

# Anti-*Toxoplasma gondii* and anti-*Neospora caninum* antibodies in sheep from Paraná state, South Brazil: prevalence and associated factors

Anticorpos anti-*Toxoplasma gondii* e anti-*Neospora caninum* em ovinos do estado do Paraná, sul do Brasil: prevalência e fatores associados

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## Abstract

The aim of this study was to evaluate the seroprevalence and factors associated with the presence of anti-*Toxoplasma gondii* and anti-*Neospora caninum* antibodies in sheep from Paraná state. The detection of antibodies for *T. gondii* and *N. caninum* was performed by homemade and commercial indirect ELISA, respectively. Multiple logistic regression analysis was used to verify the factors associated with the seroprevalence. Antibodies anti-*T. gondii* and anti-*N. caninum* were observed in 42.7% and in 17.6% of the animals, respectively. The protective factors associated to seropositive were “some level of confinement” (full or semi-extensive confinement) (OR=0.53) for *T. gondii* and “use of skilled labor” (OR=0.64) for *N. caninum*. The risk factors were “presence of cats” (OR=1.75) for *T. gondii* and “feeding of dogs with sheep placental remains” (OR=1.79) for *N. caninum*. In addition, to presenting a significant and simultaneous seroprevalence for both agents (9.9% of the animals), the results also indicate that deficiencies in management and environmental sanitation, the presence of reservoirs, and types of exploitation enhance the seropositivity. Thus, studies like this might support sanitary programs and public policies for the prevention of *T. gondii* and *N. caninum* in the sheep herds of Paraná state.

**Keywords:** Seroprevalence, *Toxoplasma gondii*, *Neospora caninum*, sheep, Parana state, Brazil, ELISA.

## Resumo

O objetivo deste estudo foi avaliar a soroprevalência e os fatores de risco associados à presença de anticorpos contra *Toxoplasma gondii* e *Neospora caninum* em ovinos do Paraná. A detecção dos anticorpos para *T. gondii* e *N. caninum* foi realizada, usando-se testes de ELISA indireto caseiro e comercial, respectivamente. A análise de regressão logística múltipla foi usada para verificar os fatores de risco associados à soroprevalência. Anticorpos anti-*T. gondii* e anti-*N. caninum* foram observados em 42,7% e em 17,6% dos animais, respectivamente. Os fatores de proteção associados à soropositividade foram “algum nível de confinamento (confinamento ou semiextensivo)” (OR=0,53) para *T. gondii* e “presença de mão de obra especializada” para *N. caninum* (OR=0,64). Os fatores de risco foram “presença de gatos” para *T. gondii* (OR=1,75) e “alimentação de cães com resíduos placentários” (OR=1,79) para *N. caninum*. Além de apresentar uma significante e simultânea soroprevalência para ambos os agentes (9,9% dos animais), os resultados também indicam que as deficiências em gestão e saneamento ambiental, a presença de reservatórios e os tipos de exploração favorecem a soropositividade. Dessa forma, estudos como estes podem auxiliar em programas sanitários e políticas públicas para a prevenção de *T. gondii* e *N. caninum* nos rebanhos ovinos do Paraná.

**Palavras-chave:** Soroprevalência, *Toxoplasma gondii*, *Neospora caninum*, ovelha, estado do Paraná, Brasil, ELISA.

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## Introduction

*Toxoplasma gondii* and *Neospora caninum*, protozoan extensively studied, are the main agents responsible for reproductive damage in sheep, goat, and bovine herds (Dubey & Lindsay, 1996; Moreno et al., 2012). Seroprevalence of 33.8% for *T. gondii* was verified in Brazilian sheep (Lugoch et al., 2019). In Paraná state was observed a mean seroprevalence of 35.9% (7.0%-51.8%) in the last decades (Ogawa et al., 2003; Moura et al., 2007; Romanelli et al., 2007; Soccol et al., 2009; Gheller et al., 2016).

The seroprevalence (9.5%) for *Neospora caninum* in the sheep in Paraná state was first described in 2007 in the herds from Guarapuava, Central-Southern region of the state (Romanelli et al., 2007). Almost ten years later, the seroprevalence in the same city was 3.7%, showing the still presence of the protozoan in the region (Gheller et al., 2016).

