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SEROLOGICAL AND MOLECULAR DETECTION OF TOXOPLASMA *GONDII* IN SHEEP IN DIYALA PROVINCE, IRAQ

MOHAMMED K. BAWI AND IBRAHIM A.H. Al-ZUBAIDI
Unit. Zoonotic Diseases, College of Veterinary Medicine, University of Baghdad

Received: 10 July 2024; **Accepted:** 2 August 2024

ABSTRACT

Toxoplasma gondii is the common cause of toxoplasmosis in both humans and animals. Sheep toxoplasmosis represents an important role in public health, causing reproductive and economic losses due to abortion and neonatal mortality worldwide. The seroprevalence and molecular detection of sheep toxoplasmosis in Diyala province, Iraq, were the aim of this study. One hundred male slaughterhouses sheep and 100 grassing dairy ewes were examined and divided into groups according to age, season, breed, importation, contact with cats, pregnancy status and abortion. *Toxoplasma gondii* infection was investigated using a *Toxoplasma* rapid test and polymerase chain reaction (PCR) test. The overall seroprevalence of toxoplasmosis in rams was 18%, while it was 14% in grassing dairy ewes. The PCR test confirmed the seropositive infection in 50% and 35.7% of rams and diary ewes, respectively. Adult dairy ewes and sheep from slaughterhouses had a statistically insignificantly greater seroprevalence of toxoplasmosis than juveniles. The prevalence of the disease was slightly higher in the winter groups in either rams or diary ewes. Sheep imported from Syria and Iran had twice as high an infection rate as native sheep from Iraq. The infection rate among dairy ewes from the Karadi and Hamdani breeds was not considerably greater than those of other breeds. Sheep with a history of abortion showed higher seroprevalence, and also toxoplasmosis was more prevalent in previously integrated sheep with cats. Therefore, continuous surveillance of sheep toxoplasmosis detection is highly recommended for further prevention.

Keywords: toxoplasmosis, Iraq, sheep, seroprevalence, *b1* gene.

INTRODUCTION

Toxoplasmosis is a common foodborne illness caused by *Toxoplasma gondii* (*T. gondii*), a common protozoan, considered a member of the phylum *Apicomplexa* (viera da silva *et al.*, 2005; Pereira *et al.*, 2010; and Darayani *et al.*, 2014). Toxoplasmosis is a common infectious agent that results in reproductive failure in sheep. It causes placentitis, abortions, and stillbirths, which over time cause significant financial losses (Ali *et al.*, 2019; Clune *et al.*, 2021). Lambs are mostly affected by eating *T. gondii* oocysts while grazing in a contaminated environment (Hassanain *et al.*, 2011; Motoi *et al.*, 2022 and Iqbal *et al.*, 2023). Only three clonally distinct forms of *T. gondii* isolates-types I, II, and III-have been identified in sheep flesh (Duby, 2002; Elfadaly *et al.*, 2017; Hill and Aleem *et al.*, 2018). It was possible for the same beef sample to contain multiple infections. The biological traits of the less virulent strains were concealed by

Corresponding author: MOHAMMED K. BAWI
E-mail address: mohammed.bawi2204m@covm.uobaghdad.edu.iq
Present address: Unit. Zoonotic Diseases, College of Veterinary Medicine, University of Baghdad

the more virulent strains (Shaapan *et al.*, 2015; Dubey, 2016; and Assim and Saheb, 2018). Sheep have higher global seroprevalences of *T. gondii* than other agricultural animals due to their higher infection rates (Stelzer *et al.*, 2019; Daher *et al.*, 2021; and Gharban and A-kaabi, 2022).

Mutton is a popular meat source, and because it has more nutrients than other red meats, it is consumed more often around the world, particularly in less developed nations, giving people sufficient dietary protein (Weiss and Dubey, 2009; Mahmoud *et al.*, 2021). *T. gondii* tissue cysts, which are present in edible animal flesh, especially sheep mutton, are the main source of human toxoplasmosis because they can survive in undercooked tissues (Toaleb*etal*, 2013; Aguirre *et al.*, 2019; and Segbedzi *et al.*, 2023). In addition to mutton, *T. gondii* tachyzoites have been found in the milk of a number of food animal species, such as sheep, goats, and cows. The infectious tachyzoites can live in fresh cheese and are expelled in milk (Munoz-Zanzi *et al.*, 2012; Dawson *et al.*, 2020; and Almeria and Dubey, 2021).

