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Molecular detection of *Toxoplasma gondii* in goat placenta in northeastern Algeria

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Summary

Toxoplasmosis, caused by *Toxoplasma gondii*, is a parasitic zoonosis of crucial medical and veterinary importance. It is diagnosed mainly by serological methods, which are insufficiently sensitive. It is therefore necessary to rely on the direct detection of the parasite. The present study aimed at the direct detection of *T. gondii* DNA in placenta fragments by PCR targeting the B1 gene of the parasite. In addition, we identified possible risk factors for infection. The study was carried out on 25 goat farms between 2019 and 2020 in four regions of the Tebessa province in northeastern Algeria and involved 503 goats that had aborted. *T. gondii* DNA was detected in 30.41% of goat placenta samples (CI: [27.12-41.53]). The on-farm molecular prevalence was 60% (CI: [53.32-72.53]). The molecular prevalence of toxoplasmosis was higher for primiparous females (53.2%) than it was for multiparous ones (15.2%) (p = 0.0001). Likewise, the level of contamination was high in farms with a sedentary management system (36.4%); this system tended to increase the prevalence of toxoplasmosis (P = 0.008). In addition, it appears that the stage of gestation, history of abortion, and season had a significant effect (p < 0.05) on the prevalence of the disease. To conclude, this study revealed that goats included in this experiment were heavily infected with *T. gondii*, which represents a major risk for consumers in the Tebessa region. Further research is needed to improve our knowledge of the different genotypes of *T. gondii* infecting populations of small ruminants.

Keywords: Algeria, goats, molecular detection, placenta, *Toxoplasma gondii*

Toxoplasma gondii is a zoonotic apicomplexan able to infect probably all warm-blooded animals, including livestock (3, 16). It has adverse effects on public health and animal production (27, 36, 42).

In recent years, increasing attention has been paid to *T. gondii* infection. According to the World Health Organization, more than 60% of infectious agents affecting humans and newly described in the last decade are caused by animals or animal products. Toxoplasmosis is one of the most common zoonotic diseases in the world (8). The high prevalence of toxoplasmosis among domesticated animals, such as cattle, sheep, and goats, can be an important cause of disease transmission to humans (12, 13).

T. gondii infection in goats is highly prevalent worldwide and is considered an important cause of reproductive disorders.

In goats, infection occurs mainly by ingestion of spore-forming oocysts from pasture or water contaminated with cat feces (45). However, congenital transplacental transmission may also contribute to the persistence and dissemination of the protozoan infection in flocks. In this species, the main clinical signs are fever, anorexia, dyspnea, embryonic resorption and mummification, abortion, and neurological signs (3, 8). In spite of the high prevalence of *T. gondii* reported in goats around the world (47), only limited data are available on the prevalence of *T. gondii* in goats in Algeria (3).

Goat farming in Algeria is undergoing profound changes, gradually transitioning from extensive farming to market-oriented breeding in order to adapt to the new context characterized by a sustained demand for goat meat and milk for sustainable development (7). It occupies a marginal place in consumption. Besides, it contributes strongly to the family economy and regional culture (46). According to FAO statistics (14), in 2017, Algeria had about 5 million head of goats, which was 2% of their total number in African countries and 28% of that in the European Union. Thus, Algeria produces 42,000 tons of goat meat per year, whereas Greece, the largest producer of meat of this species, produces 45,000 tons. The bulk of the population is distributed in steppe and sub-desert areas (24). The province of Tebessa has a goat population estimated at 110,000 head, including 77,000 females. Most of these animalas are concentrated in the southeastern part of the region, particularly in El Ma Labiodh, Ogla Malha, Bir Al Ater, and Cheria (30).

Although *T. gondii* infection causes significant economic losses in goats, its prevalence in Algeria might be underestimated or limited to small areas. Only one molecular study was performed in the central area of the Tebessa province, which found a prevalence rate of 18.7% (2). In view of the zoonotic importance of *T. gondii*, the aim of the present study was to determine the molecular prevalence of *T. gondii* infection in goats in the southeastern region of Tebessa, where these animals have a significant economic importance. The study also evaluated possible risk factors for the infection, in order to estimate the risk of toxoplasmic abortion in this region. The investigation of *T. gondii* infection in goats has important implications for the prevention and control of the disease in animals and humans in this province and elsewhere.

