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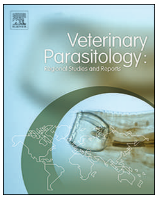

# Detection of *Toxoplasma gondii* infection in semen of rams used for natural mating in commercial sheep farms in Tunisia

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## ABSTRACT

Toxoplasmosis, a parasitic infection, disrupts sexual functions resulting in decline in reproductive and economic performance in sheep. Lacking discernible symptoms, toxoplasmosis is difficult to detect and diagnose in infected animals. Here, to estimate the prevalence of natural infection by *Toxoplasma gondii*, we used PCR to amplify and detect *T. gondii* DNA in semen from 92 rams of three breeds from four regions in Tunisia and seroprevalence was determined using a commercial ELISA kit. From the PCR amplifications, 51.09 ± 10.21% of the rams were tested positive for *T. gondii* with an overall seroprevalence of 39.13 ± 9.97%. Risk factors including ram location and number of accomplished mating seasons significantly ($p < .05$) affected the sero- and molecular prevalence of *T. gondii* in semen but, there was a fair concordance between sero- and molecular prevalence (*Kappa* = 0.33). Sequences of *T. gondii* from five positive samples were 100% identical (same haplotype). Comparison of these sequences with those archived at the GenBank showed a sequence similarity range between 95 and 100%. The haplotype defining the five Tunisian sequences was similar to the one observed in chicken, cats, European pole cat and humans from Brazil, St Kitts and Nevis, Great Britain and Tunisia, respectively. This indicates its wide geographic distribution and non-species specificity. Our findings suggest a high prevalence of toxoplasmosis in Tunisian matting rams; further studies concerning its venereal transmission capacity are needed prior to recommending a systematic screening of *T. gondii* DNA in rams' semen used for both natural breeding and artificial insemination.

## 1. Introduction

Reproductive diseases adversely affect reproductive efficiency by disrupting several key events of the reproductive cycle in all livestock species. In sheep, they can lead to delays in the onset of puberty and age at first lambing, foetal mortality, abortions and ram infertility (Fthenakis et al., 2015). Toxoplasmosis commonly occurs in livestock species (Gharbi et al., 2013; Khayeche et al., 2014) and causes reproductive complications (Blewett et al., 1982). Compared to sheep, cattle are more resistant to *T. gondii* infection making *T. gondii* the most important cause of reproductive problems in sheep as compared to all other warm-blooded animal species except humans. After the first contamination, sheep remain persistently contaminated for their whole lives; so undercooked meat is the major source of infection for humans. Contrarily, in cattle tissues very few tissue cysts develop and persist (Esteban-Redondo et al., 1999). In addition to the horizontal (Dubey et al., 2014) and vertical transmission (Weiss and Dubey, 2009), *Toxoplasma gondii* can be also sexually transmitted in many species such as rabbits (Liu et al., 2006), dogs (Arantes et al., 2009) and sheep (Lopes et al., 2009a) with consequent vertical transmission to lambs (Lopes et al., 2013). In addition to natural mating, artificial insemination is also a potential transmission route for *T. gondii* to females in goats (Wanderley et al., 2013) and sheep (de Moraes et al., 2010a,c) through fresh semen. Infective forms of *T. gondii* have previously been identified

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in semen of sheep (Spence et al., 1978), goats (Dubey and Sharma, 1980) and swine (Moura et al., 2007). There appears to be a relation between toxoplasmosis and the disturbance of reproductive parameters in males. In fact, experimentally induced acute *T. gondii* infections in male mice cause pathological changes in testes, epididymis, vas deferens, prostate and thalamus, and infertility (Yang et al., 2005). Furthermore, reductions in sperm motility and sperm concentration were reported in rats (Terpsidis et al., 2009). In rams, Lopes et al. (2009b) showed that *T. gondii*-infected rams produce smaller ejaculate volumes than non-infected animals. Due to its simultaneous effects in both females and males, toxoplasmosis can be a major impediment to satisfactory reproductive efficiency in sheep.

The aim of this study was to estimate the serological prevalence of *T. gondii* infection in comparison with the molecular prevalence of natural infection in rams' semen from different regions in Tunisia. The study also aimed to genetically characterize *T. gondii* amplicons and identify the risk factors.

