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SEROLOGICAL AND MOLECULAR DETECTION OF *TOXOPLASMA GONDII* IN MILK SAMPLES FROM SHEEP AND GOATS IN ASSIUT GOVERNORATE, EGYPT

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ABSTRACT

Toxoplasmosis is a global zoonotic disease that poses a threat to both animal and human, therefore the present investigation's aims were to detect *T. gondii* antibodies and *T. gondii* DNA in sheep and goat milk samples, and the relationship between epidemiological aspects and *T. gondii* infection. The current study was carried out on lactating sheep and goats (50 of each) from farmers' houses (El-Fateh and Abnoub) in Assiut Governorate. CMT was performed on milk samples to detect subclinical mastitis, while LAT and PCR were utilized to diagnose *T. gondii*. Among the 100 examined animals, 2 (2%) were classified as CMT (++), 9 (9%) as CMT (+), 13 (13%) as suspicious and 76 (76%) as negative. According to LAT, 26% (13/50) of sheep milk samples and 44% (22/50) of goat milk samples contained *T. gondii* antibodies. By using PCR, it was discovered that 86% (43/50) of dairy sheep and 94% (47/50) of dairy goats had *T. gondii* DNA in their milk. The *T. gondii* infection in PCR-examined dairy sheep varied significantly by location ($P<0.01$), but there was no discernible change based on age ($P>0.05$). The percentages of molecularly testing dairy goats infected with *T. gondii* did not significantly differ ($P>0.05$) based on their locality and age. In order to stop and limit the spread of toxoplasmosis in Assiut Governorate, surveillance and sufficient biosecurity measures must be implemented.

Keywords: *T. gondii*, Milk, CMT, LAT, PCR

INTRODUCTION

Toxoplasmosis is a zoonotic infection caused by the obligatory intracellular proto-protozoan parasite *Toxoplasma gondii* (*T. gondii*) (Yousaf *et al.*, 2021 and Arruda *et al.*, 2024). After being discovered for the first time by Nicolle and Manceaux (1908), *T. gondii* attracted more attention because of its significant medical and veterinary value (Razooqi *et al.*, 2022). It affects warm-blood animals, including humans (Masombuka *et al.*, 2024). While numerous species of

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animals act as intermediate hosts, domestic cats are considered definitive hosts because they are the species that excrete *T. gondii* oocysts into the environment, making them crucial to the parasite's epidemiology (Ouslimani *et al.*, 2019 and Dahmane *et al.*, 2024). Among livestock animals, sheep and goats are more commonly and severely impacted by *T. gondii*, and they exhibit elevated seroprevalences in several parts of the world, reaching up to 92% and 75%, correspondingly (Sadek *et al.*, 2015 and Daro *et al.*, 2024). During the acute phase of the disease, sheep and goats excrete *T. gondii* tachyzoites in all bodily fluids, especially milk (Sadek *et al.*, 2015 and Ossani *et al.*, 2017). Consuming raw milk from infected sheep and goats and its byproducts carries a significant risk due to the find of tachyzoites of *T. gondii* in their milk (Tavassoli *et al.*, 2013 and Daro *et al.*, 2024). *T. gondii* tachyzoites died in pasteurized milk with a temperature of 63°C for 30 minutes (Saridewi *et al.*, 2013). Although most human infections are asymptomatic, some patients might have lymphadenopathy or ocular toxoplasmosis (Dahmane *et al.*, 2024). Pregnant women who contract *T. gondii* may suffer from abortion, stillbirth or show other severe outcomes. In addition, toxoplasmosis can be lethal in patients with compromised immune systems if left untreated, and reactivating of a dormant infection can result in encephalitis that is potentially fatal (Mancianti *et al.*, 2013 and Daro *et al.*, 2024). Toxoplasmosis appears as a pregnancy disease in sheep and goats, causing stillbirth, neonatal mortality, mummified lambs, and foetal resorption (Daro *et al.*, 2024 and Elgendy *et al.*, 2024). Some investigations revealed the occurrence of tachyzoites in the milk of various species, including sheep, goat, cow, camel, and buffalo (Dubey, 1998; Dehkordi *et al.*, 2013; Sadek *et al.*, 2015; Medani and Mohamed, 2016; Saad *et al.*, 2018; Madi and Al-Samarai, 2022). Sheep and goat toxoplasmosis is very important as it results substantial financial and production harm that can be spread to humans (Saad *et al.*, 2018). The assessment of *T. gondii* can be achieved through a variety of diagnostic approaches, including serological testing (Enzyme linked immunosorbant assay (ELISA) and Latex agglutination test (LAT)), molecular techniques, cultivating of cell lines, and biological assays (Saad *et al.*, 2018 and Zeedan *et al.*, 2022). Serological tests are more often used for detection of antibodies of the parasite, while Polymerase Chain Reaction (PCR) is the most effective method for determining *T. gondii* infection (Saad *et al.*, 2018) because it has also demonstrated higher reliability, specificity, and sensitivity than other diagnostic techniques (Amairia *et al.*, 2016 and Daro *et al.*, 2024). Thus, the aims of the present investigation were to check for the presence of subclinical mastitis in sheep and goat milk samples, detect *T. gondii* in milk samples from dairy sheep and goats using serological and molecular methods, and finally examine the connection between various epidemiological risk factors, such as location and age, and the infection rate of *T. gondii* in Assiut Governorate of Egypt.