The high prevalence of toxoplasmosis observed in humid regions of the world affects one-third of the global population (Dubey and Beattie, 1988; Studeničová *et al.*, 2006; and WHO, 2015). The significance of this disease made the global epidemiological research crucial as its seroprevalence rated 40% in the USA, 30% in the UK, and 50% in southern Europe (Punda-Polić *et al.*, 2000; Diza *et al.*, 2005)**,** while in Serbia it was 20.5% (Stopić *et al.*, 2022), Albania 48.6% (Maggi *et al.*, 2009), Turkey 28.57% (Karakavuk *et al.*, 2024), Egypt 57.3% (Elaadli *et al.*, 2023), Qatar 29/8% (Abu-Madi *et al.*, 2008), Syria 56.01% (Adi and Alkhaled, 2011), Iran 30.4%, and in Pakistan 34.5%.
Serological assays that identify anti-*T. gondii* specific immunoglobulins IgM and IgG antibodies in sera samples are the primary methods used to diagnose toxoplasmosis in sheep (Shaapan *et al.*, 2008; Hassanain *et al.*, 2018; and McCall *et al.*, 2022).

Research on toxoplasmosis in Iraq has not been conducted nationwide. Several research focus on individual governorates and provide information on the seroprevalence of infection in those governorates (Al-Taie and Abdulla, 2011; Mahmood *et al.*, 2013; Al-Hindawi and Al-Shanawi, 2015; Al-Maamuri *et al.*, 2015; and Asal and AL-zubaidi, 2016; Al-Khafagi and Zainab, 2016; Ali and Al-Warid, 2021; Musa *et al.*, 2021; Alkubaisi and AL-zubaidi, 2023; and Bawi and AL-zubaidi, 2024). Therefore, this study was designed to clarify the occurrence of toxoplasmosis in sheep in Diyala province in Iraq, using serological and molecular methods, to determine the prevalence of toxoplasmosis in sheep (rams and dairy ewes), as well as to identify the associated risk factors.

MATERIALS AND METHODS

1- Ethical approval.

This experiment was conducted in compliance with the institutional rules of the University of Baghdad's College of Veterinary Medicine's Animal Care and Use Committee, Iraq. Project Number: Pg. 1-50. The herd's owners gave their verbal agreement before samples were taken.

2- Animals and study area.

An epidemiological survey was conducted in two seasons, winter and spring. The study duration was between November 2023 and February 2024 for the winter group, and between February and April 2024 for the spring one. The investigated animals were rams (5 months-5 years of age) before slaughtering in abattoirs and dairy ewes (1–6 years). The study was carried out in Diyala province (X/Y coordinates: 33.883333, 45.066667), in the eastern region of Iraq. Rams were randomly selected from various slaughterhouses

across the province, while the grazing dairy ewes were sourced from multiple herds on the farm.

3- Samples.

Blood samples were collected randomly from 100 rams, in addition to 100 blood and milk samples from multiple herds of grassing dairy ewes during the active milking period. Data was obtained about the breed, either from local breeds in Iraq or imported from other countries, contact with cats, history of abortions, availability of drinking water, and other factors.

4- Samples preparation and processing.

Approximately 10 ml of jugular vein blood was obtained from all sheep, 5 ml in EDTA tubes with anticoagulant for molecular examination, and 5 ml in gel tubes without anticoagulant for separation of serum. Only 5 ml of blood was obtained from grassing dairy ewes in gel tubes without anticoagulant for obtaining serum. Each sample was labeled using codes describing the specific animal's gender and age. Blood collected in gel tubes was left at room temperature to clot, then centrifuged at 3000 rpm for 15 minutes. Serum was separated and used in the latex agglutination test. The blood collected in EDTA tubes was stored at (-20 ºC) until used in the PCR test. 10 ml of milk samples were collected from grassing diary ewe after cleaning and sanitizing teats and removal of the first few jets of milk. Milk samples were stored at (-20 ºC) until used in the molecular analysis.

5-Serological examination.

Sheep and grassing dairy ewes' serum samples were tested using a toxoplasma rapid test (Toxo IgG and IgM combo rapid test, CTK Biotech, USA), according to the company's instructions for toxoplasmosis detection.

6-Molecular examination.

