RESEARCH ARTICLE

Molecular Detection of *Toxoplasma gondii* in Ewes Placenta in Northeastern Algeria

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Abstract: The present study aimed at the direct detection of parasitic DNA in placenta samples of ewes by PCR targeting the B1 gene of *Toxoplasma gondii*. We identified also the possible risk factors associated with the infection. A total of 307 female sheep from 23 farms were collected between 2019 and 2020 in the Tebessa region in northeastern Algeria. Data showed that *T. gondii* DNA was detected in 114 of the 307 tested females (37.1%). The on-farm molecular prevalence was 82.6%. High rates were revealed in Bir Al Ater (32.4%) and Tlidjen (43.6%). The level of contamination was high in farms applying a sedentary mode (47.6%); this system tends to have a positive effect on the prevalence of toxoplasmosis (P<0.05; 95% CI: 0.409-0.544). Moreover, it appears that the relationship between the presence of cats on the farm and the prevalence of the disease was significant (P<0.05; 95% CI: 0.445-0.597). In the same context, primiparous females were 2.54 times more likely to be infected with *T. gondii* than multiparous animals (P=0.001). To conclude, the prevalence noticed in the present study indicates a generalized exposure of sheep to *T. gondii* in the northeast of Algeria, which represents a major risk for animal and public health. Therefore, management measures should be implemented and improved in the farms of this region for better disease control and eradication.

Keywords: *Algeria, Ewes, Molecular detection, Placenta, Toxoplasma gondii*

Cezayir'in Kuzeydoğusundaki Koyun Plasentalarında *Toxoplasma gondii*'nin Moleküler Tespiti

Öz: Bu çalışmada, *Toxoplasma gondii*'nin B1 genini hedef alan direkt PCR ile koyun plasenta örneklerinde parazitik DNA'nın saptanması amaçlandı. Enfeksiyona ilişkin olası risk faktörleri de değerlendirildi. Cezayir'in kuzeydoğusundaki Tebessa bölgesinden 2019-2020 yılları arasında 23 çiftlikten toplam 307 dişi koyun örneklendi. Veriler, test edilen 307 dişi koyunun 114 (%37.1)'ünde *T. gondii* DNA'sının tespit edildiğini gösterdi. Çiftlikteki moleküler prevalans %82.6 olarak saptandı. Bir Al Ater (%32.4) ve Tlidjen'de (%43.6) pozitiflik yüksek orandaydı. Yerleşik mod uygulayan çiftliklerde kontaminasyon seviyesi yüksekti (%47.6); bu sistem, toksoplazmoz prevalansı üzerinde olumlu bir etkiye sahip olma eğilimi gösterdi (P<0.05; %95 CI: 0.409-0.544). Ayrıca, çiftlikte kedi varlığı ile hastalığın prevalansı arasında anlamlı bir ilişki saptandı (P<0.05; %95 CI: 0.445-0.597). Aynı bağlamda, primipar dişilerin, multiparlara göre *T. gondii* ile enfekte olma olasılığı 2.54 kat daha fazlaydı (P=0.001). Sonuç olarak, bu çalışmada saptanan prevalans, Cezayir'in kuzeydoğusundaki koyunların *T. gondii*'ye genel olarak maruz kaldığını göstermektedir ve bu durum, hayvan ve halk sağlığı için büyük bir risk teşkil etmektedir. Bu nedenle, hastalık kontrolü ve eradikasyonunun daha iyi yapılabilmesi için bu bölgedeki çiftlik yönetimine ait uygulamaların iyileştirilmesi ve uygulanması gerekmektedir.

Anahtar sözcükler: *Cezayir, Koyun, Moleküler tespit, Plasenta, Toxoplasma gondii*
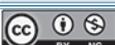
INTRODUCTION

Toxoplasma gondii is an important protozoan parasite found worldwide that potentially infects all warm-blooded vertebrates, including mammals, birds, and humans [1]. *T. gondii* cysts have been found in the tissues of pigs, sheep, goats, and other animals. They are transmitted to humans by the accidental ingestion of oocysts located in cat's feces or by eating raw or undercooked meat containing cysts [2]. *T. gondii* is the primary parasite responsible for both sheep and goat abortion [3]. These species represent a significant source of infection, mainly in those regions or countries where sheep and goat meat is routinely consumed [1]. As this parasite affects both animals and humans, food-borne transmission is one of the major sources of *T. gondii* infection by ingesting tissue cysts from under-cooked meat [4].