MATERIALS AND METHODS

1. Animals and Ethical approval

A total of 50 lactating sheep and 50 lactating goats of various ages from farmers' houses in Assiut (El-Fateh and Abnoub) in September 2023 were examined for the detection of antibodies of *T. gondii* and for the presence of *T. gondii* DNA in milk. All dairy sheep and goats employed in this research were cared for in compliance with ethical guidelines. The Research Ethical Committee of the Assiut University, Faculty of Veterinary Medicine in Assiut, Egypt, granted an ethical approval number for the current research (06/2024/0202).

2. Sampling

From each dairy sheep and goat, the teats were cleaned and 15 ml of raw milk was collected from both halves of the udder, put into a sterile falcon tube and preserved at -20°C to consequent *T. gondii* antibodies detection and DNA extraction. To diagnose

subclinical mastitis, all milk samples were examined using a California mastitis reagent (Lactotest, Cromasa-Crotales Marcados S.A.) following producer's directions (Kerstin *et al.*, 2008).

3. Serological diagnosis by LAT

Each milk sample was centrifuged in a volume of 1 ml for 10 minutes at 3000 r.p.m. to remove of the cream layer (Sadek *et al.*, 2015). LAT was done for assessing T. *gondii* antibodies in whey (supernatant) of milk samples by Toxo Latex Kit, Egyptian Co for Biotechnology-Spectrum Diagnostic, Cairo, Egypt) according to the guidelines offered by the producer.

4. Molecular assessment

4.1. Extraction of DNA

DNA was obtained from sediment of milk samples using the ABT genomic DNA mini extraction kit (Applied Biotechnology, Egypt), under the producer's recommend-dations.

4.2. Primers

The affinities of the primers (Macrogen, Seoul, Korea) targeting the B1 gene of *T. gondii* in this investigation have been previously validated (Burg *et al.*, 1989). This gene has been amplified 35 times, and it has been shown to have excellent accuracy in identifying *T. gondii* DNA (Sadek *et al.*, 2015). The following Table showed primer sequences along with their positions within the protozoan genome.