Only positive cases with the latex agglutination test were subjected to the presence of the *b1* gene of *T. gondii* using polymerase chain reaction (PCR). Blood and milk samples were processed to obtain total genomic DNA. After the samples were pelleted, 200 µL of PBS were used for dilution, before extracting total genomic DNA. A 406-bp segment of the recurrent *b1* gene (35fold repeats/genome) was the target of the PCR using specific primers (Macro Gene Company, South Korea) (Table 1). The amplified DNA was electrophoresed in 1.5% Agarose gel.

Table 1: The primers designed to detect the *b1* gene of *T. gondii*.

Primer	Sequence
Forward	5-ATACAGGTGAAATGTACCTCC-3
Reverse	5-CGATCTTCTTCTCCTGTTCTT-3

7-Statistical analysis

The System-SAS (2018) program was used to statistically analyze the data to determine the impact of different factors on the research parameters. Also, the Chi-square test and differences were performed and considered statistically significant if P-values were below 0.05.

RESULTS

1-Seroprevalence of toxoplasmosis in sheep.

The overall prevalence of toxoplasmosis in rams from slaughterhouses was 18 out of 100 (18%) using the latex agglutination test (Table 2). While, the overall incidence in grassing dairy ewes was 14 out of 100 (14%).

2-Molecular detection.

Molecular examination of seropositive cases that confirmed the infection was in 9 (50%) of the rams cases, while it was confirmed in only 5 (35.7%) seropositive cases of dairy ewes (Table 2) by amplification of the *b1* gene of *T. gondii* (Figures 1 and 2).

3-Risk factors.

Seroprevalence of toxoplasmosis was statistically insignificantly higher in adult sheep and dairy ewes than juveniles. Moreover, there was no significant

variation between winter and spring seasons in both genders, although it was a little higher in winter groups (Tables 3 and 4).

By comparing the native sheep (Iraq) and the imported ones (Syria and Iran), the difference was not statistically significant (Table 3). Both of the Karadi and Hamdani ewe's breeds had an insignificantly higher infection rate than other breeds (Table 4).

A non-significant seroprevalence was observed in ewes with a history of abortion compared to non-pregnant ones. According to our study, toxoplasmosis was significantly more common in sheep previously interacted with cats (Table 4).

Table 2: Toxoplasmosis prevalence rate in slaughterhouses' sheep and dairy ewes.

Animals	Latex agglutination test (screening)			Molecular detection (confirmation)		
	Tested no.	Positive no. (%)	Negative no. (%)	Tested no.	Positive no. (%)	Negative no. (%)
Slaughterhouses' sheep	100	18 (18)	82 (82)	18	9 (50)	9 (50)
Dairy ewes	100	14 (14)	86 (86)	14	5 (35.7)	9 (64.3)

Table 3: Seroprevalence of *T. gondii* antibody and the associated risk factors in slaughterhouses' sheep.

Factor	No. tested	Seroprevalence		*P* value
		Positive no. (%)	Negative no. (%)	
Age				
Less than one year	70	10 (14.3)	60 (85.7)	Reference
1 – 2 years	20	5 (25)	15 (75)	0.305
More than 2 years	10	3 (30)	7 (70)	0.353
Season				
Winter	50	10 (20)	40 (80)	0.795
Springe	50	8 (16)	42 (84)	
Importation				
Local from Iraq	49	6 (12.2)	43 (87.8)	Reference
Imported from Syria	19	4 (21.1)	15 (78.9)	0.448
Imported from Iran	32	8 (25)	24 (75)	0.228

Figure 1: Agarose gel electrophoresis of PCR after amplification of *the* b1 gene of *T. gondii* infection in slaughterhouse sheep. Line 1: DNA ladder 100 bp, line 2 and 3: negative samples, and lines 4 to 11: positive DNA samples with amplified product at 406 bp.

Table 4: Seroprevalence of *T. gondii* antibody and the associated risk factors in grassing dairy ewes.

Factor	No. tested	Seroprevalence		*P* value
		Positive no. (%)	Negative no. (%)	
Age				
Less than one – 2 years	27	2 (7.4)	25 (92.6)	Reference
3 – 4 years	40	7 (17.5)	33 (82.5)	0.295
5 – 6 years	33	5 (15.2)	28 (84.8)	0.442
Season				
Winter	50	9 (18)	41 (82)	0.388
Springe	50	5 (10)	45 (90)	
Breed				
Awassi	49	5 (10.2)	44 (89.8)	Reference
Karadi	27	6 (22.2)	21 (77.8)	0.183
Niamey	18	2 (11.1)	16 (88.9)	1.000
Hamdani	6	1 (16.7)	5 (83.3)	0.518
Pregnancy status				
Non-pregnant	63	8 (12.7)	55 (87.3)	1.000
Pregnant	25	3 (12)	22 (88)	Reference
Aborted	12	3 (25)	9 (75)	0.367
Previous contact with cats				
Present	10	3 (30)	7 (70)	0.145
Absent	90	11 (12.2)	79 (87.8)	