Material and methods

Ethical considerations. Permission for this study was first obtained from village authorities. Oral consent was obtained from all breeders of small ruminants in farms selected for blood sampling. All 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).

Study site and sample collection. Epidemiological monitoring was conducted in four regions of the province of Tebessa in northeastern Algeria (35°24'15.0"N 8°07'27.0" E) where sheep and goat rearing is dominant and conducted extensively under different production systems. This mountainous area, situated 960 m above sea level, is filled with high meadows containing some small shrubs. It has a semi-arid climate characterized by hot summers and cold wet winters with a rainfall averaging 363 mm per year. The four localities in the southeastern zone of the region (El Houiddjbet, El Ogla Malha, El Ma Labiodh, Bir Al Ater) (Fig. 1) were selected according to the presence

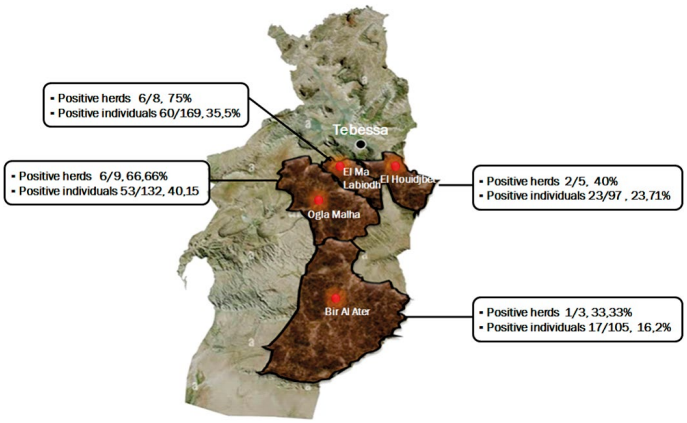

Fig. 1. Location of the farms selected for sample collection in four regions of the Tebessa province in Northeastern Algeria (El Houiddjbet, El Ogla Malha, El Ma Labiodh, Bir Al Ater)

of small ruminants and livestock activities which constitute an important basin of small ruminants.

Between 2019 and 2020, a total of 503 placenta samples from 25 farms were collected from veterinary clinics in rural districts of Tebessa, Algeria. All animal samples were collected under sterile conditions with a disposable scalpel blade. One cotyledon from each placenta was collected and stored in sterile 1.5 ml tubes at –20°C until shipped to the laboratory. Each sample was accompanied by a set of epidemiological data. Samples were generally taken from females that had recently aborted (only females that had aborted within 8 days were sampled). One criterion for herd selection was an abortion rate exceeding 5%, since a lower rate is considered normal in a farm and does not alarm the breeder (29). Five farms were located in El Houiddjbet, nine in El Ogla Malha, eight in El Ma Labiodh, and three in Bir Al Ater. The total population consisted of 503 females distributed as follows: 97 in El Houidjbet (19.3%), 132 in Ogla Malha (26.2%), 169 in El Ma Labiodh (33.6%), and 105 in Bir Al Ater (20.9%).

This was an epidemiological surveillance of abortion outbreaks in the four regions during two campaigns (2019-2020). In each case of abortion, a survey form was filled out for each animal and for each farm in order to carry out epidemiological investigations and possibly take samples of placenta fragments. Herd-level information was collected regarding the number of females that had aborted on each farm as well as farm management (breeding system, abortion history, and presence of cats in and around farming areas). The questionnaire also included information on goats that had aborted, such as age, gestational stage, and season at the time of abortion. In order to study the seasonality of the disease, herd visits were conducted over two years (September 2019 to November 2020), covering the two seasons of the year: the cold rainy season (September to January) and the hot dry season (March to May).

DNA extraction and nested PCR for the detection of *T. gondii.* Samples were partially thawed at room temperature for ten minutes. 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 DNA concentration, 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 prior to PCR analysis.