The national sheep herd was estimated at 19,715,587 million heads (IBGE, 2019). According to the register of herds of the Secretariat of Agriculture and Supply, Paraná occupies the eighth position in the nation, with approximately 556.512 thousand heads, representing 2,9% of the Brazilian herd (Paraná, 2019). Considering the lack of information and monitoring of both protozoan and the economic importance of the sheep herds to the Paraná state, it is necessary more studies that might contribute to the updating of SEAB database and which supports future actions to control. Thus, this study aimed to evaluate the seroprevalence of anti-*T. gondii* and anti-*N. caninum* IgG antibodies and the associated risk factors in the sheep herds of the Paraná state, southern Brazil.

## Material and Methods

The study is part of the EMBRAPA project "Zoosanitary characterization of goat and sheep farming in Brazil: epidemiology, risk factors, and economic impacts of diseases" and was approved by the Ethics Committee for Animal Use of the same institution (number 010/2014).

It was chosen representative sheep farms from the mesoregions: Central-North, Metropolitan of Curitiba, West, Southwest, and Center-South. To calculate the sample size (n), the OPEN EPI (v3) program was used. For both studied agents, a 95% confidence level, a 1.5 DEFF and an infinite population were adopted. A prevalence of 50.0% and an error of 2.5% were estimated for *T. gondii*, reaching the number of 1534 sampled animals. For *N. caninum*, a prevalence of 9.5% and an error of 1.5% were estimated, reaching up to the number 1,466 sampled animals. Sampling was performed by cluster; that is, within each property, the animals were stratified according to their categories, with approximately 60% of female breeders, 35% of the young (six to twelve months) and all male breeders randomly collected by visiting the property, for a total of 20 sheep.

Information about each property from 24 municipalities and their animals were obtained using a questionnaire. The information (126 questions) raised was - infrastructure, characteristics of production and commercialization, technological profile, sanitation, presence of other species on the property (domestic and/or wild) sex, age, and breed of the animals (Romanelli et al., 2020). Due to the great number of variables evaluated (questions), the authors opted to describe in the Results only those statically significant in the univariate analysis.

In each visited property during 2016, blood sample of 20 animals (a total of 1,609 sheep) was obtained by jugular puncture, collected in a tube without anticoagulant, and the serum obtained after centrifugation were kept at -4°C until analysis. The detection of anti-*T. gondii* IgG antibodies was performed by the indirect ELISA technique as described by Garcia et al. (2007). The optimal conditions for the antigen concentration, serum titration and conjugate dilution were established by the highest ratio of the mean absorbance of positive samples to the mean absorbance of negative samples. The standardized *T. gondii* antigen concentration was 2.5 µg mL<sup>-1</sup>, the sera were diluted to 1:100, and the conjugate anti-sheep IgG antibodies (Sigma Aldrich, USA®) was diluted to 1:4,000. All samples were tested in duplicate. The cutoff point of each plate was obtained according to Garcia et al. (2006), using the mean value of optical densities of the negative controls plus three standard deviations. For detection of the anti-*N. caninum* antibodies, commercial indirect ELISA kits (ELISAI - IMUNODOT®, Jaboticabal, Brazil) were used. The cutoff index (CI) was calculated by averaging the optical density of the negative controls multiplied by the factor 2.5. Positive samples were found to have a bright yellow color and an optical density equal to or greater than the CI, as described by the manufacturer.

The EpiInfo 7.1.5.2 program (Dean et al., 1996) was used to tabulate the variables that made up the epidemiological questionnaire along with the serological results. The variables of the epidemiological questionnaire were analyzed by a Yates-corrected chi-square test and Fisher's exact test. Those with  $p < 0.05$  were selected for multiple logistic