Toxoplasmosis is relatively common in small ruminants [5]. Although most infections in these species are asymptomatic, there can be abortions, fetal mummification, stillbirths, and birth of weak lambs and kids [6]. The exposure of a pregnant ewe to primary infection with *T. gondii* may, depending on its stage of gestation when the infection occurs and the infective dose, result in the death of the fetus and lead to significant losses in affected flocks as a result of barrenness, abortion, and stillbirths. The birth of clinically normal, infected lambs usually results from a primary infection contracted during the latter part of pregnancy [7]. Once infected, ewes develop good protective immunity against the parasite, which protects against disease in subsequent pregnancies [8].

Infectious abortion is one of the major flock health problems faced by sheep farmers and has a significant financial impact on production. Toxoplasmosis is one of the main causes of infectious reproductive failure in small ruminants in the world [8,9].

Small ruminant production is one of the most important sources of meat in Algeria and plays a vital role in the country's food security [10]. However, the prevalence of *T. gondii* infection in these animals in Algeria remains largely unknown. Only a few data on the infection of small ruminants are available, not updated or just limited to small areas. However, some epidemiological studies as part of final theses and doctorates in medical sciences have provided an idea of seroprevalence in sheep that varied from 8.28% to 35.37% [11,12]. Only one molecular study of blood samples was conducted with a recorded prevalence of 35.2% in ewes [13].

Therefore, this current work aimed to report the results of a cross-sectional survey on the molecular prevalence of *T. gondii* infection in placenta samples of ewes in the northeastern part of Algeria, where these animals have great economic importance. The study also assessed the possible risk factors associated with the infection to estimate the risk of toxoplasma abortion in this region.

MATERIAL AND METHODS

Ethical Statement

All the animal studies were conducted with the utmost regard for animal welfare, and all animal rights issues were appropriately observed. No animal suffered during the work. All the experiments were carried out according to the guidelines of the Institutional Animal Care Committee of the Algerian Higher Education and Scientific Research (Agreement Number: 45/DGLPAG/DVA.SDA. 14).

Period and Type of Study

Our work covered the farrowing and abortion seasons from September to January and March to May for one year. This is a descriptive exploratory study conducted from September 2019 to October 2020.

Study Sites and Materials

The study took place in the steppe of Tebessa in the northeastern of Algeria (35°24'15.0"N 8°07' 27.0" E), which constitutes the predilection area of sheep and goat breeding. This zone is mountainous and is 960 m above sea level. It has a semi-arid climate characterized by hot summers and cold, wet winters with a rainfall averaging 363 mm per year. The study area can be broadly divided into three departments of the central zone of Tebessa, including Bir Al Ater, Tlidjen, and Negrine *(Fig. 1)*.

The work was conducted on ewes that had abortion problems within 8 days. Flock selection was based on abortion rates exceeding 5% since a lower rate is considered normal in a flock and does not attract the attention of the farmer [14]. A total of 307 female sheep from 23 farms were collected between September 2019 and October 2020. Eight farms were located in Bir Al Ater, 10 in the Tlidjenarea, and 5 in Negrine.

Data Collection

In each case of abortion, the commemoratives were noticed for each animal and each farm. Data were collected on age and the reported stage of pregnancy at the time of the abortion. In addition, herd-level information was collected (breeding system, abortion history, and presence of stray cats in the farm) and used as explanatory variables.

