts that excrete the environmentally resistant stage, the oocyst, in their feces (16). Horses can be infected by *T. gondii* when they ingest contaminated food or water with oocysts released by cats (33, 16). Horses have high resistance to *T. gondii* infection among domestic animals, while clinical signs, such as abortion, ocular infection, hyperirritability and incoordination have been recorded (18). Due to the special relationship between horses and humans, *Toxoplasma gondii* could transmit from horse to humans. In some countries consumption of horse meat is common, so that due to eating of meat of the horse some cases of toxoplasmosis have been recorded (10,28). In pregnant sheep, abortions and stillbirths are common; abortions tend to occur in the last 3-4 weeks of pregnancy and the rate may be as high as 50% (12). The only clinical syndrome recognized with any regularity in the field is abortion and neonatal mortality in sheep but may associated with the occurrence of embryonic death and absorption, fetal death and mummification and stillborn (15).

Abortion and neonatal mortality occur when sheep acquire a primary infection during pregnancy. Most of the sheep that had abortion due to *T. gondii* develop protection against future toxoplasmosis induced abortion, but the protection is not absolute. Early diagnosis of infection is of great consequence for reducing the severity of the disease and the risk of congenital toxoplasmosis (19). The objective of this study to demonstrate the seroprevalence of *T. gondii* in ewes and horses in Duhok governorate and to demonstrate the role of the age, present of cats, history and number of abortions in ewes. The role of the age has also been studied with the prevalence of toxoplasmosis in horse.

MATERIAL AND METHODS

Study design and sampling In the current analytical cross-sectional study, A total of 700 blood samples from ewes and 62 blood samples from horses were collected in Duhok province between November 2016 and March 2017.

Data collection

The data were collected through two different stages. The general information of the subjects was collected from the owner of the animals through the self-reported technique. In the second stage, the diagnostic information of *T. gondii* was collected through the laboratory diagnostic tests, including serological and molecular methods. Three ml of blood were added to non-additive tubes for ELISA test and 2 ml was added to EDTA tubes for molecular tests. The free tubes were then left for 30 minutes at room temperature after collection allowing blood to clot. Serum obtained by ordinary centrifugation at 5000 rpm spanned for 5 minutes and the sera were stored at -20ºC until examination by ELISA. Whole blood in EDTA tubes preserved in -20ºC for DNA extraction.

Questioner performance

A questionnaire on personal information was prepared, asking the owner of the animals about animal's age, contacting with cats, number of abortion and abortion history.

Serological test (ELISA)

Indirect ELISA performed to detection of IgM (ID VET innovative diagnosis, France) antibodies directed against *T. gondii* in sera according to the manufacturer's instruction.

Molecular confirmation of Toxoplasmosis

The DNA was extracted randomly from some blood samples that were found positive by ELISA. Amplification and detection of *T. gondii* DNA was performed by conventional PCR. The primers, targeting the *B1* gene, were used according to (24), the forward TOX4 (CGCTGCAGGGAGGAAGACGAAAGTTG) and the reverse TOX5 (CGCTGCAGACACAGTGCATCTGGATT).

The master mix used was 2X HS Prime Taq Premix (G-7100, GeNet Bio,Korea) which is composed of 1 unit/10 ul Taq-Pol, 2X reaction buffer 75 mM Tris-HCL (pH 9), 4 mM MgCl2, and 0.5 mM of each dNTP.

The total PCR volume was 25 µl.The reaction mixture contained 12.5 ul of 2X master mix above, 10 pmol/ul of each forward and reverse primers, 2 µg DNA template (50ng/ul), and 8.5 ul RNase free water to a total volume of 25 ul DNA. The cycler condition of PCR was set up at initial denaturation 95 °C for 4 minutes, and then followed by 35 cycles of denaturation 95°C 45 s, annealing 55 °C for 45s, extension 72 °C 45s and final extension at 72 °C for 5 minutes. Finally, 10 µl of PCR products were electrophoresed on 1% agarose gel and visualized under UV.

Sequencing of a partial *B1* gene fragment

The PCR products of *B1* gene of five samples from each group were sent off to Korean (Macrogen) company for sequencing using primers Toxo4 and Toxo 5. The qualities of the sequences have been tested and the bad sequences have been trimmed out using BioEdit sequence alignment editor 7.0.0 (Isis Pharmaceuticals, Inc., Carlsbad, USA). All sequences were applied to NCBI Nucleotide blast for determination the identity and similarity of the samples. Finally, the sequences were submitted to NCBI to get the GenBank accession Numbers using BankIt portal (horse MK704513 and sheep MK693029).