## 2. Materials and methods

### 2.1. Study area and samples

Semen and serum samples were collected from 92 rams used for natural mating from 4 farms, prior to and during the mating season, between April and September 2015. Samples were collected from rams of different age groups and breeds. Among the sampled animals, 82 (89.1%) were of the main sheep breed in the country (Barbarine) and 10 (10.9%) from two other less common breeds (Noire de Thibar and Queue Fine de l'Ouest) found in the North of Tunisia. Samples were collected from four locations in Tunisia: Bou Salem (district of Jendouba), Mouhamadia (district of Ben Arous), Saouef (district of Zaghouan) and Oueslatia (district of Kairouan) (Table 1).

Rams usually start their first mating season aged between 18 and 24 months. The number of mating seasons was therefore calculated based on the age of the animal at sampling. This was determined from the records of the different targeted farms. Whole blood was collected in dry tubes via jugular venipuncture; the sera were separated and stored at −20 °C until analysed. Simultaneously, semen samples were collected from each ram using an artificial vagina, kept in sterile tubes, and transported immediately to the National School of Veterinary Medicine of Sidi Thabet and stored at −20 °C until analysed. To avoid cross contamination, new liners and cone for the artificial vagina were used for each sampled ram.

### 2.2. Serology

All serum samples were examined for IgG antibodies against *T. gondii* infection using a commercial ELISA kit (ID Screen® Toxoplasmosis Indirect Multi-species, Montpellier, France) according to the manufacturer's instructions.

### 2.3. DNA extraction

The frozen semen samples were thawed and a volume of 500 μl was used for DNA extraction. After centrifugation at 13000 g for 20 min, a volume of 300 μl of the lysis buffer (1% sodium dodecyl sulfate, 100 mM NaCl, 10 mM Tris–HCl pH 8.0, 25 mM disodium EDTA pH 8.0, 10 mg/ml proteinase K) was added to the pellet and vortexed (Keid et al., 2007). After 3 cycles of freezing and thawing (−20 °C and + 50 °C, respectively), 10 μl proteinase K was added and the samples were then incubated overnight at 37 °C. The aqueous phase containing nucleic acids was extracted from the lysates using a commercial DNA extraction kit (Wizard® Genomics DNA Purification Kit; Promega, Madison Wisconsin, USA) according to the manufacturer's instructions.

A universal set of primers (forward: 5′-AACCTGGTTGATCCTGCC AGT-3′ and reverse: 5′-GGCACCAGACTTGCCCTC-3′) was used to verify the presence and quality of DNA in each extract prior to performing PCR. The universal PCR was carried out in 25 μl reaction volumes consisting of 1× PCR buffer, 2 mM $MgCl_2$, 10 μM of each primer, 0.2 mM of each dNTP, 2 U Taq polymerase (Vivantis, Chino, California), and 1.5 μl of DNA template. The DNA amplification was performed in Esco Swift Max Pro® thermocycler using the following cycling profile: initial denaturation for 5 min at 94 °C, followed by 25 cycles (94 °C; 59 °C and 72 °C for 50 s each) and a final extension at 72 °C for 10 min (Wang et al., 2014).

### 2.4. PCR amplification of the B1 gene of Toxoplasma gondii

The 114 base-pairs of *T. gondii* B1 gene was amplified via PCR using two primers, a forward one: B22 (5′- AACGGGCGAGTAGCACCTGAGG AGA-3′) and reverse one: B23 (5′- TGGGTCTACGTCGATGGCATGAC AAC-3′). The PCR reaction was carried out in a final volume of 25 μl consisting of 10 × PCR buffer, 2.5 mM $MgCl_2$, 200 μM of each dNTP, 0.6 mg BSA, 10 pmol of each primer, 1U Taq polymerase and 6 μl of sample DNA. The PCR amplification profile involved two initial preliminary steps of 2 and 6 min at 50 °C and 95 °C, respectively followed by 40 cycles of 30 s at 94 °C, 30 s at 57 °C and 1min at 72 °C adding 1 s/cycle. A final elongation step of 7 min at 72 °C completed the PCR run. PCR products were resolved in 3% agarose gel and visualized under UV light (Boughattas et al., 2014) (Fig. 1).