Samples Collection

In the selected farms, samples concerned only females with abortion. Placentas were collected under sterile conditions using a disposable scalpel blade. One cotyledon from each placenta was collected and stored in sterile 1.5 mL tubes at -20°C until analysis in the laboratory.
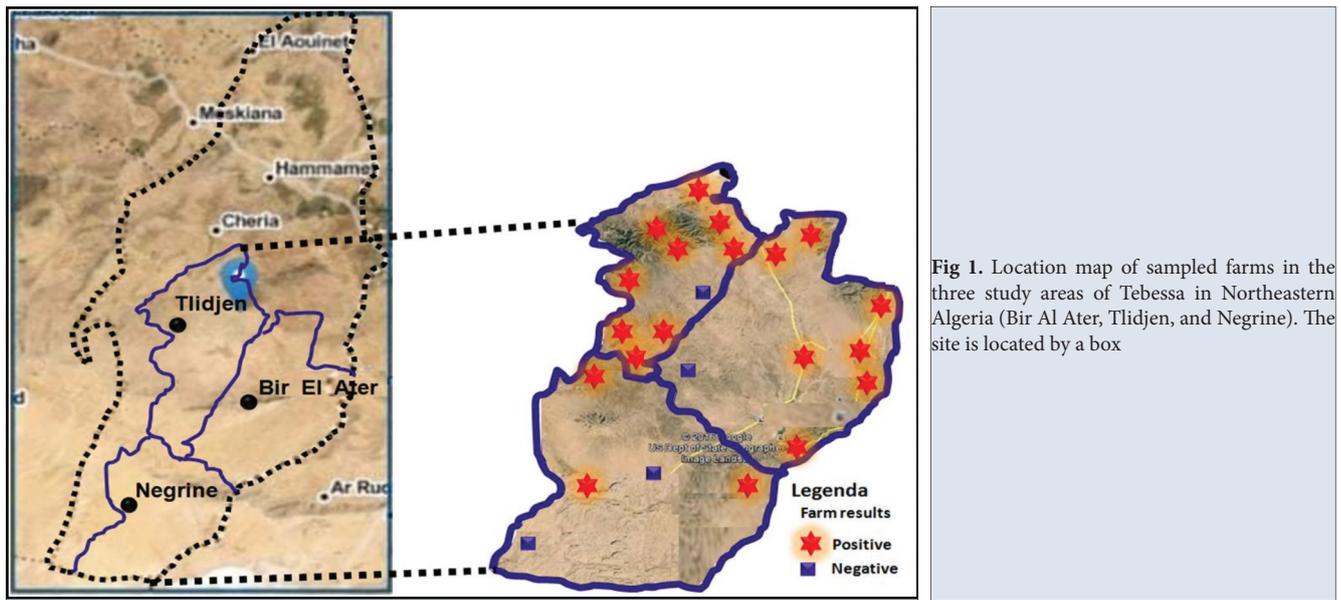
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Fig 1. Location map of sampled farms in the three study areas of Tebessa in Northeastern Algeria (Bir Al Ater, Tlidjen, and Negrine). The site is located by a box

DNA Extraction and Nested PCR for the Detection of *T. gondii*

Samples were partially thawed at laboratory temperature for 10 min. DNA was extracted from 100 mg of homogenized cotyledon using a commercial QIAmp DNA tissue Mini Kit (Qiagen, France) according to the manufacturer's protocol. Due to the high concentration of DNA, the purified DNA samples were resuspended in ultrapure water. DNA concentrations were determined by spectrophotometric analysis, and all samples were diluted to a final concentration of 300 ng/µL and stored at -20°C before PCR analysis.

The presence of *T. gondii* was detected by conventional PCR using the nucleotide sequence of the B1 gene as a target. A pair of primers, JW63: (5'-GCACCTTTC GGACCTCAACCG-3') and JW62 (5'-TTCTCGCCTCA TTTCTGGGTCTAC-3') were used to amplify a 286 bp fragment of the gene target as described by Pelloux et al.[15]. The PCR reaction was performed in 50 µL of a mixture containing 5 µL of sample DNA diluted with 17.5 µL of water of injections, 1.25 µL of each primer, and 25 µL of Master Mix. The latter is a prepared solution containing: 1x Taq polymerase buffer supplemented with $MgCl_2$ (3 µM), 1.6 µM of each dNTP, and 50 Units/mL Taq DNA polymerase (GoTaq®, Promega). Amplification was performed on a thermal cycler (Applied Biosystem 2700) by incubation in 4 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 1 min at 55°C, 1 min at 72°C, and final 5 min at 72°C. As a positive control, *T. gondii tachyzoites* (BALBcstrain) were obtained from ascites of previously infected mice. Negative controls (double distileed water) were included in each set of PCR reactions. Amplified products were analyzed by 2% agarose gel electrophoresis and visualized on a UV screen by ethidium bromide staining.

Statistical Analysis

A herd was considered positive if at least one animal in the herd was tested positive. The prevalence was calculated by dividing the number of positives by the number of tested animals. The p-value and the confidence interval of 95% (95% CI) were also calculated. The analysis was conducted globally (at the level of the herd) and then individually. Pearson's Chi-square was used to test the different variables. All questionnaire responses were included in the statistical analysis as independent variables.

Statistical analyses were finalized via logistic regression using XLSTAT software version 2016.02.28451. First, univariate logistic regression analysis was performed for all hypothesized risk factors to investigate possible relationships between *T. gondii* prevalence and the different risk factors. Second, all variables were entered into a multivariate model, developed by backward elimination until all remaining variables were significant (P≤0.05).