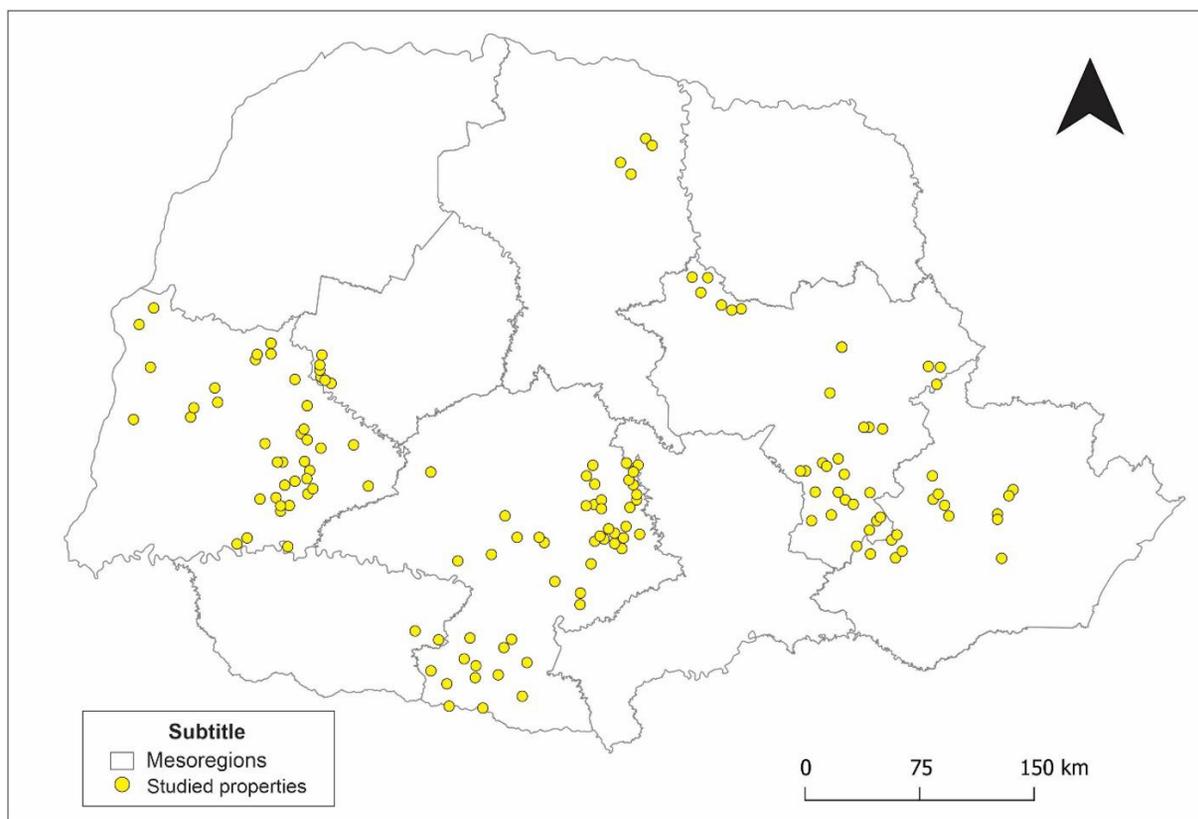
regression analysis, and the association strength was measured using the odds ratio (OR) values and the respective 95% confidence interval. The properties were mapped using a global positioning system (GPS). Shapefiles were built using the ArcGIS® program (ESRI, 2011). The point distribution and kernel density were built using QGIS.

### Results and Discussion

The study covered six of ten mesoregions in Paraná state, including 80 rural properties distributed in 24 municipalities (Figure 1), totaling 1609 animals. In all mesoregions studied, 92.5% (74/80) and 80.0% (64/80) of the properties showed animals *T. gondii* and *N. caninum* seroreactive, respectively (Table 1). For *T. gondii*, all the regions, except the metropolitan one (26.2%), had seropositivity close to or greater than 50% (40.7 - 72.1%), and the largest being in the Central-Northern mesoregion (Table 1). Whereas for *N. caninum*, the prevalence was smaller (7.5-32.7%), with a major rate of positivity in the Central-Eastern region (Table 1). Presence of positive animals for both protozoan was observed in 75.0% (60/80) of the studied properties. It is important to highlight that the present study showed the prevalence in most of the mesoregions of Paraná state, differently of previous studies that represented fewer regions. In addition, these results demonstrated the existence of favorable conditions for the spread of both agents in sheep in whole Paraná state.

The prevalence of anti-*T. gondii* IgG antibodies in sheep was 42.7% (688/1609; 95% CI 40.36-45.19). Studies on the occurrence or prevalence of toxoplasmosis in sheep in Paraná have been performed by other authors. The occurrence in the northern region of Paraná ranged from 47.8 to 54.6% (Ogawa et al., 2003; Garcia et al., 1999) and from 40.7 to 51.5% in the midwestern region (Romanelli et al., 2007; Gheller et al., 2016).

A recent review reported 196 studies around the world (2009-2020) and approximately 45% of them used ELISA assays to detect anti-*T. gondii* antibody in sheep (Dubey et al., 2020). The higher occurrence was observed in Parma, Italy (100%) (Vismarra et al., 2017), El Fayoum and Assiut, Egypt (98 and 86%, respectively) (Ghoneim et al., 2010; Kuraa & Malek, 2016), Montserrat, Caribbean islands (89.3%) (Hamilton et al., 2014), and in Nationwide, Belgium (75.7%) (Verhelst et al., 2014). In the South Atlantic, only one study showed a prevalence of 41.1% in sheep from Several, Costa Rica (Villagra-Blanco et al., 2019).