Table 1: The B1 gene of *T. gondii's* nucleotide sequence of the primers employed, and the size of the products developed following PCR

Gene	Primer	Sequencing of nucleotides	Size of product (bp)
B1 gene	Forward:1	5′-TCG GAG AGA GAA GTT CGT CGC AT-3′	96
	Reverse: 2	5′-AGC CTC TCT CTT CAA GCA GCG TA-3′	

4.3. PCR (Ghoneim *et al.*, 2009)

The control positive sample containing *T. gondii* DNA was obtained from the Veterinary Research Institute, National Research Center, Cairo, Egypt. PCR was used to amplify the *T. gondii* genome's B1 gene, with 96 bp PCR products length produced. PCR was performed in a thermocycler for PCR (Peqlab, Germany) using the following components: 20 µl of the final volume included 10 µl of 2X ABT red master mix (Applied Biotechnology, Egypt), 1 µl of each primer (10 pmol), 5 µl DNA and 3 µl molecular grade water for PCR. In short, there was one initial denaturation at 95°C for 2 minutes, followed by 35 cycles of denaturation at 95°C for 20 seconds, an annealing step at 55°C for 30 seconds, and an extension step at 72°C for 45 seconds, and then a final extension at 72°C for 10 minutes. For visualizing, 5 µl of amplified PCR products were subjected to 55 minutes of gel electrophoresis at 90 V and 155 mA in a 2% agarose gel dyed with ethidium bromide (10 mg/ml). A UV transilluminator (Syngene, UK) was used to view the amplicons after the using of 100 bp size DNA ladder (Applied Biotechnology, Egypt) to determine their size.

5. Statistical analysis

Relative risk and chi-square tests were performed using the statistical package for the social sciences (SPSS) Statistics software (Version 16, 2007) to ascertain the influence of every aspect independently on *T. gondii* molecular detection in the sheep and goats milk under study (i.e., locality and age). 95% confidence intervals (95% CI) and odds ratios were applied to obtain and evaluate the data. A probability value (P-value) of $P<0.05$ was deemed statistically substantial.

RESULTS

1. Detection of subclinical mastitis by CMT

According to Table (2), A prevalence of subclinical mastitis was revealed by CMT analysis of milk samples from sheep and goats.

Table 2: Prevalence of subclinical mastitis in the examined milk samples of sheep and goats based on the result of CMT

Number of examined samples	Degrees of reaction on samples							
	Strong positive (++ve)		Light positive (+ve)		Suspicious (±ve)		Negative (-ve)	
100 (50 sheep and 50 goats)	No.	%	No.	%	No.	%	No.	%
	2	2	9	9	13	13	76	76

2. Serological diagnosis of *T. gondii* antibodies by LAT

Proportion of *T. gondii* antibodies in both sheep and goat's milk samples by LAT was shown in (Table 3). Positive results indicated by clear agglutination of latex particles in the tested samples.

3. Molecular diagnosis of *T. gondii*

The extracted DNA samples were subjected to PCR to detect *T. gondii* targeting the B1 gene with an expected 96bp product (Figure 1). Table (3) demonstrated the frequency of *T. gondii* DNA in milk samples from sheep and goats.

4. Relative sensitivity and specificity of LAT and PCR in diagnosis of *T. gondii* infection

The results of PCR were more sensitive and specific than LAT (Table 3 and 4). In the present investigation, it was observed that 32 samples were positive by both LAT and PCR, 7 samples were negative by both LAT and PCR, 3 samples were positive by LAT but negative by PCR, and 58 samples were negative by LAT but positive by PCR. Thus, the relative sensitivity and specificity of LAT to PCR were 35.6% and 70%, correspondingly. The overall agreement between the two tests was 65%.

5. Correlation between *T. gondii* infection and CMT

It is clear from the data observed in (Table 5) that there was a relationship between *T. gondii* infection and CMT scores.

6. Connection between the percentage of *T. gondii* infection in sheep and goat and possible risk factors

A few potential aspects that affected the frequency of *T. gondii* infection in sheep and goats were assessed in the present investigation (Table 6 and 7).