The presence of *T. gondii* was detected by nested PCR using the nucleotide sequence of the B1 gene as target. A pair of primers, JW63: (5'-GCACCTTTCG-GACCTCAACCG-3') and JW62 (5'-TTCTCGCCT-CATTTCTGGGTCTAC-3') were used to amplify a 286 bp fragment of the target gene as described by Ait Issad et al. (3). The PCR reaction was carried out in 50 µl of a mixture containing 5 µl of sample DNA diluted with 17.5 µl of H_2O, 1.25 µl of each primer, and 25 µl of Master Mix. Master Mix is a prepared solution containing 1 × Taq polymerase buffer supplemented with $MgCl_2$ (3 µM), 1.6 µM of each dNTP, and 50 Units/ml of Taq DNA polymerase (GoTaq®, Promega). The amplification was carried out on a thermocycler (AppliedBiosystem 2700) by 4 min incubation at 94°C, followed by 35 cycles of 30 sec at 94°C, 1 min at 55°C, 1 min at 72°C, and 5 minutes at 72°C. *T. gondii* tachyzoites (strain BALBc) used as a positive control were obtained from ascites of previously infected mice, and negative controls (double distillation of water) were included in each series of PCR reactions. The amplification products were analyzed by electrophoresis on 2% agarose gel and visualized on a UV screen by staining with ethidium bromide. To avoid false positive reactions, DNA extraction, PCR, and electrophoresis were performed in separate rooms with different sets of instruments, and aerosol protective caps and disposable gloves were used.

Statistical analysis. A herd was considered positive if at least one animal tested positive. The prevalence was calculated by dividing the number of animals positive by the total number tested. The p-value and 95% confidence interval were also calculated. The analysis was carried out globally 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 performed by logistic regression using XLSTAT software version 2016.02.28451. First, a univariate logistic regression analysis was carried out for all putative risk factors in order to study the possible links between the prevalence of *T. gondii* and the different risk factors. Second, all variables were entered into a multivariate model, expanded by backward elimination until all remaining variables were significant ($p \leq 0.05$).

Results and discussion

In our study, we analyzed molecular data obtained from placenta samples of female goats. All results are presented in Table 1. We were able to estimate prevalence for the total population, represented by 503 goats from 25 farms. Figure 2 summarizes the main data for the 25 farms surveyed. We also noted that there were large variations in prevalence within different herds. In fifteen of the twenty-five farms, *Toxoplasma gondii* coexisted within the herd, which showed a regular circulation of this pathogen within the breeding herd. *T. gondii* infection was confirmed in 60% of the goat herds (95% CI: 53.32-72.53%). Individually, animals positive for toxoplasmosis were very numerous: 30.41% (153/503) with a confidence interval of 95%, [i.e. 27.12-41.53]

Intra-herd prevalence for infected farms ranged from 28% to 64.7%. *T. gondii* was most prevalent in herds 2, 6, and 11, with a rate greater than 60 [46.3-91.1]%.

The prevalence of *T. gondii* infection was almost identical in herds 5 and 7, where it amounted to 42.8 [29.9-90.1]% and 42 [35.4-84.6]%, respectively, followed by herds 3, 4, 10, 12, 14, and 15, with prevalence rates of 40.6 [31.3-73.5]%, 40.7 [25.1-69.7]%, 39.1 [26.7-54.3]%, 39.1 [28.5-51.3]%, 39.3 [21.7-54.1]%, and 39.4 [23.7-51.1]%, respectively. The least infected were herds 1, 9, 17, and 22, with prevalence rates of 28 [22.2-46.8]%, 29.4 [14.8-62]%, 29 [19.2-46.8]%, and 29.4 [17.3-36.1]%, respectively.

A significant association was demonstrated between the rates in different farms ($p = 0.025$).

Our results confirm that toxoplasmosis of small ruminants is widespread in the four regions, with fifteen of the twenty-five herds estimated positive and therefore presumed infected. The presence of *T. gondii* was higher in the El Ogla Malha and El Ma Labiodh regions (40.15% (53/132), 35.5% (60/169)) than it was in the El Houidjbet and Bir Al Ater regions (23.71%

Tab. 1. Rates of *T. gondii* infection and the confidence interval at 95% in the studied population

	Animal			Herd		
	Tested animals (n)	Positive animals (n/%)	95% CI	Tested animals (n)	Positive animals (n/%)	95% CI
Goats	503	153 (30.41%)	[27.12-41.53]	25	15 (60%)	[53.32-72.53]