**Figure 1.** Local (studied properties) of sheep blood collection in the Paraná state, southern Brazil, to evaluate the occurrence of antibodies anti-*Toxoplasma gondii* and anti-*Neospora caninum*.

**Table 1.** Frequencies of anti-*Toxoplasma gondii* and anti-*Neospora caninum* IgG antibodies (indirect ELISA) on sheep and on sheep breeding properties in six mesoregions of Paraná state, Brazil - 2016.

Mesoregion	<i>T. gondii</i> Property Positive/Total (%)	<i>T. gondii</i> Animals Positive/Total (%)	<i>N. caninum</i> Property Positive/Total (%)	<i>N. caninum</i> Animals Positive/Total (%)
Western	18/19 (94.7)	154/387 (39.8)	14/19 (73.7)	49/387 (12.7)
Southeast	8/8 (100.0)	81/170 (47.6)	7/8 (87.5)	13/169 (7.7)
Central-Southern	19/22 (86.4)	168/419 (40.1)	14/22 (63.6)	43/419 (10.3)
Central-Eastern	21/23 (91.3)	214/453 (47.2)	22/23 (95.6)	148/453 (32.7)
Central-Northern	3/3 (100.0)	34/60 (56.7)	3/3 (100.0)	13/60 (21.7)
Metropolitan	5/5 (100.0)	26/100 (26.0)	4/5 (80.0)	13/99 (13.1)
<b>Total</b>	<b>74/80 (92.5)</b>	<b>688/1609 (42.7)</b>	<b>64/80 (80.0)</b>	<b>283/1607 (17.6)</b>

In Brazil, most part of the studies used indirect fluorescent antibody test (IFAT) as a diagnose method (28/34) (Dubey et al., 2020). Considering studies that use iELISA to detect anti-*T. gondii* antibodies was observed a mean prevalence of 44.8% (22.1-67.5%) (Dubey et al., 2020) and the higher prevalence (67.5%) was observed in Teresina, Piauí state (Rêgo et al., 2016). Thus, the seroprevalence to *T. gondii* found in the present study, in Paraná state, were similar to those observed in other Brazilian states.

The seroprevalence to *N. caninum* in the sheep of the present study was 17.6% (283/1607; 95% CI 15.83-19.55). In previous studies on Paraná state were observed occurrences of 3.7% (3/81) (Gheller et al., 2016) and 9.5% (29/305) (Romanelli et al. (2007) in the Central-Southern region of the state. In compare with our study, the Central-Southern region had 21.7% of seropositivity (Table 1). The higher seroprevalence might be due to the number of animals and properties in the present study.

It was observed that 9.9% (160/1607) of the animals were positive for both agents concurrently. Thus, control measures should be directed at both agents on most sheep farming properties of the state of Paraná.

The evaluations of the management, production, reproduction, and environmental characteristics with the results of the presence of anti-*T. gondii* and anti-*N. caninum* IgG antibodies are shown in the Tables 2 and 3, respectively.

The variables age, sex, use of skilled labor on the property, production system with some level of confinement, adequate food storage facilities, water supplied in off-site installations, and presence of cats on the property showed significant associations with a higher chance of seropositivity for *T. gondii* in the univariate analysis. Other variables evaluated, such as abortion, did not have statistical significance in the seroprevalence. Thus, those do not were shown in the text.

According to revision by Dubey et al. (2020), age, breed, geographic location, rainfall, flock size, grazing area, climate, and farm management might influence on the prevalence of *T. gondii* in sheep. The model obtained in the multiple logistic regression analysis that best explained the data behavior for *T. gondii* contained the variables "production system with some level of confinement" ( $p < 0.0001$ ) and the "presence of cats on the property" ( $p < 0.0001$ ). The last variable increased the chance of sheep being seropositive for toxoplasmosis by 1.75-fold; on the other hand, the "production systems with some level of confinement (full confinement or semi-extensive confinement)" were found to be a protective factor against *T. gondii* infection (OR = 0.53) (Table 2).

The variables that were significantly associated ( $p < 0.05$ ) with the highest chance of seropositivity to *N. caninum* in the univariate analysis were age (higher than one year; OR 1.78), sex (OR 2.60), free access of animals to water sources (OR 1.36), water supplied in off-site containers (OR 2.08), use of skilled labor on the property (OR 0.69), and feeding dogs with placental remains of sheep (OR 1.71) (Table 3).