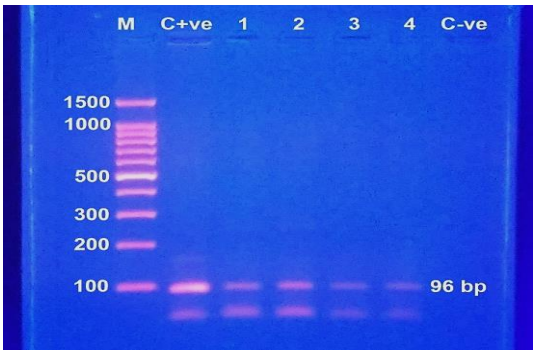

Figure 1: PCR agarose gel electrophoresis following *T. gondii B1* gene amplification in milk samples. Lane M: DNA marker (100 bp), lane C+ve: Control positive sample, lanes 1,2,3,4 positive milk samples with amplified product at 96 bp, and lane C-ve: Control negative (distilled water).

Table 3: Prevalence of *T. gondii* in milk samples of sheep and goats based on LAT and PCR

Species	No. of samples	LAT		PCR	
		+ve (%)	-ve (%)	+ve (%)	-ve (%)
Sheep	50	13 (26%)	37 (74%)	43 (86%)	7 (14%)
Goat	50	22 (44%)	28 (56%)	47 (94%)	3 (6%)
Total	100	35 (35%)	65 (65%)	90 (90%)	10 (10%)
P-value		0.06		0.18	

Table 4: Sensitivity and specificity of LAT with PCR for detection of *T. gondii* infection

Test		PCR		Total	Sensitivity (%)	Specificity (%)	Overall Agreement (%)
		Positive	Negative				
LAT	Positive	32	3	35	35.6%	70%	65%
	Negative	58	7	65			
	Total	90	10	100			

Table 5: Correlation between LAT and PCR for determination of *T. gondii* infection and CMT scores

Method	No.	%	CMT score							
			Strong positive (n=2)		Light positive (n=9)		Suspicious (n=13)		Negative (n=76)	
			No.	%	No.	%	No.	%	No.	%
LAT	35	35%	0	0%	2	5.71%	5	14.29%	28	80%
PCR	90	90%	2	2.22%	9	10%	12	13.33%	67	74.45%

Table 6: Relationship between potential aspects and *T. gondii* infection in sheep's milk established by the PCR findings.

Variable		No. of investigated animals	PCR		Odds ratio	95% CI	P-value
			Positive No. (%)	Negative No. (%)			
Locality	El-Fateh city	25	25(100%)	0 (0%)	0.419	0.29-0.60	0.004**
	Abnoub city	25	18 (72%)	7 (28%)			
	Total	50	43 (86%)	7 (14%)			
Age	1.5-2.5 years	42	36 (85.71%)	6 (14.29%)	0.857	0.09-8.27	0.894
	>2.5-4 years	8	7 (87.50%)	1 (12.50%)			
	Total	50	43 (86%)	7 (14%)			

**Highly significance differences (P<0.01).

Table 7: Relationship between potential aspects and *T. gondii* infection in goat's milk established by the PCR findings.

Variable		No. of investigated animals	PCR		Odds ratio	95% CI	P-value
			Positive No. (%)	Negative No. (%)			
Locality	El-Fateh city	25	25 (100%)	0 (0%)	0.468	0.35-0.64	0.07
	Abnoub city	25	22 (88%)	3 (12%)			
	Total	50	47 (94%)	3(6%)			
Age	1.5 -2.5 years	44	41 (93.18%)	3 (6.82%)	0.872	0.78-0.97	0.51
	>2.5 - 4 years	6	6 (100%)	0 (0%)			
	Total	50	47 (94%)	3(6%)			