Statistical analysis

The mean and standard deviation were used for numerical and frequency and percentage for categorical characteristics of ewes and horses. The difference in sero-positivity of the parasite in different characteristics of the cases was examined in Pearson Chi-Square and Fishers' Exact tests. The P-value of less than 0.05 was used to reject the null hypothesis. The statistical package for social sciences (SPSS version 25:00l IBM: USA) was used for statistical calculation (20).

RESULTS AND DISCUSSION

Out of 700 ewes sera examined by ELISA, the seropositivity of anti-*Toxoplasma gondii* IgM antibodies was 28.9%. The sero-positivity of antibodies to *Toxoplasma* was showed no statistical different baqetween different age groups of ewes, history of abortion and numbers of abortion cases in ewes as show in Table 1. On the other hand, the highest seropositivity (63.2%) observed in ewes in the fields that have direct contact with the cats, while the seropositivity was lower (9.4%) in fields in which there were no cats. Statistical analysis of the results showed the presence of highly significant (P≤ 0.001) difference between the two groups (Table 1).

Table1. Prevalence and association of general characteristics between the ewes with and without toxoplasmosis

Ewes Characteristics (n=700)	Results	P-value (two-sided)
	IgM Positive	
Antibody reaction	**202(28.9%)**	
Age (year)		
Less Than 4	**150 (29.7%)**	**0.427***
More Than 4	**52 (26.7%)**	
Abortion		
Aborted	**117 (28.3%)**	**0.675**
Non Aborted	**85 (29.7%)**	
Abortion Number		
No Abortion Before	**85 (29.7%)**	**0.740**
1 Abortion	**111 (28.0%)**	
More Than 1 Abortion	**6 (35.3%)**	
Cat Contact		
Contact With Cats	**160 (63.2%)**	**<0.001**
Not Contact With Cats	**42 (9.4%)**	

The result of this study highlighted some similarity with the study conducted in Pakistan by Hanif and Tasawar (23) at which the prevalence rate of toxoplasmosis was 27.4% by ELISA. Furthermore, this result shares some similarity with the result of the study implemented in Misan-Iraq, where the prevalence rate of toxoplasmosis was 25% by using ELISA (27). The results of present study, showed that the incidence of toxoplasmosis in ewes was lower (28.9%) among ewes in Duhok Province compared with the prevalence rate of other studies in different area of Iraq, such as studies conducted in Duhok by Al-Barwary and Mikail (6), in Mosul by Al-Sim'ani (11) and in Sulaimani by Abdulla and Al-Taie (2), at which the prevalence rates were 67.31%, 42.7% and 57%, respectively using Latex agglutination test (LAT). These results also found to be lower than those recorded in Duhok by Al Hamada *et al.,* (4), in Sulaimani by Abdulla and Al-Taie (2) and in Nineveh by Al-Dabagh *et al.,* (7) in which the prevalence were 42.1%, 51.7% and 32.8%, by using ELISA respectively. However, it has been noticed that seropositivity in ewes in this study was higher than other studies implemented in Iraq, such as in Duhok, Baghdad, Thiqar, Al-Muthanna and Al-Basra where the prevalence rates were 22.9%, 16.66%, 12.71%, 16.6% and 18.63%, respectively using ELISA (34,27,1). Comparing with the other countries, this study indicated that the results of the present study are higher than those reported in Pakistan 18.16%, by Ahmad *et al.,* (3) and in Saudi Arabia 22% by Al-Mohammed (9). The variability of the seropositivity might be due to the diagnostic techniques and methods used for detection and identification of *Toxoplasma* in different studied. This has also been noticed by Tenter *et al.,* (36) when stated that the variability of prevalence rate in the different countries could be due to the methods of diagnosis. The prevalence rate varies from location to another, depending on several factors such as climate, geographical area, cat population's sanitary conditions and management, environmental condition of oocyst, size of sample collection and susceptibility of animals (35,36). Regarding to the frequency of abortion, this study shows that, the seropositivity in ewes with single abortion 28% (111/397) was higher than those with history of two or more abortions 35.3% (6/17) (Table1). This is approved by Mikail (34), in which ewes with single abortion (136/365) 37.26% was higher than those of 2 abortion (12/39) 30.77%. This may be due to the following infection with *T. gondii*, sheep develop humoral and cell-mediated immune responses against the parasite that provides effective protection against disease in subsequent pregnancies (15). Concerning to the age groups, we found that the seropositivity of *Toxoplasma* was high in age group<4years 150(29.7%) than old ≥4 years 52(26.7%) (Table1). This result has been supported by Lashari and Tasawar (29) in Pakistan, they show that the prevalence decreased as age of sheep increased from 28 months. This could be due to the fact that younger ewes have less resistance and also, they can be actively exposed to oocyst and have been more chance to acquire infection. On the other hand Leyva *et al.,* (31), reported that natural infection with *T. gondii* generally leads to a state of long-lasting protective immunity. The current results indicated that there is no significant difference between those ewes that were not aborted before or aborted once or more than one times when estimated of the antibodies in sera by ELISA 385(29.7%), 111 (28%) and 6(35.3%), respectively according to the frequency of abortion (Table1). This is also approved by Mikail (34). In this study, the seropositivity of anti-*Toxoplasma gondii* antibodies was 63.2% (160/253) and 9.4% (42/447) in ewes had a contact with cats and not those had not contact with cats, respectively. This has been confirmed by Ibrahim *et al.,* (25) in Egypt, by Sheppa (35) in Tanzania, by Lopes *et al.,* (32) in Brazil and by Ahmad *et al.,* (3) in Pakistan that showed the presence of cats in the farms was strongly associated with occurrence of toxoplasmosis. Cats, as the definitive host, are considered the major source of toxoplasmosis to animals and humans through excreting the oocysts with its feces (18). Regarding to the horse toxoplasmosis, out of 62 horses sera were tested by ELISA, the prevalence of positive sample was 17.7%. The study did not show the significant difference of sero-