### 2.5. DNA sequencing and phylogenetic analyses

Five positive amplicons from three regions (Bou Salem, Mouhamadia and Saouef) were randomly selected for sequencing and phylogenetic analysis. These were purified using the ExoSAP-IT™ PCR Product Cleanup Reagen (ThermoFisher), according to the manufacturer's instructions. The purified PCR products were sequenced in both directions with the two PCR primers (B22 and B23), using the BigDye® Terminator v3.1 Cycle Sequencing Chemistry (Applied Biosystems, ThermoFisher) on an ABI Prism 3500 DNA analyser.

### 2.6. Statistical analyses

The Chi-square Mantel-Haenszel test with Epi Info 6 at a threshold of 5% (Schwartz, 1993) was used to compare the infection prevalences within and between different groups. The level of agreement between the PCR and ELISA results was assessed with the *Kappa* test (Toma et al., 2007). The interpretation of the *Kappa* values was as follows: poor (< 0); slight (0.01–0.20); fair (0.21–0.40); moderate (0.41–0.60), substantial (0.61–0.80), almost perfect (0.81–1.00) (Landis and Koch, 1977).

For each of the five samples sequenced, two fragments were generated for the B1 gene. These were edited manually using ChromasPro software (version 1301.7.4) and subsequently joined to reconstruct a fragment of 114 bp of the B1 gene. The fragments were aligned using the MEGA 7 software (Tamura et al., 2011) which was also used to perform multiple sequence alignments. Furthermore, the generated sequences were compared with *T. gondii* amplicons deposited in the GenBank using the Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information (NCBI)[1]. A phylogenetic tree was constructed using the Neighbour-joining (NJ) algorithm (Saitou and Nei, 1987) as implemented in MEGA 7 following 1000 bootstrap replications.

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[1] See https://blast.ncbi.nlm.nih.gov/Blast.cgi

**Table 1**
Characteristics of the studied area (Climate data.org, 2016; Dateandtime.info, 2016; Ministry of agriculture, 2014).

| Locality | District | Localization | Mean annual rainfall (mm) | Mean temperature (°C) | Latitude | Longitude | Total sheep population (thousands) |
|---|---|---|---|---|---|---|---|
| Bou Salem | Jendouba | North-West | 542 | 9.8–27.9 | 36°30′ N | 8°46′ E | 232 |
| Mouhamadia | Ben Arous | North | 455 | 10.6–26.6 | 36°45′ N | 10°13′ E | 45 |
| Saouef | Zaghouan | North-East | 477 | 9.4–26.8 | 36°24′ N | 10°08′ E | 366 |
| Oueslatia | Kairouan | Centre-East | 293 | 10.4–27.9 | 35°40′ N | 10°05′ E | 718 |

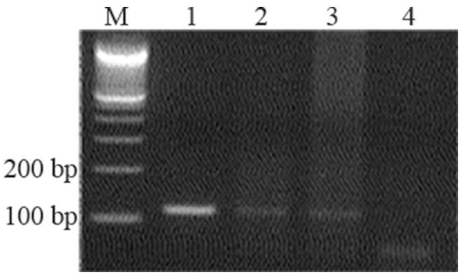

**Fig. 1.** Agarose gel electrophoresis of *Toxoplasma gondii* infection PCR.
M: 100 bp ladder; 1: positive control; 2, 3: positive PCR products and 4: negative control.

## 3. Results

### 3.1. Seroprevalence of *Toxoplasma gondii*

The overall seroprevalence of *T. gondii*, which differed significantly between the four study locations ($p < .001$), was 39.1 ± 10.0%. It was higher in Bou Salem and Mouhamadia (63.2 ± 21.7 and 68.2 ± 19.5%, respectively) compared to Saouef and Oueslatia (21.4 ± 12.4 and 0.0 ± 0.0%, respectively) ($p < .001$).

Finally, seroprevalence was significantly associated with the number of mating seasons in that a higher seroprevalence was observed in older rams that had accomplished three or more mating seasons compared to younger rams in their first or second mating season ($p = .05$). *Toxoplasma gondii* infections were very high in rams that had gone through six mating seasons (90.0 ± 18.6%) (Table 2).

### 3.2. Prevalence of *Toxoplasma gondii* in semen

The overall molecular prevalence of *T. gondii* in rams' semen averaged 51.1 ± 10.2%. Similar to seroprevalence, the molecular prevalence also differed significantly between locations ($p < .001$). The highest prevalence was recorded in Mouhamadia (72.7 ± 18.6%) and the lowest in Saouef (30.9 ± 14.0%) (Table 2).