DISCUSSION

The current study showed the high prevalence of *T. gondii* in sheep's milk (86%) and goats' milk (94%). The high prevalence of *T. gondii* infection in investigated sheep and goats suggested that they were continuously exposed to infection due to ecological exposure to excreted oocysts from stray cats with inadequate managemental circumstances (Sadek *et al.*, 2015). In this study, the prevalence of *T. gondii* antibodies was 26% in sheep milk samples and 44% in goats' milk samples by LAT. A higher percentage was emphasized by Sadek *et al.* (2015), who reported that prevalence of *T. gondii* antibodies was 39.66% in sheep milk samples. Lower percentages were obtained by Dehkordi *et al.* (2013) and Da Silva *et al.* (2015), who determined that the prevalence of *T. gondii* antibodies was 5.94 % and 3.76% in sheep milk samples, respectively. Compared to the obtained findings, a greater outcome was recorded by Abdel-Rahman *et al.* (2012), who recorded that 58.90% of the investigated goat milk samples had *T. gondii* antibodies. However, other studies concluded lower prevalence rates, where Dehkordi *et al.* (2013) observed *T. gondii* antibodies in 8.88% of goat milk samples, Da Silva *et al.* (2015) referred to *T. gondii* antibodies in 5.78% of goat milk samples, and Sadek *et al.* (2015) noted *T. gondii* antibodies in 38.30% of goat milk samples. Since molecular methods can detect the parasite's DNA rather than the antibodies produced against *T. gondii*, they are more precise, sensitive, and specific assays than serological methods (Saad *et al.*, 2018). Several molecular methods were used to identify *T. gondii* DNA by propagation of the target *B1* gene, a gene sequence that is repeated 35 times. The amplification of this gene demonstrated great accuracy for the identification of *T. gondii* DNA (Sadek *et al.,* 2015). The findings of this investigation concluded that 86% and 94% of sheep and goats' milk samples contained DNA of *T. gondii*. The DNA in the current study was higher than those previously reported, where *T. gondii* DNA was determined in milk samples of 4.63% sheep and 1.07% of goat (Tavassoli *et al.,* 2013) and of 6.83% sheep and 7.79 % of goat (Zeedan *et al.,* 2022). The variation in these findings may be due to various factors, such as the study areas, breeding conditions, management, and distribution and behavior of cats (Sadek *et al.*, 2015). From the current findings, lactating goats showed a greater proportion of *T. gondii* infection than lactating sheep. The lower rate of *T. gondii* infection in sheep compared to goats, which was possibly explained by the animals' different feeding habits and susceptibilities to *T. gondii* (Sadek *et al.*, 2015). In the current study, LAT findings did not match the PCR findings, since not all serologically positive animals excrete the parasite in their milk. A number of milk samples that tested positive for PCR had negative LAT findings. The animal's immunity and the stage of infection determine this elimination. The positive milk samples by LAT and the negative by PCR imply that parasite shedding may occur intermittently (Razooqi *et al.*, 2022). It is possible that the negative milk samples tested by LAT but positive by PCR could indicate that these animals were in the early stages of the infection, when there was not enough antibody present for serological test to identify the infection (Sadek *et al.*, 2015). Furthermore, the current investigation showed that *T. gondii* was being excreted in the milk of sheep and goats, which implies the chance of *Toxoplasma* transmission to people by consuming of raw milk and its unpasteurized derivatives, which could affect public health.

The results of the CMT scores of the tested milk samples from sheep and goats revealed that 2 (5.71%) of 35 positive milk samples by LAT, and 11 (12.12%) of 90 positive milk samples by PCR may suffer from subclinical mastitis. This finding may be attributed to *T. gondii* infection is not

directly linked to mastitis, but it causes widespread systemic inflammation or affects organs involved in immune response, it could indirectly contribute to an elevated SCC in affected animals.

