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RESEARCH NOTE

# Evaluation of frequency of antibodies against *Toxoplasma gondii, Neospora caninum* and *Sarcocystis* spp. and transmission routes in sheep from Humid Pampa, Argentina

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## Abstract

The aim of this study was to describe the frequency of ovine specific antibodies to *Toxoplasma gondii*, *Neospora caninum* and *Sarcocystis* spp. and to estimate different transmission routes of these infections. One hundred and thirty Texel sheep and their 117 Texel lambs were included in the study. Serum samples were tested for antibodies to *T. gondii*, *N. caninum* and *Sarcocystis* spp. using IFAT. *Toxoplasma gondii* seroprevalence was 10.00% in sheep ($IC_{95\%}$: 4.80–15.20%), being higher in adult sheep (≥12 year) than in younger sheep (OR 1.30; 95% CI, 1.10–1.50). *N. caninum* and *Sarcocystis* spp. seroprevalences were 1.54% ($IC_{95\%}$: 0.00–5.70) and 72.09% ($IC_{95\%}$: 67.70–82.70), respectively, with no association between age and seropositivity in sheep ($P>0.05$). *T. gondii* seroprevalence in lambs was 4.27% ($IC_{95\%}$: 0.61–7.94). No association between *T. gondii* serological status in sheep and their lambs was detected ($P = 0.07$). Two *T. gondii* and *Sarcocystis* spp. seropositive lambs were euthanized and *T. gondii* and *Sarcocystis* spp. DNA was detected by PCR in their tissues. In conclusion, the increase of *T. gondii* seropositivity in relationship with sheep age and the lack of association between sheep-lamb serological status, suggest that horizontal infection is the main transmission route in this flock as reported before. Due to the low number of *N. caninum*-seropositive ewes no assumptions can be done about the impact of this parasite in this flock. According with previous reports, the main transmission route for *Sarcocystis* spp. in this species in the present study was horizontal.

## Keywords

*Toxoplasma gondii*, sheep, lamb, seroprevalence, *Neospora caninum*, *Sarcocystis* spp.

## Introduction

There are 15,000,000 sheep in Argentina. Over 80% of them are raised in the Patagonia and 13.60% located in the Humid Pampa region (*Ministerio Nacional de Agricultura*, *Ganadería y Pesca* 2010). Between these two regions, 16,579 and 76,014 tons of meat and wool are produced annually, respectively (*Ministerio Nacional de Agricultura*, *Ganadería y Pesca* 2010). Sheep industry has an important socio-economic impact in Argentina. However, information regarding reproductive losses in this species is limited to other abortifacient agents as *Brucella ovis* and *Leptospira* spp. but scarce information about protozoan infections are published (Hecker *et al.* 2013).

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*Toxoplasma gondii* is one of the most common causes of reproductive failure in goats and sheep worldwide and toxoplasmosis is an important zoonosis (Dubey 2009). Serological findings in sheep have revealed prevalence of *T. gondii* infections that range from 3% to 97% depending on the region of the world and the serological test applied (Dubey 2009). Although anti-*T. gondii* antibodies have been investigated in Argentina (Hecker *et al.* 2013), clinical disease is scarcely reported. Additionally, small ruminant's tissues have been identified as a primary source of *T. gondii*-infection for humans in Europe and North America (Dubey 2009), however its role in the epidemiology of human toxoplasmosis in Argentina remains unclear.

In addition, infections with the apicomplexan protozoa *Neospora caninum* and *Sarcocystis* spp. could cause abortion in livestock and should be considered as differential diagnoses of *T. gondii*. Although neosporosis has shown to be a major cause of abortion in cattle, its role as a cause of abortion and neonatal loss in sheep is still under study (Dubey *et al.* 2017). On the other hand, *Sarcocystis* spp. is one of the most prevalent protozoan parasites in sheep. If the ovine meat is heavily *Sarcocystis*-infected may be condemned as unfit for human consumption for its zoonotic potential, generating important economic losses (Dubey *et al.* 2016). Considering the relevance of *T. gondii* as an abortifacient agent of sheep and the lack of regional information concerning *N. caninum* and *Sarcocystis* spp. in this species, this study aimed to describe the frequency of these protozoan infections and to estimate different transmission routes in a Texel flock located at the Humid Pampa region, Argentina.