The molecular prevalence was significantly ($p < .001$) lower in younger rams with one mating season (20.0 ± 20.2%) and highest in animals with 6 mating seasons (90.0 ± 18.6%). Among the PCR positive 47 rams, only 26 were seropotive. The Kappa coefficient between PCR and ELISA test was 0.33 indicating a regular agreement according to the table for interpreting kappa values (Landis and Koch, 1977).

### 3.3. Phylogenetic analyses of *Toxoplasma gondii*

The BLAST comparison of the partial sequences (114 base pairs) of the B1 gene revealed 100% homology between the five sequenced amplicons from Tunisia. The PCR amplification of the genomic DNA recovered from the 92 semen samples corresponded to *T. gondii* thus confirming the infection of the rams' semen.

The amplicons showed a 100% sequence homology with recently reported sequences of *T. gondii* from Tunisia (GU249509.1), Brazil (JX218225.1), Saint Kitts and Nevis (KU599369.1) and Great Britain (KC928250.1) archived at the GenBank. They shared 99% homology with sequences from Puerto Rico (KU599371.1) and 98% homology with four human isolates (GU139479.1, KU599432.1, KU599427.1 and KT310098.1). The amplicon also shared 97% sequence homology with many others from Tunisia, Gabon, Ethiopia, Portugal, France, Turkey and USA. A homology of 96% was shared with an amplicon from French Guiana (KU599429.1) and 95% with samples from Tunisia (GU139472.1), Austria (KU599330.1), Turkey (KU599480.1) and China (KU599377.1 and KU599387.1). A phylogenetic tree incoporating the *T. gondii* amplicon from the current study and sequences of other amplicons downloaded from the GenBank was constructed (Fig. 2). It confirms that the five sequences form a single haplotype that

**Table 2**
Association between the *Toxoplasma gondii* infection in sheep and different parameters based on PCR.

| Parameter | | ELISA | | | PCR | | |
|---|---|---|---|---|---|---|---|
| | | Positive/examined (% ± SE[a]) | Stratified OR[b] [95% CI][c] | *p* value | Positive/examined (% ± SE) | Stratified OR [95% CI] | p value |
| Locality (district) | Bou Salem (Jendouba) | 12/19 (63.2 ± 21.7) | 7.0 [2.8; 17.7] | < .001** | 13/19 (68.4 ± 20.9) | 4.63 [2.1; 10.0] | < .001** |
| | Mouhamadia (Ben Arous) | 15/22 (68.2 ± 19.5) | | | 16/22 (72.7 ± 18.6) | | |
| | Saouef (Zaghouan) | 9/42 (21.4 ± 12.4)[d] | | | 13/42 (30.9 ± 14.0)[d] | | |
| | Oueslatia (Kairouan) | 0/9 | | | 5/9 (55.6 ± 32.5) | | |
| Number of mating seasons | 1 | 0/15 | 2.6 [1.0; 7.6] | .051* | 3/15 (20 ± 20.2)[d] | 5.24 [2.5; 12.0] | < .001** |
| | 2 | 0/8 | | | 5/8 (62.5 ± 33.5) | | |
| | 3 | 13/26 (50 ± 19.2) | | | 16/26 (61.5 ± 18.7) | | |
| | 4 | 4/9 (44.4 ± 32.5) | | | 3/9 (33.3 ± 30.8) | | |
| | 5 | 7/15 (46.7 ± 25.2) | | | 7/15 (46.7 ± 25.2) | | |
| | 6 | 9/10 (90 ± 18.6) | | | 9/10 (90 ± 18.6) | | |
| | 7 | 3/9 (3.3 ± 30.8)[d] | | | 4/9 (44.4 ± 32.5) | | |
| Overall | | 36/92 (39.1 ± 10.0) | | | 47/92 (51.1 ± 10.2) | | |

[a] Standard error.
[b] Odds ratio.
[c] Confidence interval.
[d] Reference.
* Significant (.001 ≤ p ≤ .05).
** Highly significant (p < .001).