positivity in male and female horses (18.6% and 15.8%, respectively; P=0.784) (Table 2).

Table 2. Seroprevalence of anti-Toxoplasma gondii IgM antibodies in horses according to sex

Horse (n=62)	Results	P-value (two-sided)
	IgM Positive	
Antibody	**11 (17.7%)**	
Sex		
Male	**8 (18.6%)**	**0.784***
Female	**3 (15.8%)**	

Boughattas *et al.*, (14) in which the seroprevalence of infection was 17.92% (110/614) by Indirect hemagglutination test (IHA) test and 17.7 (28/158) by Modified agglutination test (MAT) respectively. The prevalence of *T. gondii* in the present study was higher than the studies conducted in Turkey (26) and Korea (30) which were 7.2% and 2.9% using Dye test (DT) and ELISA, respectively. While, the prevalence rate of this study in horses was lower than the study done in Mosul/ Iraq by Alshahery and Mansour (10) which was 77.8% by LAT. and also lower than those in Iran (21) and Saudi Arabia (5) which were 71.2% and 31.6% Using MAT and DT, respectively. These differences may be related to the present of cats in the living area, size of sample, sampling time, animal's susceptibility and different diagnostic techniques. Regarding the sex, this study showed that the prevalence of *T. gondii* in horse was higher in male 18.6% (8/62) than female 15.8% (3/62). This result are supported by a study conducted in Mosul (10) were the male 77.8% (7/9) and female 71-4% (50/70) by LAT and agreed with those that achieved in China (37) were male 19.38% (56/289) and female 16.62% (54/325) by IHT and also to the study in Turkey (26) were male 10%(5/50) and female 5.3%(4/75) using Sabin-Feldman dye test (SFDT) The sero-prevalence rates variation to *T. gondii*. in horses between our results and those previously reported may be due to the serologic test used, the virulence of *T. gondii* strains, the immune status, age and management of investigated animals in different localities. The seropositive samples of both ewes and horses have been randomly selected for PCR to validate the results of ELISA. In horse, all samples were found to be positive by ELISA, while in case of sheep only 60 samples were found positive by ELISA. These samples were randomly selected to be confirmed by amplifying *B1 gene* using PCR. The PCR result shows 2 positive samples out of 11 in horse at rate (18.2%) and clearly showed amplicon size of 529 bp as in Figure 2, and 13 positive samples out of 60 was positive in sheep at rate (21.7%)and clearly showed amplicon size of 529 bp (Figure1). The low positive rate by PCR compared to the ELISA results is suggested to be due to the absence of the parasite itself in the blood at the collection time and the parasite has already localized within the body of the host as a tissue cysts, tachyzoites and/or bradyzoites. Also, the reason may be that the acute infection subsides or converted to chronic infection (13). The DNA sequence was aligned to NCBI and it was 100% identical and similar to (KX270384) and (KX270382) for horse and sheep respectively. Then the sequences submitted to NCBI, Gene Bank and sequences accepted under accession numbers (MK704514) and (MK693029) for horse and sheep respectively