## Materials and Methods

A longitudinal study was conducted in a Texel flock belong to INTA-Balcarce, Argentina from November 2014 until March 2015. The flock located in the Southeast of Buenos Aires province, Argentina (37°48′26″S 58°17′38″W) was raised for meat production under an extensive, pasture-based system with no supplementation, and maintained a good body condition throughout the experiment. Seroprevalence study involved 130 Texel sheep of 3 (n = 29), 4 (n = 35), 5 (n = 29), 6 (n = 17) and 12 (n = 20) years old. Sheep were mated by Texel rams. The pregnancy rate of studied flock was 94.20% (130/138), the abortion rate was 4.61% (6/130) whereas the perinatal mortality rate was 12.09% (15/124). Despite reproductive losses were detected at the end of the lambing season (ewes with no lambs), they were not observed during the study. One hundred and nine out of 130 pregnant sheep delivered 117 lambs between August 1 and September 30, 2014 (101 sheep with single gestation and 8 sheep with double gestation). All animals were handled in strict accordance with good animal practice and the conditions defined by the Animal Ethics Committee (CICUAE) at INTA Balcarce under protocol number 008/2015.

A single blood sample was obtained from jugular vein of 130 sheep on December 2014 when they were between 65 to 125 days post-partum. A single sampling was made in their 117 lambs when they had an average of 193 days-old (range 163–223 days-old). Serum samples were tested for *T. gondii*, *N. caninum* and *Sarcocystis* spp. antibodies using IFAT as previously described by Hecker *et al.* (2013) and Moré *et al.* (2008). A cut-off titer of ≥1:50 was defined for all IFATs and serial dilutions were performed and tests were considered positive when total peripheral parasite fluorescence was demonstrated (Hecker *et al*. 2013; Moré *et al.* 2008). The highest serological dilution with complete peripheral positive reactions was considered the end-point titer. Positive control sera were obtained from *T. gondii*-, *N. caninum*- and *Sarcocystis* spp.– experimentally infected sheep. Negative control sera were obtained from *T. gondii*-, *N. caninum*- and *Sarcocystis*-free sheep (Soares *et al.* 2009).

In order to confirm the infection, two *T. gondii* and *Sarcocystis* spp. seropositive lambs born of *T. gondii* and *Sarcocystis* spp. seropositive sheep, respectively, were slaughtered and tissue samples collected for DNA extraction and histopathological analysis as previously mentioned (Katzer *et al.* 2014). No *N. caninum*-seropositive lambs born of *N. caninum*-seropositive sheep were registered and the seroprevalence of *N. caninum* was low compare with *T. gondii* and *Sarcocystis.* Therefore, *Neospora* seropositive lambs were not tissue sampled. Samples of central nervous system, heart, tongue, forelimb and hind limbs muscles and prescapular ganglion were analyzed. Tissue samples were frozen and stored at −20°C until DNA was isolated using a commercially available kit according to the manufacturer's recommendations (DNeasy Tissue Kit, QIAGEN Group, Germany). DNA concentration was measured using an Epoch micro-volume spectrophotometer system (Epoc, Bioteck® Instruments, Inc., Vermont, USA). PCR assay was used to detect *T. gondii* DNA as described by Moré *et al.* (2010). *T. gondii* genotyping was carried out by multilocus RFLP using the PCR-amplified markers nSAG2, SAG3, BTUB, GRA6, c22-8, L358, PK1, c29-2 and Apico as described previously (Moré *et al.* 2010). *N. caninum* DNA was assessed by a nested-PCR on the internal transcribed spacer (ITS1) region with four oligonucleotides as described by Buxton *et al.* (1998). *Sarcocystis* DNA was amplified by PCR using primers SarcoFext and SarcoRext proceeding as previously reported (Moré *et al.* 2013). *Sarcocystis* amplicons obtained were purified using a commercial kit (Wizard SV clean up system, Promega) according to manufacturer instructions, and submitted for sequencing to the Genomic Unit, Biotechnology Institute CICVyA – CNIA – INTA, Argentina. Positive (purified *T. gondii*, *N. caninum* and *Sarcocystis cruzi* DNA), negative and no template controls were used for each assay. The products were visualized by 1.5% agarose gel electrophoresis and SYBR™ Safe DNA gel staining under UV light.