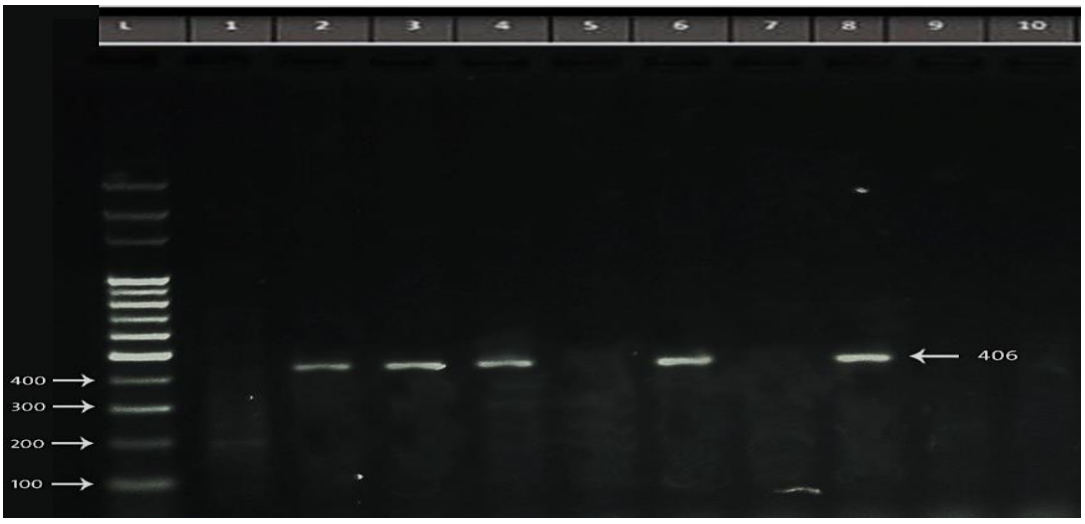

Figure 2: Agarose gel electrophoresis of PCR after amplification of the *b1* gene of *T. gondii* infection in grassing dairy sheep. Line 0: DNA ladder 100 bp, line 1, 5, and 7: negative samples, and lines 2, 3, 4, 6, and 8: DNA samples with amplified product at 406 bp.

DISCUSSION

Being sheep an important reservoir for many pathogens with public health significance, this study was conducted to investigate the seroprevalence and molecular recognition of sheep toxoplasmosis in Diyala province, Iraq. The overall seroprevalence of toxoplasmosis in rams was 18%, while it was 14% in grassing dairy ewes. This result agreed with Deyhimi *et al.* (2019), who recorded a

prevalence of 27.31% in Iran, while it disagreed with Abdel-Aziz *et al.* (2020) in Egypt, who recorded 45.8%, and Ishaku *et al.* (2018) in Nigeria, who documented 35.3% occurrence percentage. This variation may be related to the type of the collected sample, as Deyhimi *et al.* (2019) study was on blood samples and the two other studies were on meat samples. This confirms that *T. gondii* may persist in the blood for a short time but remain in tissues for a longer duration (Almeria and Dubey, 2021).

Molecular examination of seropositive cases had confirmed the infection in 9 (50%) of the ram cases, while it was confirmed in only 5 (35.7%) of the seropositive cases of dairy ewes. This result agreed with a study in Egypt (62.6%) by Elaadli *et al*. (2023). However, it disagreed with the result of 6.5% in Salvador (de Santana Rocha *et al.*, 2015), 5.4% in Brazil (Camossi *et al.*, 2011), and 28% of Slovak Republic-sourced sheep (Luptakova *et al.*, 2015). Various factors, such as farm management, environmental conditions, diagnostic techniques employed, and the presence of cats in the herds could be responsible for the discrepancy in results (Amairia *et al*., 2016).

Seroprevalence of infection was higher in adult slaughterhouses' sheep and dairy ewes than juveniles, although it was statistically insignificant. This confirms previous investigations showing higher rates of exposure to toxoplasmosis infection with increasing age of sheep (Ibrahim *et al.*, 2017; Subedi *et al.*, 2018).