RESULTS

T. gondii DNA was found in 19 (82.6%; 95% CI: 90-100%) of the 23 farms. At the individual level, *T. gondii* was detected in 114/307 placenta samples indicating an overall molecular prevalence of 37.1% *(Table 1)*. The spatial distribution of positive farms is shown in *(Fig. 1)*. Intra-herd prevalence for infected farms ranged from 18.2% to 61.9%. Negative animals were found on all farms. The infection rate was more than 50% in 17.4% of the herds (4 herds), and in 14 farms (60.9%), more than 25% or less than 45% of animals were positive. Only 1 farm had a percentage below 20% *(Fig. 2)*.

The results obtained from the univariate analysis of the risk factors are presented in *Table 2*. The molecular prevalence

Table 1. *Toxoplasma gondii infection rates and confidence interval (CI) at 95% in the studied population*

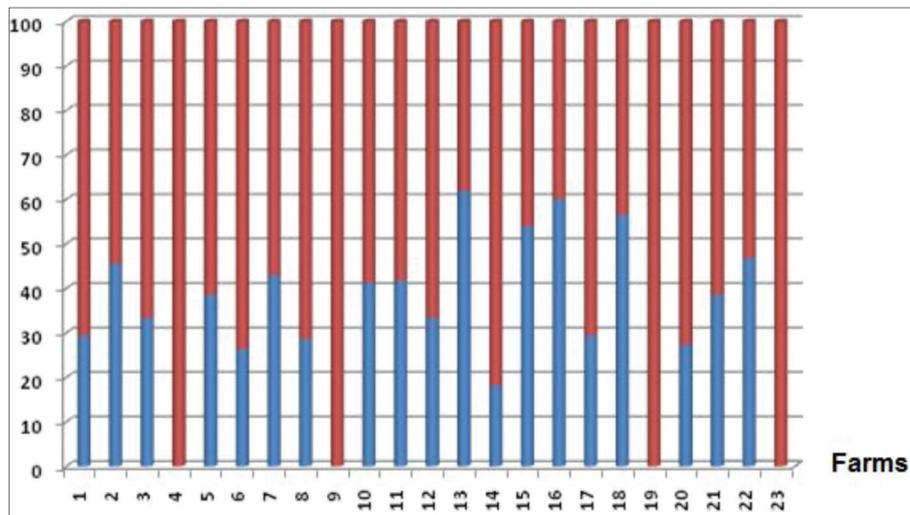| Animals			Herds		
Number of Tested Animals	Number of Positive Animals (n) (%)	95%CI	Number of Tested Herds	Number of Positive Herds (n) (%)	95%CI
307	114 (37.1%)	[31.72-42.53]	23	19 (82.6%)	[67.11-98.09]

Fig 2. The proportion of *T. gondii*-positive *(blue)* and negative *(red)* animals in the 23 sheep farms

Table 2. *Descriptive statistics and univariate analysis of the effect of different variables on toxoplasmosis prevalence*

Variable	Category	Number of Tested Animals	Number of Positive Animals	%	95% CI	P-Value
Collection area	Bir Al Ater	105	34	32.4	0.234- 0.413	[1] VS [2] Chi2 = 3.25 P=0.07
	Tlidjen	149	65	43.6	0.357-0.516	[1] VS [3] Chi2 =0.27 P=0.6
	Negrine	53	15	28.3	0.162-0.404	[2] VS [3] Chi2 =3.76 P=0.05
Age	Primiparous	137	77	56.2	0.479-0.645	Chi2 = 36.41 P<0.05
	Multiparous	170	37	21.8	0.156-0.280	
Abortion stage	Early gestation (1-90 days)	139	61	43.9	0.356-0.521	Chi2 = 4.92 P<0.05
	End of gestation (90-145 days)	168	53	31.5	0.245-0.386	
Breeding mode	Sedentary	210	100	47.6	0.409-0.544	Chi2 = 27.66 P<0.05
	Transhumant	97	14	14.4	0.074-0.214	
History of abortions	Yes	236	99	41.9	0.357-0.482	Chi2 = 9.66 P<0.05
	No	71	15	21.1	0.116-0.306	
Presence of cats	Yes	167	87	52.1	0.445-0.597	Chi2 = 32.86 P<0.05
	No	140	27	19.3	0.128-0.258	
Statistically significant variables are indicated by bold typing; [1] Bir Al Ater, [2] Tlidjen, [3] Negrine						

in both sites; Bir Al Ater (32.4%) and Tlidjen (43.6%) was higher. This shows that ewes from the Negrine area influence the prevalence of *T. gondii* (P=0.05; 95% CI: 0.162- 0.404). Regarding age, the highest prevalence was recorded in primiparous females with 56.2%, compared to multiparous females (21.8%). It appears that there

is a significant difference (P<0.05; 95% CI: 0.156- 0.280) in Toxoplasma prevalence between the two age groups. Besides, statistically significant results were revealed according to the gestational stage (P<0.05; 95% CI: 0.356-0.521). Regarding the variable "rearing mode", the level of contamination was high in farms applying a sedentary