In order to investigate the possible influence of sheep age on *T. gondii*, *N. caninum* or *Sarcocystis* spp. seroprevalence, and to estimate protozoan transmission in their lambs, logis-

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**Table I.** Frequency of antibodies to *Toxoplasma gondii*, *Neospora caninum* and *Sarcocystis* spp. in ewes according to their age

| Protozoa | Animals sampled | Number of positives (n/%) | Range of antibody titer | Positive to more than one parasite | Seroprevalence according to the age (years) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | 3 (n = 29) | 4 (n = 35) | 5 (n = 29) | 6 (n = 17) | 12 (n = 20) | OR ($IC_{95\%}$) | P–Value |
| *T. gondii* | 130 | 13 /10% | 50–1600 | 1 (*N. caninum*-*Sarcocystis* spp.)<br>9 (*Sarcocystis* spp.) | 0.0% | 5.7% | 10.3% | 11.8% | 30.0% | 1.3 (1.1–1.5) | <0.01 |
| *N. caninum* | 130 | 2/1.54% | 200–400 | 1 (*T. gondii*-*Sarcocystis* spp.) | 3,5% | 0.0% | 0.0% | 5.9% | 0.0% | 1.0 (0.7–1.4) | 0.98 |
| *Sarcocystis* spp. | 129* | 93/72.09% | 50–400 | 1 (*T. gondii*-*N. caninum*)<br>9 (*T. gondii*) | 79.3% | 77.1% | 64.3% | 76.5% | 80.0% | 1.1 (1.0–1.2) | 0.28 |

*One sheep was not available to analyze *Sarcocystis* spp. antibodies

tic regression models were used (LOGISTIC procedure, SAS Studio v3.6, SAS Institute Inc., Cary NC, USA). Firth adjustment was used when necessary. The magnitude of the association was estimated by calculating the odds ratio (OR). All tests were conducted at a significance level of 0.05.

## Results and Discussion

### Seroprevalence study in sheep

Estimated *T. gondii* seroprevalence in sheep was 10.0% ($IC_{95\%}$: 4.80–15.20%) (Table 1). Similar *T. gondii* seroprevalence (12.60%) was reported in extensive grazing systems in Patagonia (Robles *et al.* 2014). Furthermore, similar *T. gondii* seroprevalence was detected in flocks from other countries (Dubey 2009). However, Hecker *et al.* (2013) described higher *T. gondii* seroprevalence (17.3%) in dairy sheep flocks of the Humid Pampa region, probably be due to more intensive husbandry conditions and the more probable exposition to contaminated feed or water with oocysts of *T. gondii* (Dubey 2009)

Four out of 13 *T. gondii* seropositive sheep (30.76%) had >1:400 titers, possibly indicating active infections. Similarly, Dubey *et al.* (2008) isolated *T gondii* from 46.1% of lambs with MAT titers of ≤1:100 and from 87% of lambs with titers ≥1:200. They hypothesized that lambs with higher titers are probably on acute stage suggesting recently acquired infection.

Adult sheep (≥12 year) were more likely to be *T. gondii*-seropositive in comparison with younger ewes (OR 1.30; 95% CI, 1.10–1.50) (Table I). This result is in accordance with previous studies (Dubey 2009) probably indicating that horizontal transmission by ingestion of sporulated oocysts is the most important route of *T. gondii* infection in this flock.

*Neospora caninum*-seroprevalence in sheep was 1.54% ($IC_{95\%}$: 0.00–5.70), similar to the prevalence reported by Soares *et al.* (2009) in Brazil. In contrast, other authors reported higher *N. caninum*-seroprevalences in Slovakia, Brazil and Jordan (Figliuolo *et al.* 2004; Dubey 2009). Interestingly, Hecker *et al.* (2013) detected a similar *N. caninum*-seroprevalence in dairy flocks from the same region evaluated in this study. *N. caninum* infected sheep had <1:400 titers and no significant association was detected between age and seropositivity (OR 1.00; $CI_{95\%}$ 0.70–1.40; $P = 0.98$) (Table I). However, due to the low number of *N. caninum*-seropositive ewes, no assumptions can be done about this finding.