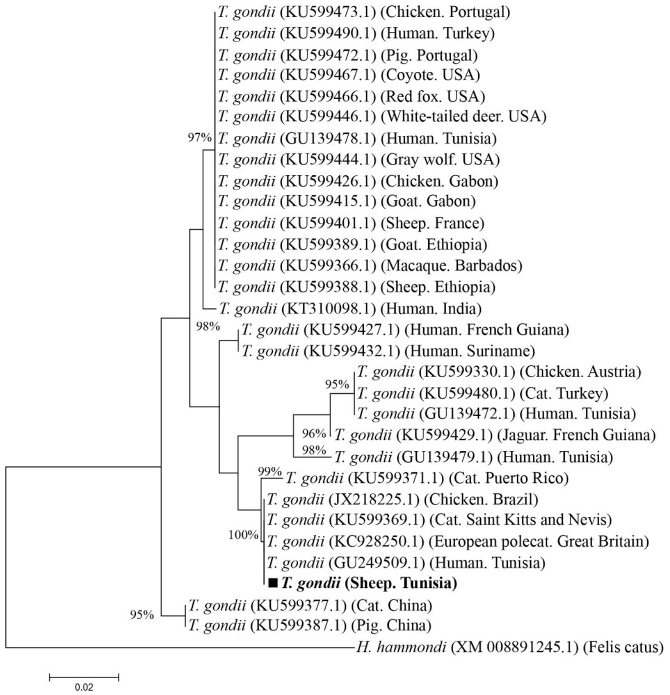

**Fig. 2.** Phylogenetic tree of B1 gene for *Toxoplasma gondii* isolated from rams' semen and the main *T. gondii* species isolates deposited in GenBank.

has also been isolated from chicken, cats, European pole rat and humans in Brazil, St Kits and Nevis, Great Britain and Tunisa, respectively.

## 4. Discussion

In this study, the authors investigated the presence of *T. gondii* infection in 92 rams from 4 different farms in Tunisia. Infection was investigated by means of: i) presence of specific antibodies detected by a commercial ELISA kit; and ii) detection of *T. gondii* DNA in semen with an in house PCR. To avoid contamination, drastic measures were taken along both sampling and all the PCR runs. In fact, a negative (Water DEPC-treated) and a positive control were used. The negative control was always handled the last. The positive control used in the DNA assay of this study is a sample that was confirmed as positive for *T. gondii* in another study performed in the laboratory. In addition PCR bench was regularly cleaned using bleach. Furthermore, high contamination risks are observed in the two-step nested PCR which is not the case for this B1 gene conventional PCR.

Semen experimental infection by *T. gondii* has been studied in sheep (de Moraes et al., 2010a) and in many other species such as goats (Liu et al., 2006), rabbits (Arantes et al., 2009), dogs (Wanderley et al., 2013) and mice (Asgari et al., 2015), but the prevalence of *T. gondii* in semen of naturally infected rams remains little investigated to date. Using the ELISA test, our study found a seroprevalence of 39.1%. This is higher than that reported by Gharbi et al. (2013) in sheep from West-central and northern Tunisia (19 and 1.8%, respectively). Using the Modified Agglutination Test (MAT), Lahmar et al. (2015) found antibodies against *T. gondii* in 82 out of 204 sheep (40.2%) from southwest Tunisia; this was similar to the seroprevalence found in the present study. Taking reports from other countries into consideration, the seroprevalence found in our study was higher than that reported in Brazilian sheep using the Indirect Fluorescent Antibody Test (IFAT) (9.2%; Bezerra et al., 2014; de Moraes et al., 2010b). However, the seroprevalence was lower than that reported in Serbia with the MAT (84.5%; Klun et al., 2006) but was similar to that reported in Ghana (33.2%) and Iran (35%) using ELISA and IFAT, respectively (Van der Puije et al., 2000; Sharif et al., 2007). Such differences could be explained by several factors: environmental conditions, differences in diagnostic methods' performances and the age of sampled animals.

In the present study, *T. gondii* DNA was detected in 51.1% of the analysed semen samples. Using the same diagnostic technique, lower prevalence's were reported by Bezerra et al. (2014) in Brazilian sheep where only 22.2% of the samples were positive. This difference could be attributed to differences in the sampling approach. In the Brazilian survey, all the rams came from artificial insemination centres with no previous direct contact with ewes. The higher prevalence observed in the current study could be related to breed differences and to a less ingestion risk of oocysts as a main source of infection.