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Table 3. *Multivariate logistic regression analysis data of the supposed risk factors in relation to toxoplasmosis*

Variable	Category	Odds Ratio	95% CI	P-Value
Age	Primiparous	2.540	1.436-4.495	0.001
Abortion stage	Early gestation (1-90 days)	1.155	0.641-2.081	0.631
Breeding mode	Sedentary	3.972	2.038-7.742	< 0.0001
Presence of cats	Yes	3.188	1.725-5.892	0.0001
Statistically significant variables are indicated by bold typing				

mode (47.6%); this system tends to have a positive effect on the prevalence of toxoplasmosis (P<0.05; 95% CI: 0.409-0.544). Concerning the risk factor "previous abortion"; in fact, the prevalence of toxoplasmosis increased with the existence of a history of abortion (P<0.05; 95% CI: 0.357-0.482). Moreover, it appears that the relationship between the presence of cats on the farm and the prevalence of the disease is significant (P<0.05; 95% CI: 0.445-0.597).

Four risk factors for toxoplasmosis were retained in the multivariable model, with three variables significantly associated with a higher probability of herds being positive *(Table 3)*. For the farm management variables, the risk of toxoplasmosis positive was 3.97 times higher in sedentary livestock (P<0.0001). Furthermore, the presence of a cat on the farm was significantly associated with a high rate of toxoplasmosis (OR=3.18, P=0.0001). Regarding the abortive syndrome, only age was identified as a potential risk factor for *T. gondii* infection. In this study, primiparous animals were 2.54 times more likely to be infected with *T. gondii* than multiparous animals (P=0.001). In contrast, the variable stage of gestation was not significantly related to the risk of developing the disease (OR = 1.15, P=0.63).

DISCUSSION

Toxoplasma gondii is a food- and waterborne parasite that infects nearly all mammals, including humans [16,17]. Sheep and goats are important sources of infection for humans, representing an important public health role. In addition, toxoplasmosis is an important cause of neonatal mortality in sheep and goats, resulting in reproductive and economic losses worldwide [18,19]. Epidemiological data on *T. gondii* infections in animals for human consumption are not regularly collected and the current lack of standardization of diagnostic techniques and protocols should be taken into account when comparing seroprevalence data [20]. Molecular analysis of *T. gondii*, which detects the circulating parasites would be useful for the final diagnosis. Serological findings are only an indication of infection, while molecular detection of *T. gondii* in blood or other samples provides the presence of the parasite in the body [21,22].

Previously in Algeria, the prevalence of *T. gondii* infection in animals was assessed by serological tests [17]. In our present work, the detection of *T. gondii* in ewes placenta was carried out by molecular analysis.

Data revealed a herd prevalence of 82.6%. This rate is among the previously reported values in Algeria, obtained from the study of toxoplasmosis seroprevalence in sheep ranged from 57.89%- 100% [23,24]. Other works conducted in European countries have shown variable seroprevalence: 98.4% and 87.5% of sheep flocks were positive in surveys conducted in Southern Spain in 2020 [25], and in Northern Italy in 2015 [1], respectively.

In the present study, *T. gondii* DNA was detected in 114 females out of 307 placenta samples (37.1%) by conventional PCR based on B1 gene amplification. In a previous study of blood samples, similar values were reported in sheep from the same region (35.2%) [13]. Our results were higher than that of a molecular report confirming the presence of *T. gondii* in 1.69% of sheep carcasses in Northern India [26] and that found by Prasad Sah et al.[27] in Bangladesh (15.52%) in sheep tissue samples. Other previous studies conducted on placental tissue samples have shown different rates. Indeed our result was higher than that found in ewes in Italy (3.5%) [28]. It is also consistent with other data found by PCR in ovine abortion products (fetuses and placenta) in the same country (31.5%) [29].

The divergence in toxoplasmosis prevalence may be explained at first glance by the differences in methodology, in sample size and sampling techniques [30], and in climatic variations and feline density [31,32].