The correlation amongst various potential factors and the frequency of *T. gondii* infection was studied in the present investigation. These factors included locality, age, and health status. According to the study's location, the percentage of *T. gondii* infection in the lactating sheep and goats obtained from El-Fateh city was higher than that from Abnoub city. This might be possible because lack of awareness of farmers about raising of sheep and goats through grazing in unhygienic areas (trash) with lots of cats, which increased their exposure to *T. gondii* oocyts. In terms of age susceptibility, the percentage of *T. gondii* infection in the age groups of sheep and goats didn't vary significantly. This result supported the conclusions of Jabar and Jori (2013), who observed that the age groups of sheep and *T. gondii* infection did not vary statistically significantly, and Amairia *et al.* (2016), who found that there were no statistically significant effects between the age groups of goats and *T. gondii* infection. One explanation for our findings might be that sheep and goats of all ages had the same risk of infection with *T. gondii*.

CONCLUSION

The obtained results indicated that sheep and goats' milk samples in Assiut Governorate, Egypt, had a high proportion of *T. gondii*. Depending on the collected findings, it is possible to recommend sufficient pasteurizing or boiling milk before consuming it to reduce the possibility of parasites transmission to milk consumers. Additionally, keeping street cats away from other animals will help to prevent the cats' feces from contaminating the animals' feed. Furthermore, routine testing of infected sheep and goats' milk with serological and molecular techniques aids in disease surveillance and, consequently, helps in limiting the transmission of infections to other animals and humans. So, it is advised to use more than one technique in diagnosis of *T. gondii* infection in sheep and goats. Finally, farmers need to be aware of grazing sheep and goats in clean areas.

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الكشف المصلي والجزيئي عن التوكسوبلازما جوندي في عينات ألبان الأغنام والماعز في محافظة أسيوط ، مصر

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داء المقوسات مرضٌ مشترك عالمي، يُشكل خطرًا على كلٍّ من الحيوان والإنسان، لذلك هدفت الدراسة الحالية إلى الكشف عن الأجسام المضادة للتوكسوبلازما الجوندية والحمض النووي لها في عينات ألبان الأغنام والماعز، والعلاقة بين الجوانب الوبائية والإصابة بالتوكسوبلازما الجوندية. أُجريت الدراسة الحالية على الأغنام والماعز الحلوب (٥٠ رأسًا من كلٍّ منهما) من بيوت الفلاحين (الفتح وأبنوب) في محافظة أسيوط. أُستخدم اختبار كاليفورنيا لالتهاب الضرع على عينات الألبان للكشف عن التهاب الضرع تحت الاكلينيكى، بينما استُخدم اختبار الاتكس وتفاعل البلمرة المتسلسل لتشخيص التوكسوبلازما جوندي. أظهرت نتائج اختبار كاليفورنيا لالتهاب الضرع إيجابية قوية, إيجابية خفيفة, مشتبه بها وسلبية في ٢ (٢٪) و٩ (٩٪) و١٣ (١٣٪) و٧٦ (٧٦٪)، على التوالي. كما وجد أن ٢٦٪ (٥٠/١٣) من الأغنام الحلوب و٤٤٪ (٥٠/٢٢) من الماعز الحلوب تحتوي على أجسام مضادة للتوكسوبلازما جوندي وفقًا لاختبار الاتكس. واكتشف أن ٨٦٪ (٥٠/٤٣) من الأغنام الحلوب و٩٤٪ (٥٠/٤٧) من الماعز الحلوب تحتوي على الحمض النووي للتوكسوبلازما جوندي في ألبانها طبقا لتفاعل البلمرة المتسلسل،. وقد تباينت عدوى التوكسوبلازما جوندي في عينات ألبان الأغنام التي تم فحصها باستخدام تفاعل البلمرة المتسلسل بشكل كبير حسب مكان جمع العينات ولكن لم يكن هناك فرق واضح بناءً على العمر. وقد لوحظ أن نسب ألبان الماعز التي تم اختبارها جزيئيًا والتي كانت مصابة بالتوكسوبلازما جوندي لم تختلف بشكل كبير بناءً على موقعها وعمرها. ومن الدراسة تبين انه يجب وضع تدابير كافية للمراقبة والأمن الحيوي من أجل وقف انتشار داء المقوسات والحد منه في محافظة أسيوط.