Additionally، there was no significant variation between winter and spring seasons in both rams and diary ewes, and generally, the prevalence was a little higher in winter groups. This higher prevalence in winter may be related to climate conditions and the age of the sheep included in this study, because in this area people always slaughter old sheep during the winter season.

Comparing the native sheep (Iraq) and the imported ones (Syria and Iran), the difference was statistically insignificant. Sharif *et al*. (2015) documented the variation in prevalence of toxoplasmosis, which was at a higher rate in Iran than Iraq. Syrian sheep had an infection rate of 56.01%, which is likewise high. This clarifies why the infection rates of Syrian and Iraqi sheep differed (Adi and Alkhaled, 2011).

Dairy ewes of the Karadi and Hamdani breeds had an insignificant higher infection rate than other breeds. The two possible explanations for this could be as follows: first, sheep like Hamdani and Karadi mainly breed in regions close to Iran's borders, which puts them at high risk of infection. The second factor, currently under investigation, relates to how the immune competence of these breeds compares to the Iraqi Awassi breed.

A non-significant seroprevalence was observed in ewes with a history of abortion compared to non-pregnant ones. The animal might still be infected due to the chronic nature of the disease, the possibility that it had the parasite for a long time, and the fact that abortion did not completely eliminate the parasite (Ali *et al*., 2019).

According to our study, toxoplasmosis was significantly more common in sheep previously interacted with cats. Cats, the definitive host of *T. gondii*, are a major source of infection for humans and animals due to shedding of oocysts into the environment (Frenkel *et al.*, 1970). Thus, transmission of infection can occur via drinking contaminated water and consumption of contaminated food (Robert-Gangneux and Dardé, 2012).

CONCLUSION

The study confirmed the presence of toxoplasmosis infection in Diyala province Iraqi sheep. The prevalence of the disease was higher in adult sheep imported from Iran and Syria, and in adult abortive Karadi and Hamdani ewes, especially in the winter months.

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الكشف المصلي والجزيئي لطفيل التوكسوبلازما جوندي *TOXOPLASMA GONDII* في الأغنام في محافظة ديالى، العراق

محمد خليل ، إبراهيم الزبيدى

Email: mohammed.bawi2204m@covm.uobaghdad.edu.iq Assiut University web-site: www.aun.edu.eg

يعد طفيل التوكسوبلازما جوندي هو السبب الشائع لداء المقوسات في كل من البشر والحيوانات. ويمثل داء المقوسات في الأغنام دورا هاما في الصحة العامة، حيث يسبب خسائر إنجابية واقتصادية بسبب الإجهاض ووفيات الأطفال حديثي الولادة عالميا. كان الهدف من هذه الدراسة هو الانتشار المصلي والكشف الجزيئي لداء المقوسات في الأغنام في محافظة ديالى، العراق. تم فحص مائة خروف من المسالخ و١٠٠ نعجة حلوب وتم تقسيمها إلى مجموعات حسب العمر والموسم والسلالة والاستيراد ومخالطة القطط وحالة الحمل والإجهاض. تم التحقيق في عدوى التوكسوبلازما باستخدام اختبار التوكسوبلازما السريع واختبار تفاعل البلمرة المتسلسل. بلغت نسبة الانتشار المصلي لداء المقوسات في الكباش ١٨%، في حين بلغت ١٤% في النعاج الحلوب. أكد اختبار تفاعل البلمرة المتسلسل الإصابة الإيجابية للمصل في ٥٠% و٣٥,٧% من الكباش والنعاج على التوالي. كان لدى الكباش المجازر والنعاج البالغة المنتجة للألبان معدل انتشار مصلي لداء المقوسات أكبر بشكل غير ملحوظ إحصائيا مقارنة بالأحداث. كان انتشار المرض أعلى قليلا في المجموعات الشتوية سواء في ذكور الأغنام أو النعاج. وشهدت الأغنام المستوردة من سوريا وإيران معدل إصابة أعلى بمرتين من الأغنام المحلية من العراق. لم يكن معدل الإصابة بين النعاج الحلوب من سلالتي الكرادي والهمداني أكبر بكثير من السلالات الأخرى. أظهرت الأغنام التي لديها تاريخ من الإجهاض معدل انتشار مصلي أعلى، كما وجد أيضًا أن داء المقوسات أكثر انتشارًا في الأغنام المختلطة سابقًا مع القطط. ولذلك، يوصى بشدة بالمراقبة المستمرة للكشف عن داء المقوسات في الأغنام لمزيد من الوقاية.