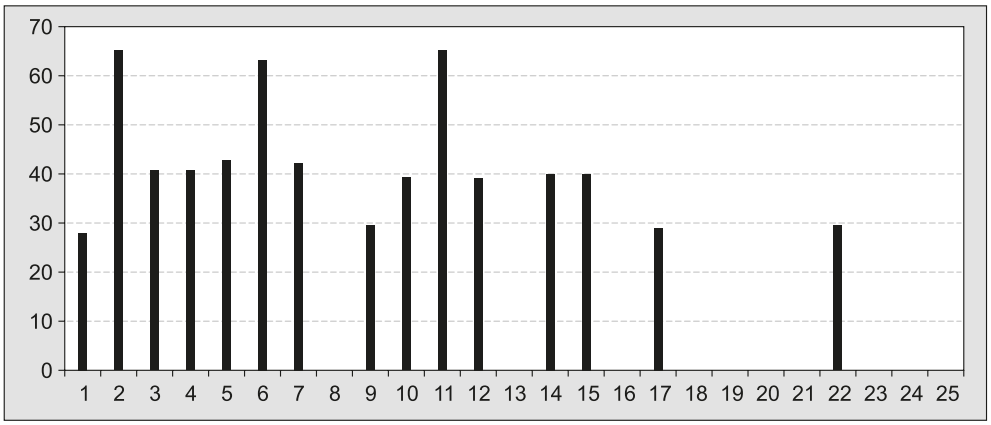

Fig. 2. Proportion of *T. gondii*-positive animals in 25 goat farms in 4 study areas

(23/97), 16.2% (17/105)). Thus, there is a significant relationship between the first two locations (El Ogla Malha, El Ma Labiodh) and toxoplasma prevalence (p = 0.0001, p = 0.04).

Data obtained from the univariate analysis of the risk factors for goats are given in Table 2. The factor „age" had a significant effect (p = 0.0001; 95% CI: [46.33-60.13]) on the prevalence of toxoplasmosis. Females positive for toxoplasmosis constituted 53.2% of the primiparous group and 15.2% of the multiparous group. Regarding gestational stage, positive females were 46.2% of those with early gestational abortions and 20.3% of those with late gestational abortions. It appears that there is a significant difference (p = 0.0001; 95% CI: [39.23-53.15]) in Toxoplasma prevalence between the two stages of gestation. Besides, the type of breeding affected the prevalence of toxoplasmosis, with a p-value of 0.008; 95% CI: [30.99-41.85]. The rate was high in farms applying a sedentary system 36.4%. In addition, a history of abortion in the herd was a high risk factor for toxoplasmosis (p = 0.03; 95% CI: [29.91-40.79]). Beyond that, statistically significant results were observed depending on the season (p = 0.002; 95% CI: [32.11-43.22]).

In the multivariable model, five risk factors for toxoplasmosis were retained, all of the five variables being significantly associated with a higher probability of herds being positive (Tab. 3).

The current study is the second report on *T. gondii* DNA detection in ruminant placenta in Algeria based on a previous analysis of blood samples. We investigated the results of the second cross-sectional epidemiological survey conducted in the Tebessa region, designed to estimate the prevalence of *T. gondii* and identify risk factors associated with its circulation. We analyzed molecular data obtained from placenta samples from female goats kept in 25 farms that had abortion problems between 2019 and 2020 in four districts of Tebessa where abortions have been observed in goats. This information is necessary to formulate measures against toxoplasmosis, which has a significant impact on the performance of small ruminants.

Tab. 2. Descriptive statistics and univariate analysis of the effect of different factors on *T. gondii* prevalence

Variable		Tested	Positive (%)	95% CI	P-value
Sampling area	El Houidjbet [1]	97	23 (23.71)	[15.24-32.17]	[1] ESR [2] Chi^2 = 3.49, **p = 0.06** [1] ESR [4] Chi^2 = 1.2, p = 0.27
	Ogla Malha [2]	132	53 (40.15)	[31.78-48.51]	[1] ESR [3] Chi^2 = 2.14, p = 0.14 [2] VS [4] Chi^2 = 15.59, **p = 0.0001**
	El Ma Labiodh [3]	169	60 (35.5)	[28.28-42.71]	[2] VS [3] Chi^2 = 3.92, **p = 0.04**
	Bir Al Ater [4]	105	17 (16.2)	[9.14-23.23]	[3] VS [4] Chi^2 = 1.2, p = 0.27
	Total	503	153 (30.41)	[26.39-34.43]	
Age	Primiparous	201	107 (53.2)	[46.33-60.13]	Chi^2 = 42.31, **p = 0.0001**
	Multiparous	302	46 (15.2)	[11.17-19.28]	
Stage of Gestation 'days'	Early gestation [1-90]	197	91 (46.2)	[39.23-53.15]	Chi^2 = 19.65, **p = 0.0001**
	Late gestation [90-145]	306	62 (20.3)	[15.75-24.76]	
Breeding system	Sedentary	302	110 (36.4)	[30.99-41.85]	Chi^2 = 7.05, **p = 0.008**
	Transhuman	201	43 (21.4)	[15.72-27.06]	
History of abortions	Yes	297	105 (35.3)	[29.91-40.79]	Chi^2 = 4.53, **p = 0.03**
	No	206	48 (23.3)	[17.52-29.07]	
	No	153	16 (10.4)		
Season	Cold (autumn and winter)	292	110 (37.7)	[32.11-43.22]	Chi^2 = 9.47, **p = 0.002**
	Hot (spring and summer)	211	43 (20.4)	[14.94-25.81]	