Compared to the other districts, both *T. gondii* seroprevalence and molecular prevalence were significantly higher in Bou Salem (63.2 and 68.4, respectively) and Mouhamadia (68.2 and 72.7, respectively) ($p < .001$). This could be attributed to the climatic conditions in these two localities characterized by high rainfall and relatively low temperature which enhances oocysts' viability in the environment (Yan et al., 2016; Rouatbi et al., 2019). As expected, the number of mating seasons was significantly correlated with infection rates. Lower infections prevalences occurred in younger rams with less than two mating seasons while older rams having more mating seasons, showed significantly higher infection rates. Our ELISA findings were similar to the results of Boughattas et al. (2014) who found with MAT anti-*Toxoplasma* antibodies in 38.2% of young sheep and 73.6% of adult sheep. Moreover, in France, anti-*T. gondii* antibodies were found in 22.0 and 65.6% of lambs and ewes, respectively (Dumètre et al., 2006). Higher infection rates in older animals could be explained by the cumulative persistent infections over time and an expected consequence of a longer period of time of exposure for older animals and not the increased number of mating episodes which is a less well recognized route of exposure. Thus, the association between the number of mating seasons and prevalence could be biased by the factor age.

The present study highlighted the association between the number of mating seasons and infection prevalence with lower infection rates observed in younger rams compared to older animals that have accomplished more mating seasons during their reproductive careers. This however, does not suffice in confirming the likelihood of sexual transmission. Further studies, such as experimental infections by *T. gondii*, are necessary to confirm this observation since even though some authors proved *T. gondii* sexual transmission in both rams and ewes, the reverse transmission pathway is yet to be confirmed (Lopes et al., 2013) and it remains an important researchable issue.

Surprisingly, the molecular prevalence of *T. gondii* in semen was higher than the seroprevalence in our rams. As in this study, de Moraes et al. (2010b) detected a higher prevalence in semen compared to serology. Contrastingly, Bezerra et al. (2014) found that all seropositive rams (109/109) were also *T. gondii* PCR positive in semen.

In this study, PCR was used to detect *T. gondii* by targeting B1 repetitive gene which is a very useful method in diagnosis (Burg et al., 1989). In fact, when comparing bioassay of mice, capture ELISA, immunoblotting and PCR after inoculation of tachyzoites, authors found that PCR was the most sensitive technique when compared to other techniques (Hafid et al., 2001). In addition, another study showed that targeting B1 gene was more sensitive compared to P30 gene since after PCR, the later gave more false negative results than B1 gene. In conclusion, the fair agreement between the two techniques (*Kappa* = 0.33) with a higher molecular prevalence in semen may be due to the difference between ELISA and PCR as a sensitive and specific molecular tool for *T. gondii* infection identification (Riaz et al., 2016). The fair concordance between PCR and ELISA and the higher molecular prevalence in semen found in this study could also be explained by the fact that to escape the immune system, T. *gondii* bradyzoïtes hides in organs weakly exposed to the immune system effectors such as the eyes, the brain and the genital organs.

To confirm the infection of semen with *T. gondii*, 5 amplicons from the 47 positive amplicons were sequenced. All the five sequences were

100% identical and formed a single haplotype. They also shared between 95% and 100% sequence identity with amplicons from other amplicons deposited with the GenBank and corresponding to the SAG3 gene. The haplotype amplified in the present study clustered with another Tunisian amplicon from humans and had a 95%, 97% and 98% sequence homology with three other amplicons from Tunisia. This reflects most likely relatively high diversity of *T. gondii* occurring in Tunisia, sequencing more samples will be necessary to ascertain this hypothesis. The fact that our haplotype was also reported in chicken, cats, European pole rat and humans in Brazil, St Kits and Nevis, Great Britain and Tunisia, respectively indicates that the *T. gondii* haplotype is neither species nor geographic specific.

## 5. Conclusions

Rams' semen *T. gondii* infection is a real issue since it causes reproductive problems in both females and males, resulting in a decline in flock productivity. Through its national sheep breeding program, over the last three decades, Tunisia has invested in establishing a large network of improved rams' and artificial insemination centres. Given the high prevalence of *T. gondii*, as reported in this study, it is recommended to set up a systematic screening programme in these centres to detect *T. gondii* DNA in semen. Furthermore, rather than culling infected animals from breeding, it would be more appropriate to prevent toxoplasmic infections in lambs destined to reproduction.

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## Ethical approval

The study received ethical approval from the Association Tunisienne des Sciences des Animaux de Laboratoire (ATSAL). Ethical concerns were taken into account by adhering to local animal welfare regulations and practices and conformed to ethical guidelines for animal usage in research of the ATSAL.

## Declaration of Competing Interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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