For *N. caninum* the multiple logistic regression model that best explained the prevalence are the variable "use of skilled labor" ( $p = 0.0025$ ) as a protective factor against *N. caninum* infection (OR = 0.64) and "feeding of dogs with sheep placental remains" ( $p = 0.0001$ ) as a risk factor that increased the chance of the animal being seropositive by 1.79- fold.

To prevalence of *N. caninum* in older animals might be more likely to infection due to longer exposure to agents in the environment, as reported by many studies (Jittapalapong et al., 2005; Pinheiro et al., 2009; Anderlini et al., 2011; Díaz et al., 2014). On the other hand, Arraes-Santos et al. (2016) reported a 3.7 higher probability of occurrence of

**Table 2.** Results of logistic regression analysis of the management, production and reproductive factors associated with seroreactivity for *Toxoplasma gondii* in sheep from Paraná state, Brazil.

Variables	Positive/total (%)	Univariate		Multiple logistic regression	
		p	OR	p	Adjusted OR
			(CI 95%)		(CI 95%)
<b>Age range (&gt;1 year)</b>					
Yes	286/524 (54.6)	< 0.0001	2.98	UR	
No	119/415 (28.7)		(2.27-3.92)		
<b>Sex</b>					
Female	366/809 (45.2)	0.0015	1.92	UR	
Male	39/130 (30.0)		(1.29-2.87)		
<b>Skilled labor</b>					
Yes	244/640 (38.1)	0.0067	0.74	0.1471	
No	400/885 (45.2)		(0.60-0.91)		
<b>Production system</b>					
Confinement			0.54		0.53
semi-confinement	261/725 (36.0)	< 0.0001	(0.44-0.68)	<0.0001	(0.42-0.66)
Extensive	346/684 (50.6)				
<b>Adequate food storage facilities</b>					
Yes	470/1129 (41.6)	0.0043	0.71	0.4539	
No	210/421 (49.9)		(0.57-0.89)		
<b>Water provided supplied in off-site installations</b>					
Yes	464/968 (47.0)	0.0010	2.07	UR	
No	430/100 (30.0)		(1.32-3.23)		
<b>Cats on the property</b>					
Yes	449/968 (46.3)	0.0015	1.42	<0.0001	1.75
No	197/522 (37.7)		(1.14-1.77)		(1.38-2.21)

p:p value; OR: odds ratio; CI: confidence interval; UR: unrealized - variable not included in the multiple logistic regression analysis due to a loss of questionnaire filling greater than 20%.

*N. caninum* in young animals when compared to that of adult animals. Although in the horizontal transmission of neosporosis the time of exposure of the animals (age) to sources of infection is very important, this factor is less relevant in vertical transmission, so the higher prevalence in young animals might be easily accepted in properties where vertical transmission is more important than horizontal transmission. The fact that females are kept in the herd longer when compared to males, which are slaughtered earlier, justifies the greater exposure of these animals and consequently the higher seroprevalence. However, Uzêda et al. (2004) suggested greater susceptibility of females due to decreased immunity during pregnancy and lactation.

Regarding the age and sex of the animals there was a significant association for both agents. The variable "use of skilled labor" was significantly associated with seropositivity for both agents studied, proving to be a protective factor. The importance of skilled labor is discussed by Viana & Silveira (2009), who studied the profile of sheep farming in the southern half of Rio Grande do Sul. According to the authors, specialized technicians follow up on sick animals; however, the permanent workforce in the properties do not exclusively work with sheep, and thus,

**Table 3.** Results of logistic regression analysis of the management, production and reproductive factors associated with seroreactivity for *Neospora caninum* in sheep from Paraná state, Brazil.