Additionally, *Sarcocystis* spp. seroprevalence in this flock was 72.09% ($IC_{95\%}$: 67.70–82.70). Similar *Sarcocystis* spp. seroprevalences were previously reported in small ruminants ranging from 70 to 100% (Bittencourt *et al.* 2016; Dubey *et al.* 2016) confirming the endemic presence of this parasitosis worldwide. *Sarcocystis* spp. infected sheep had <1:400 titers. Although horizontal transmission of sarcocystosis is the most common mode of infection (Dubey *et al.* 2016), no age effect was confirmed in this study (OR 1.10; $CI_{95\%}$ 1.00–

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1.20; P = 0.28) (Table 1), probably because the animals get infected at an early age after ingestion of sporocysts in food or water (Dubey *et al.* 2016). The main feeding source of these animals was native pasturelands; the high *Sarcocystis*-prevalence detected could be associated with their close contact with definitive hosts, like domestic dogs, present in the production system. Although high frequency of *Sarcocystis*-infection (91.6%) was reported in cattle by gross and histopathological examinations in Argentina (Moré *et al.* 2008), more studies are need in order to determine the importance of the high seroprevalence detected in this flock.

*Toxoplasma gondii*, *N. caninum* and *Sarcocystis* spp. coinfections were detected in the flock (Table I). Recently, Gondim *et al.* (2017) reviewed the serological cross-reactivity among *T. gondii*, *Hammondia* spp., *Neospora* spp., *Sarcocystis* spp. and *Besnoitia besnoiti* in cattle. These authors mentioned serological cross reactivity between *T. gondii* and *N. caninum* and between *Sarcocystis* spp. and *N. caninum* but no serological cross-reactions were showed between *T. gondii* and *Sarcocystis* spp. In accordance with these authors, Kalita *et al.* (2015) reported the absence of serological cross-reaction between the species *Sarcocystis hirsuta* and *T. gondii.* More studies are needed in order to validate serological test for *T. gondii* in sheep and to exclude potential cross-reactivity with antibodies to *Sarcocystis* spp.

**Seroprevalence and molecular studies in lambs**

*T. gondii* seroprevalence in lambs was 4.27% ($IC_{95\%}$: 0.61–7.94) with 2 positive lambs born from positive *T. gondii* ewes and 3 positive lambs born from negative *T. gondii* ewes (Table II). There was no significant association between ewes and lambs *T. gondii* serostatus ($P = 0.07$), suggesting that vertical transmission of toxoplasmosis in the flock is low, being horizontal transmission the main route of infection. In accordance with this result, previous report mentioned that lambs usually are infected at a young age (less than 223 days of age) (Figliuolo *et al.* 2004).

In the present study, two *T. gondii* seropositive lambs with IFAT titers of 1:400 were born from seropositive ewes. One of these lambs was also seropositive to *Sarcocystis* spp. Although previous reports confirmed that *T. gondii* vertical transmission can occur more frequently than what was previously mentioned (Innes *et al.* 2009, Dubey 2009), vertical transmission of these two animals was not confirmed since no pre-calostrum bleeding was performed.

Only one lamb was *N. caninum* seropositive (IFAT<1:200) (0.85%, $IC_{95\%}$: 0.00–2.52), and was born from a negative *N. caninum* ewe (Table II). All other lambs born from *N. caninum* seropositive sheep were *N. caninum* seronegative. The low *N. caninum*-seropositivity detected in sheep as well as the lack of association between ewe-lamb serostatus could exclude vertical transmission of neosporosis in this flock, probably being the ingestion of oocysts from feed or water the predominant route of infection (Dubey *et al.* 2017). No cross-reactions between *N. caninum* and *T. gondii* were detected.

*Sarcocystis* spp. infection was confirmed by IFAT in 67.57% of lambs ($IC_{95\%}$: 58.40–75.56) with 57 positive lambs born from seropositive *Sarcocystis* spp. ewes and 18 positive lambs born from negative *Sarcocystis* spp. ewes (Table II). No association was detected between *Sarcocystis* serological status of ewes and their lambs (P = 0.19). All lambs presented end titers <1:200. Since at that sampling point the colostrum antibodies should be lower than detection levels, it is possible to assume that lambs were effectively *Sarcocystis*-infected. Considering the rare occurrence of congenital transmission of *Sarcocystis* in sheep, we considered that most of the lambs were horizontally infected when they start to graze on natural pastures (Dubey *et al.* 2016).