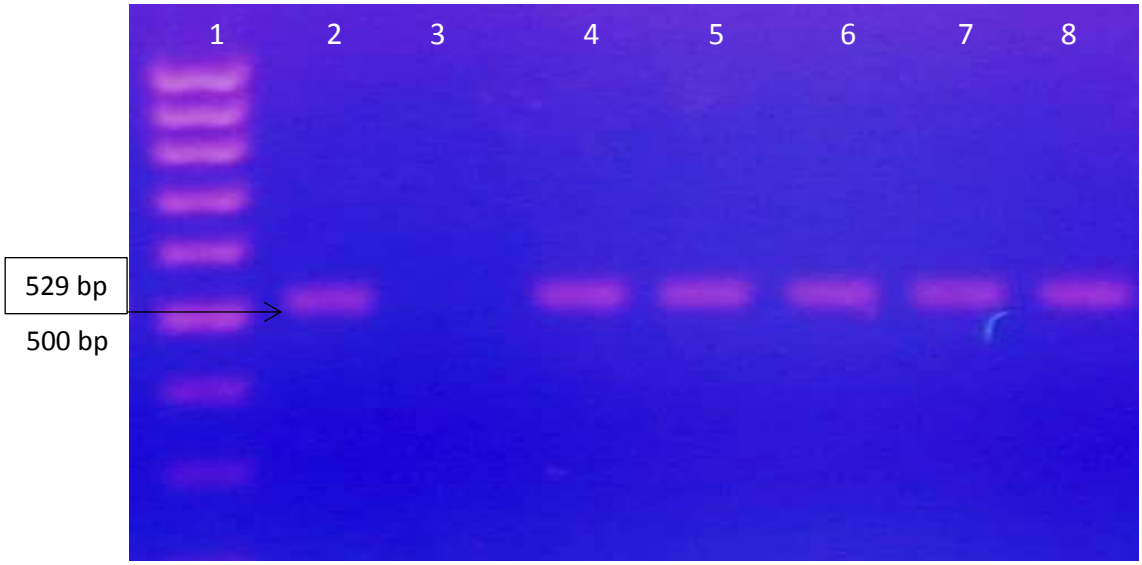

Figure1. PCR products of *T. gondii* in ewes on 1% agarose. Lane 1 100 bp (GeNet Bio, Korea) ladder, lane 2 positive control (previously isolated strain from Dicla university, Diyarbakir/ Turkey), lane 3 negative control and lanes 4-8 tested samples

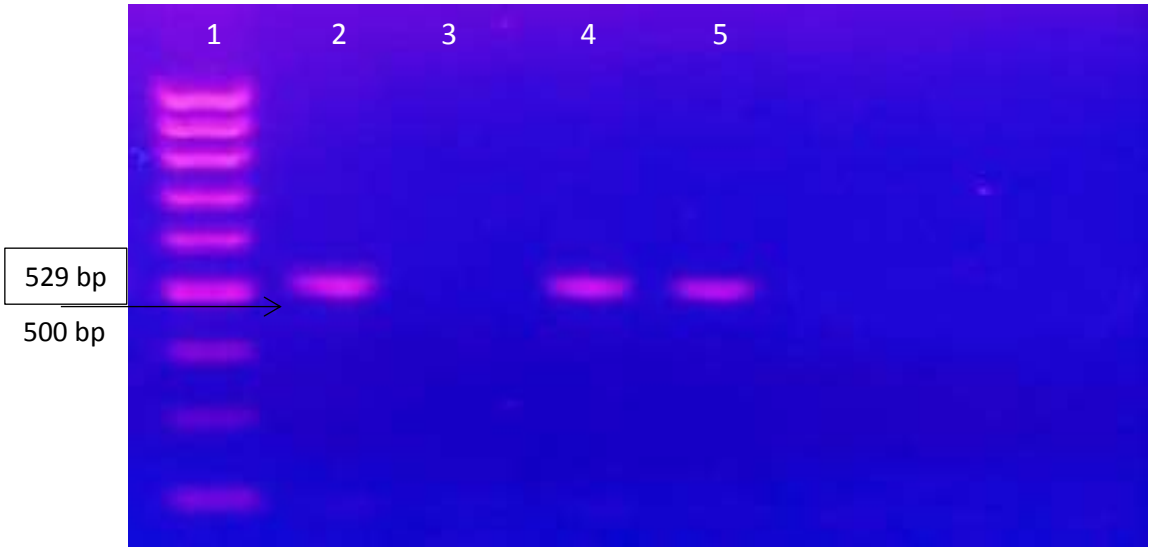

Figure 2. PCR products of *T. gondii* in horse on 1% agarose. Lane 1:100 bp (GeNet Bio, Korea) ladder, lane 2 positive control (previously isolated strain from Dicla university, Diyarbakir/ Turkey), lane 3 negative control and lanes 4 and 5 tested samples.

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