In our study, the "site" effect was associated with the prevalence of *T. godii* in the univariate analysis. Females collected in the regions of Bir Al Ater and Tlidjen showed a high molecular prevalence. A statistical relationship was found between the prevalence of infection and females from the Negrine area, characterized by a high presence of cats. *T. gondii* oocysts excreted by cats remain infective for years under favorable conditions (adequate humidity and temperature) [33].

In tested females, molecular prevalence was positively correlated with age, as reported in previous studies [34,35]. The higher risk of *T. gondii* infection in primiparous ewes suggests that once infected and aborted females generally

do not abort upon re-exposure to the parasite, even though the parasite survives as a cyst until the end of the mother's life. The ewe then harbors bradyzoites and becomes immune after the first infection [36,37].

In the present study, although stage of gestation was not indicated as a risk factor associated with *T. gondii* infection in the multivariate analysis, it was positively associated with the prevalence of toxoplasmosis in the univariate analysis. Females with early abortions had higher prevalence. This variable was also identified as a risk factor in a previous study which is consistent with our finding [9]. In another study performed in Brazil, Silva Filho et al.[38] reported that all abortions occurred in the last months of gestation. Therefore, it has been mentioned that the immune system of the ovine fetus can respond to *T. gondii* at 60 days of gestation or shortly thereafter. Thus, infection before 40 days of gestation is probably due to local suppression of immune mechanisms in the maternal placenta and the immaturity of the fetal immune system. In contrast, infection between 40 and 120 days may be attributed to insufficient immunity to confer protection until the last month before birth [39].

Consistent with our results on husbandry, the multivariate model analysis detected two risk factors for toxoplasmosis. Indeed, extensive management was certainly an important risk factor associated with infection. The high rate was observed in farms with sedentary breeding. This result is in agreement with that of Heidari et al.[32] who reported that sedentary managed animals are much more able to acquire Toxoplasma. In a completely enclosed farm, the infection risk is almost limited to the introduction of new animals and the presence of vectors such as rodents or insects. In contrast, in extensive farming, where animals are potentially in contact with animals from other farms, wildlife, or a contaminated environment, the infection risk is much higher [40]. Also, the presence of felines on farms increases the risk of toxoplasmosis. These findings are in agreement with the biology of *T. gondii* and emphasize that the presence of cats plays a central role in *T. gondii* infection. This may indicate high contamination of production areas (both pastures and containment facilities) with *T. gondii* oocysts, thus necessitating sanitary control and prevention measures on farms. The significant correlation with the prevalence of toxoplasmosis has been cited several times [41]. Finally, and consistent with our previous observations, the variable "history of abortions" was identified as a risk factor for the disease in the univariate analysis. Molecular prevalence was positively associated with the existence of a history of abortions in the farms, which was previously revealed by Gharekhani [31]. Therefore, the infection is likely to reappear after a few years due to the increased number of susceptible animals in the replacement generations [13].

Toxoplasma gondii infection has occurred with a high molecular prevalence in ewes. The results of this study show that toxoplasmosis is present in the sheep population of Tebessa, confirming that this species could be an important source of *T. gondii* among consumers in this area. Hygiene and dietary advice should be disseminated to consumers of sheep meat and particularly to vulnerable individuals (pregnant women and immunocompromised patients not yet immunized for this disease), including the importance of consuming sufficiently cooked meat. The data obtained further underestimates the risk factors associated with *T. gondii* infection and the relationship between the parasite and these small ruminant hosts. Such information may be useful for veterinarians and farmers to develop or improve toxoplasmosis control plans in herds in the study areas and in areas where farming systems are similar to those described in the current work. In addition, given that Algeria raises sheep for domestic consumption, isolation of *T. gondii* from sheep with molecular characterization of isolates will be necessary to assess better and understand the risk of ovine toxoplasmosis to human health. If isolates of virulent genotypes are found, this will increase the risk of potentially serious infestation to humans.

AVAILABILITY OF DATA AND MATERIALS

The datasets during and/or analyzed during the current study available from the corresponding author (N. Ait Issad) on reasonable request.

COMPETING INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

AUTHOR CONTRIBUTION

NAI: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Writing - Original Draft, Writing, Review and Editing. KA, SB, RB, HHA, TGAH, NM: Resources, Methodology, Validation, Formal Analysis, Investigation, Project administration. DD: Statistical analysis. RK: Investigation, Writing - Review & Editing, Project administration. DK: Supervision, Investigation, Writing - Review & Editing, Project administration.

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