Two seropositive *T. gondii* and *Sarcocystis* spp., of seropositive *T. gondii* and *Sarcocystis* sheep, respectively, were euthanized in order to confirm *T. gondii*, *N. caninum* and/or *Sarcocystis* spp. infection. No parasite cysts, gross and histological lesions were observed following post mortem examination. It was not detected DNA of *N. caninum* in any sample of both lambs. Central nervous system, heart, tongue, forelimb and hind limbs muscles samples resulted positive to *T. gondii* DNA using specific PCR in lamb #1 (Fig. 1A) and central nervous system, prescapular ganglion and forelimb muscles samples resulted positive to *T. gondii* DNA in lamb #2 (data no shown). Unfortunately, the low load of *T. gondii*-DNA present in the infected lambs did not allow genetic characterization of the infecting strain. Detection of *T. gondii* DNA in sheep tissues strongly confirmed the potential risk of transmission of this parasite to humans and other animals through the consumption of ovine meat.

In addition, *Sarcocystis* spp. DNA was detected in one forelimb muscle sample of lamb #2. The amplicon was sequenced,

**Table II.** *Toxoplasma gondii*, *Neospora caninum* and *Sarcocystis* spp. serostatus of ewes and their lambs

| ***Toxoplasma gondii*** | | | ***Neospora caninum*** | | | ***Sarcocystis* spp.** | | |
|---|---|---|---|---|---|---|---|---|
| | Sheep | | | Sheep | | | Sheep | |
| Lambs | Positive | Negative | Lambs | Positive | Negative | Lambs* | Positive | Negative |
| Positive | 2 | 3 | Positive | 0 | 1 | Positive | 57 | 18 |
| Negative | 11 | 101 | Negative | 2 | 114 | Negative | 23 | 13 |

*Only 111 lamb serum samples were available to analyze

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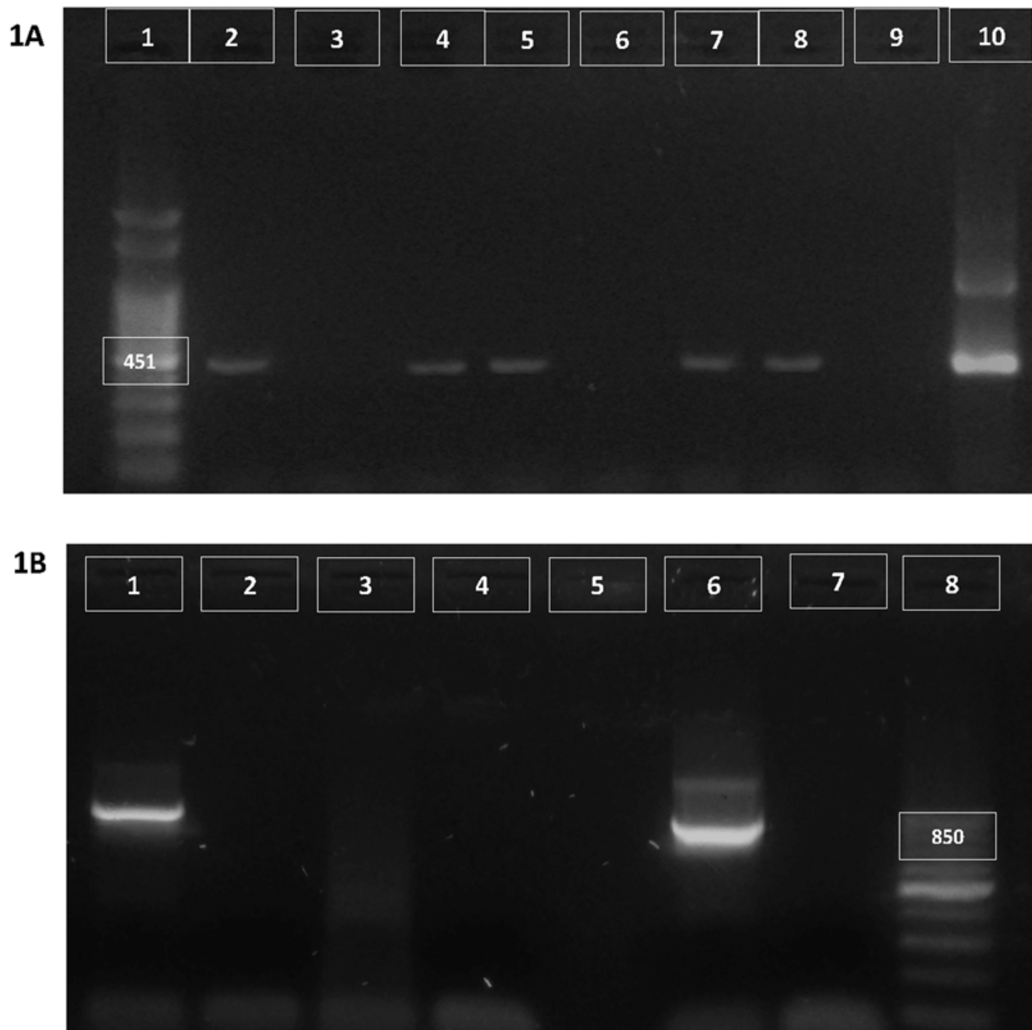