Variables	Positive/total (%)	Univariate		Multiple logistic regression	
		p	OR	P	Adjusted OR
			(CI 95%)		(CI 95%)
<b>Age range (&gt;1 year)</b>					
Yes	138/524 (26.3)	0.0004	1.78	UR	
No	69/414 (16.7)		(1.29-2.47)		
<b>Sex</b>					
Female	193/808 (23.9)	0.0012	2.60	UR	
Male	14/130 (10.8)		(1.45-4.63)		
<b>Free access to water sources</b>					
Yes	165 /841 (19.6)	0.0230	1.36	0.1324	
No	113/746 (15.2)		(1.05-1.77)		
<b>Water provided supplied in off installations</b>					
Yes	185/984 (18.8)	0.0400	2.08	UR	
No	10/100 (10.0)		(1.06-4.08)		
<b>Skilled labor</b>					
Yes	94/639 (14.7)	0.0106	0.69	0.0025	0.64
No	176/884 (19.9)		(0.52-0.91)		(0.48-0.85)
<b>Feeding of dogs with sheep placental remains</b>					
Yes	100/426 (23.5)	0.0001	1.71	0.0001	1.79
No	164/1080 (15.2)		(1.29-2.26)		(1.34-2.38)

p:p value; OR: odds ratio; CI: confidence interval; UR: unrealized - variable not included in the multiple logistic regression analysis due to a loss of questionnaire filling greater than 20%.

the constant technical orientation of the employees has an impact on the adoption of good production practices, leading to abandonment and a gradual increase in the need to hire new laborers.

The more technologically advanced production systems, the full confinement and semi-confinement systems, when compared to the extensive system, presented as protective factors against the occurrence of *T. gondii* seropositivity. The observed data were corroborated by Pinheiro et al. (2009) when they found that sheep farming properties where the production systems were extensive presented 2.3-fold greater chances of occurrence of toxoplasmosis when compared to those where the system was intensive. However, Gazzonis et al. (2016) observed that properties that adopted semi-extensive production systems presented a 3.5-fold higher probability of occurrence of ovine neosporosis. As described by Dubey & Schares (2011), in the semi-extensive production system, animals were supplemented with fodder and grains, which were stored in barns that were easily accessible to dogs, a practice that poses a high risk of infection.

Cats on farms often have free access to pastures, livestock facilities, food and supplement stores, and water sources and reservoirs, where they can eliminate *T. gondii* oocysts when defecating. The presence of cats on the properties is the main factor of environmental contamination and, consequently, of infection in humans and animals (Weigel et al., 1999). For the free access of animals to natural sources of water and the supply of water to animals in containers outside the premises, the possibility of contamination occurs due to the access of dogs and cats to these areas. Gondim et al. (2005) showed that dogs can eliminate *N. caninum* oocysts more than once in their lives, contaminating food and water. Cavalcante et al. (2008) found that *T. gondii* infection is associated with the low frequency of sanitation measures for goat drinkers in Ceará state and emphasized the impossibility of

the sanitation of the facilities used for capturing and storing large volumes of water, which thus become modes of transmission to animals.

Although the dog is the definitive host of *N. caninum*, the variable presence of dogs on the properties was not significantly associated with seropositivity for this parasite; the presence of dogs was even observed on 71.4% of the properties (60/84). In Brazil, there is a cultural practice of feeding dogs with viscera from slaughtered sheep or placental remains, which may contain parasites and infect dogs, which in turn end up in feces, thereby contaminating the environment. Dubey et al. (2007) warned against the implementation of preventive measures to combat neosporosis, such as restricting the access of dogs to the areas of production animals and the correct disposal of placental remains.

*Toxoplasma gondii* is known to cause fetal resorption, miscarriages, and neonatal deaths of lambs and has been pointed out as the main parasite responsible for abortion in sheep and goats (Buxton et al., 2007, Moreno et al., 2012). In the present study, the occurrence of abortion on the property was not statistically associated with seropositivity for *T. gondii* or *N. caninum*, suggesting that the causes of abortion on these properties may not be the studied parasites or may be from other factors in conjunction with them. Approximately in 1.4 to 3.9% of the world's sheep flocks are losses due to reproductive effects, mainly abortion, associated with toxoplasmosis during pregnancy (Freyre et al., 1999). However, it is known that females that had abortion on account of *T. gondii* infection might also have protection against future reproductive problems due to toxoplasmosis (Dubey et al., 2020). In another study at Paraíba state, northeast of Brazil, it was observed that around 50% of the sheep herd was seronegative at the age of 1 year, period of the first pregnancy (Valencio et al., 2020), making it highly susceptible to reproductive problems caused by this parasite.

The study conducted allowed the knowledge of the *T. gondii* and *N. caninum* distribution in the state of Paraná in sheep, with a higher prevalence of toxoplasmosis. The study of the variables associated with these parasites allowed us to characterize the deficiencies of environmental management and sanitation, the presence of reservoirs and the types of exploitation. It can also be used to support health programs and as a basis for future public policies related to the prevention of *T. gondii* and *N. caninum*.

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