Explanations: Statistically significant variables are indicated by bold typeface; [1] El Houidjbet, [2] Ogla Malha, [3] El Ma Labiodh, [4] Bir Al Ater; 95% CI: 95% Confidence Interval

Tab. 3. Multivariate analysis of the effect of different factors on *T. gondii* prevalence

Variable		P-value	Odds ratio	Odds ratio Lower bound (95%)	Odds ratio Upper bound (95%)
Age	Primiparous	< 0.0001	8.619	4.989	14.889
Stage of gestation	Early gestation (days 1-90)	< 0.0001	0.188	0.104	0.337
Season	Cold	< 0.0001	0.216	0.122	0.383
Breeding system	Sedentary	< 0.0001	4.418	2.432	8.026
History of abortions	Yes	< 0.0001	7.059	3.684	13.527

Explanations: Statistically significant variables are indicated by bold typeface; 95% CI: 95% Confidence Interval

Epidemiological data on *T. gondii* infections in animals for human consumption are not collected regularly, and the current lack of standardization of diagnostic techniques and protocols should be taken into account when comparing prevalence data (43). The PCR technique was chosen as a laboratory diagnostic method because of its high sensitivity. The good sensitivity of PCR in diagnosing Toxoplasma has already been reported by several studies (33, 37). According to Owen et al. (33), who related abortion to hyperthermia which accompanies the primary infection, cases of false negative diagnosis occur during abortions less than 14 days after infection. Molecular analysis of *T. gondii*, which detects 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 confirms the presence of the parasite in the body (6, 35).

Given the nature of samples used in the search for *Toxoplasma gondii*, any positive result can, in our opinion, allow the veterinarian to link abortion to the presence of the parasite.

The present study established a herd prevalence rate of 60%. The results showed the presence of *T. gondii* in fifteen goat farms. These results differ from those already reported from Algeria by studies aimed at evaluating the seroprevalence of toxoplasmosis in sheep, which found rates ranging from 66.66% to 87.2% (10, 32). Other studies conducted in European countries have shown variable seroprevalence. As much as 96.6% of goat herds in northern Italy and and 93.7% in southern Spain tested positive (17, 23). Regarding the 15 herds where positive cases were found, we can affirm that the intra-herd infection could have been related to environmental factors, including the herd management system in those farms.

In the present study, 30.41% of goat females tested positive. This result was higher than that reported by a previous survey carried out on blood samples from goats in the same region (18.68%) (2). It exceeds prevalence reported from several other countries. *T. gondii* DNA was detected in 15.52% of tissue samples from goats that had aborted in Bangladesh (34), and in 14.3%, 25%, and 15.4% of placenta and blood samples from female goats in Bangladesh, Italy, and the Republic of Korea, respectively (20, 28, 31). The divergence may be explained by differences in methodology, sample size, sampling technique (19), climatic variation, and the density of felines (18, 22). However, our findings could suggest that animals from the study area may be more susceptible to toxoplasmosis or simply overexposed to the parasite.

The PCR targeting the B1 gene made it possible to obtain an overall prevalence rate of 30.41%, which varied according to the site. The prevalence rate was higher in El Ogla Malha (40.15%) and El Ma Labiodh (35.5%). A statistical relationship was demonstrated between the prevalence of infection and females in those localities ($p = 0.0001$, $p = 0.04$), which could be explained by the local farming method for this species, that is, group management of herds belonging to several breeders from the same village. These factors favor the rapid transmission of infection. In addition, those localities are characterized by a strong presence of cats. *T. gondii* oocysts excreted by cats remain infectious for years under favorable conditions (i.e. adequate humidity and temperature) (44).