**Fig. 1.** Agarose gel showing positive PCR amplifications. Fig. 1A showing positive PCR amplifications of *T. gondii* in lamb #1. Lanes 1 is showing the molecular weight marker. Line 2, 4, 5, 7, 8 are showing positive samples from the central nervous system, heart, tongue, forelimb and hind limbs muscles. Lanes 9 and 10 are sowing the negative and positive control, respectively. Fig. 1B showing positive PCR amplification of *Sarcocystis* spp. in lamb #2. Line 1 is showing positive sample from the forelimb muscles. Lanes 6 and 7 are showing the positive and negative control, respectively. Lanes 8 is showing the molecular weight marker. Line 2, 3, 4, 5 are showing negative samples from the central nervous system, heart, tongue and hind limbs muscles

and the assembled sequence obtained revealed 100% identity with sequences of *S. tenella* reported in GenBank (KP263759 and KC209734) by BLASTn. All the PCR results confirmed that this lamb was co-infected with *T. gondii and S. tenella*, supporting the IFAT results. The obtained sequence was reported in the GenBank with the accession number MF401626. *Sarcocystis tenella* is the most pathogenic of the *Sarcocystis* species in sheep causing anorexia, fever, decreased weight gain, anemia and death in experimentally infected lambs although the majority of animals infected are asymptomatic and parasites are observed mainly as an incidental finding at necropsy (Dubey *et al.* 2016). Canids are the definitive hosts of *S. tenella* shedding sporocysts after 7–10 days post ingestion of sheep muscles containing sarcocysts. This species has apparently no impact in human health. Humans are definitive host for *Sarcocystis suihominis* found in pork and *Sarcocystis hominis* found in beef, both reproduces sexually in the humans intestines. Additionally, humans can be intermediate hosts for a variety of other *Sarcocystis* species and can be affected by potential toxins of sarcocysts of *S. aucheniae* (muscles of South American camelids) and *S. fayeri* (horse muscles) as a "food poisoning" (Dubey *et al.* 2016). Although *S. tenella* was confirmed in one lamb, the origin of the seropositivity of lambs in the present study is uncertain, since other *Sarcocystis* infections could not be discarded, probably producing positive results in a genus-specific IFAT technique (Moré *et al.* 2010). Detailed morphological and/or molecular analyses are required to identify the different *Sarcocystis* spp. potentially present in this flock (Moré *et al.* 2013; Dubey *et al.* 2016). Bittencourt *et al.* 2016 evaluated the frequency of infection to *Sarcocystis* spp.

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in muscular tissues by morphological, ultrastructural, and molecular tests in Brazil. Unfortunately, only frozen muscle samples of two lambs were available in the present study to confirm these findings. More studies are needed in order to evaluate the losses due to meat confiscation for this parasite infection in the region.

In conclusion, the positive correlation of *T. gondii* seropositivity and sheep age and the lack of association between ewe-lamb serological status, suggest that horizontal infection is the main transmission route of *T. gondii* in this flock. On the other hand, due to the low number of *N. caninum*-seropositive ewes and that the animals did not present abortions in the moment of the collection of samples, no assumptions can be done about the impact of this parasite in this flock. Finally, according with previous reports, the main transmission route for *Sarcocystis* spp. in this flock was horizontal. Further investigations at a larger scale are required to provide detailed information and establish the impact of toxoplasmosis, neosporosis and sarcocystosis on sheep reproductive losses of the region.

**Conflict of interest statement**

There are no financial or personal relationships with other people or organizations that could inappropriately influence this work.

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