The results of the study reveal a variation in the prevalence rate according to the age of goats: 53.2% in primiparous females and 15.2% in multiparous ones. Thus, primiparous females were more frequently infected with toxoplasmosis. In fact, age was significantly associated with *T. gondii* infection in primiparous animals ($p = 0.0001$). According to this study, primiparous goats were 8.619 times as likely to be infected with *T. gondii* as multiparous ones. This has also been reported previously (2, 4, 21). The higher risk of *T. gondii* infection in primiparae suggests that, once infected, females that have previously aborted generally do not abort upon further exposure to the parasite, even if the parasite survives as a cyst until the end of the mother's life. The goat then harbors bradyzoites and becomes immunized after the first infection (38, 40).

The analysis of the results according to the period of reproductive loss shows that the prevalence rate was higher for females having early abortions (46.2%). The gestational stage significantly influenced the rate of toxoplasmosis ($p = 0.0001$). Indeed, females having abortions at the beginning of gestation were also at higher risk of toxoplasmosis, with OR = 0.188, $P < 0.0001$. This is also consistent with results of PCR in abortion products of the goat species in France (40). In contrast, studies conducted by Silva Filho et al. (39) in Brazil and by Ait Issad et al. (2) in Algeria indicated that all abortions took place during the last months of gestation. In fact, it has been reported that the ovine fetal immune system may respond to *T. gondii* at or shortly after day 60 of gestation. Thus, infection before day 40 of gestation is likely due to the local suppression of immune mechanisms in the maternal placenta and the immaturity of the fetal immune system, whereas infection between days 40 and 120 may be attributed to immunocompetence being insufficient to confer protection until the last month before birth (9).

With regard to farm management, extensive breeding was found to be statistically significantly associated with the prevalence of toxoplasmosis in univariate analysis ($p = 0.008$) and tested as a risk factor for toxoplasmosis in the binary model (OR = 4.418, $P < 0.0001$). A high rate was observed in farms applying a sedentary system. These results converge with those of Heidari et al. (22) and of Freycon (15), who

reported that animals under sedentary management are much more likely to acquire Toxoplasma. This contrasts with a previous study that found statistically insignificant associations in extensively managed herds (17). In completely closed off-ground farming, the risk of infection is limited almost entirely to the introduction of new animals into the farm and the presence of vectors, such as rodents or insects, whereas in extensive farming, where animals are potentially in contact with those from other farms, wildlife, or a contaminated environment, the risk of infection is much higher (5).

Furthermore, the prevalence of toxoplasmosis increases with a history of abortion ($p = 0.03$). The analyses revealed higher infection rates for herds with a history of abortion (35.3%). In addition, this variable is a risk factor for *T. gondii* infection (OR = 7.059, $P < 0.0001$), which has already been reported (18).

Finally, the high proportion of positive results during the cold, very wet season can only be explained by climatic conditions, which constitute an important factor in the persistence of oocysts (11, 25). In our study, the same results were obtained for female goats, more of which were infected in rainy periods than in hot periods. The "site" effect was clearly associated with the prevalence of the disease ($p = 0.002$). Indeed, females that had abortions in the cold season were also at greater risk of toxoplasmosis (OR = 0.216, $P < 0.0001$), which is in agreement with findings of other studies (2, 26). In addition, small ruminants are at risk of ingesting large quantities of oocytes and brucella because of the density of vegetation at that time. However, the association of the cold season with pathogen positivity results from the occurrence of abortions with increased shedding of parasites.

This study confirmed the circulation of the etiological agent of toxoplasmosis (*Toxoplasma gondii*) in goat herds in Tebessa. Our results revealed that goats are heavily infected with *T. gondii*, confirming that the goat species could be an important source of *T. gondii*. This represents a major risk for consumers in this area, especially for pregnant women, who need to be made aware of this risk and preventive measures they should take (avoid consumption of undercooked meat). Several factors influence the molecular prevalence of *T. gondii* in goats: it was higher in primiparous females and during the cold season. The DNA of *T. gondii* was isolated mainly from goats which had aborted during the first three months of gestation, and the level of contamination was high in farms applying a sedentary system and for herds with a history of abortions. Such information may be useful for both veterinarians and livestock keepers in developing or improving toxoplasmosis control programs for herds in the study area and/or for those under similar farming systems. Further studies are needed to improve our knowledge on the different genotypes of *T. gondii* infecting Algerian goat herds.

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