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Neospora caninum and *Toxoplasma gondii*: seroprevalence and associated factors in cows from milk farms of Toledo, Parana, Brazil

Neospora caninum e *Toxoplasma gondii*: soroprevalência e fatores associados em vacas leiteiras de Toledo, Paraná, Brasil

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Highlights:
Nowadays, neosporosis is the major cause of abortion in cattle.
Toxoplasmosis is one of the most common parasitic infections in humans and animals.
Serology is an important tool to diagnose the disease.

Abstract

Neospora caninum and *Toxoplasma gondii* are important protozoa in cattle production. Neosporosis is responsible for reproductive failures in cattle, constituting one of the main causes of abortion and leading to significant economic losses in dairy farming. Toxoplasmosis is a cosmopolitan disease of great importance in the context of public health and animal production. Considering the lack of available data on the subject for the studied region, the objective of the present study was to detect the seroprevalence of antibodies against *N. caninum* and *T. gondii* in dairy cows in the municipality of Toledo, Western Paraná, Brazil and to assess how to detect factors associated with risk or protection. Serology was performed with serum samples from 390 dairy cows from 58 farms by indirect immunofluorescence (IFI) reaction using cutoff point 100 for *N. caninum* and 64 for *T. gondii*. The analysis of associations between seropositivity and the variables detected in the properties was also performed. The seroprevalence for *Neospora caninum* was 15.4% (60/390; CI 95% 12.4-19.3) and for *Toxoplasma gondii* was 38.7% (151/390; CI 95% 34.0-43.6). The prevalence of co-infection was 8.72% (34/390; CI 95% 6.31-11.94). For *N. caninum*, the presence of abortion was observed as a risk factor (OR= 5.32). With *T. gondii*, the presence of abortion was found to be a risk factor (OR= 2.09) and properties with 10 or more lactating animals was found to be a protection factor (OR= 0.56). Abortion was also detected as a factor associated with the risk of co-infection (OR= 11.80). Through serological diagnosis, the present study showed that protozoa are widely distributed among dairy cattle in the region, and control and prophylaxis measures should be adopted in the studied animal population. Because abortion is a risk

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associated with *N. caninum*, *T. gondii*, and the two agents surveyed (co-infection), all reproductive disorders detected in cattle should include neosporosis and toxoplasmosis as a differential diagnosis.
Key words: Antibodies. Dairy Cattle. Neosporosis. Toxoplasmosis.

Resumo

Neospora caninum e *Toxoplasma gondii* são protozoários importantes na cadeia produtiva dos bovinos. A neosporose é responsável por falhas reprodutivas nesta espécie, constituindo uma das principais causas de abortamento, levando a significativas perdas econômicas na pecuária leiteira. A toxoplasmose é uma doença cosmopolita, com grande importância no contexto de saúde pública e produção animal. Considerando a escassez de dados disponíveis sobre o tema para a região estudada, o objetivo do presente estudo foi detectar a soroprevalência de anticorpos contra *N. caninum e T. gondii* em fêmeas de bovinos leiteiros no município de Toledo, Oeste do Paraná, Brasil, bem como detectar os fatores associados ao risco ou proteção. A sorologia foi realizada com amostras de soro de 390 fêmeas bovinas leiteiras provenientes de 58 propriedades rurais, pela reação de Imunofluorescência Indireta (IFI) utilizando ponto de corte 100 para o *N. caninum* e 64 para o *T. gondii*. Também foi procedida as análises das associações entre a soropositividade e as variáveis detectadas nas propriedades. A soroprevalência encontrada para o *Neospora caninum* foi de 15,4% (60/390; IC 95% 12,4-19,3) e para o *Toxoplasma gondii* foi de 38,7% (151/390; IC 95% 34,0-43,6), sendo que a co-infecção detectada foi de 8,72% (34/390; IC 95% 6,31-11,94). Para o *N. caninum* foi observado como fator associado ao risco a presença de aborto (OR= 5,32). Já para o *T. gondii*, como fatores associados ao risco, foi encontrado a presença de aborto (OR= 2,09) e como fator associado à proteção, foi detectado propriedades com 10 ou mais animais em lactação (OR= 0,56). O aborto foi também detectado como um fator associado ao risco da co-infecção (OR= 11,80). Através do diagnóstico sorológico o presente estudo evidenciou que os protozoários estão amplamente distribuídos entre os bovinos leiteiros da região e medidas de controle e profilaxia devem ser adotadas na população animal estudada. Devido ao aborto ser uma associação de risco para o *N. caninum*, para o *T. gondii* e para os dois agentes pesquisados (co-infecção), todas as desordens reprodutivas detectadas em bovinos devem incluir a neosporose e a toxoplasmose como diagnóstico diferencial.
Palavras-chave: Anticorpos. Bovinocultura leiteira. Neosporose e Toxoplasmose.

Introduction

Dairy cattle are considered an important production chain in Brazilian agribusiness, and Brazil is home to one of the largest productive herds in the world, with 23 million lactating cows and a production of 34.5 billion liters of milk in 2017 (Ministério da Agricultura, Pecuária e Abastecimento [MAPA], 2017). Paraná is considered the second state in the country's dairy production ranking, and Toledo is the fifth milk producing city in the state.

Currently, neosporosis is considered one of the main causes of reproductive problems (Cerqueira-Cézar, Calero-Bernal, Dubey, & Gennari, 2017). Since its discovery, *Neospora caninum* has been associated with miscarriages, new insemination or coverage costs, reduced cow life, and reduced milk production (Melo, Silva, Ortega-Mora, Bastos, & Boaventura, 2006). Vertical transmission or transplacental is the most important source of infection in the disease, where agent transmission is from mother to fetus (Dubey, Hemphill, Calero-Bernal, & Schares, 2017). Protozoa can also be acquired after birth, through horizontal transmission by ingestion of food or water contaminated by sporulated oocysts that are eliminated by the definitive hosts (Paré, Thurmond, & Hietala, 1997).

Toxoplasmosis is a form of zoonosis caused by the protozoan *Toxoplasma gondii* and, unlike *N. caninum*, involvement in reproductive problems in cattle has not been conclusively demonstrated

(Dubey, 2010). It is also of little epidemiological importance in terms of infection in humans through consumption of milk and beef (Dubey, 1986). A point to note in cattle infection is that pastures contaminated with oocysts are the main route of transmission to these animals (Marana, Venturini, Freire, Vidotto, & Navarro, 1994), indicating that extensive rearing of cattle breeding is a sentinels of oocysts shedding, through the contact of cattle with the pastures contaminated by feline feces.

The epidemiological study of *N. caninum*, as well as *T. gondii* is essential to know the real prevalence of the disease and subsequently to enable measures for the control and eradication of the disease. Besides, serological screening of the herd can be useful in the strategic control of diseases. (Dubey & Schares, 2011) Thus, the objective of the present study was to detect the seroprevalence of antibodies against *N. caninum* and *T. gondii* in dairy cows in the municipality of Toledo, Western Paraná, Brazil, as well as to detect the factors associated with risk or protection.

Materials and Methods

All procedures performed involving animals in this study were approved by the Animal Use Ethics Committee of the Pontifical Catholic University of Paraná under certificate 809/2013.

Study area and sampling

The city of Toledo (24°43'11.12"S/ 53°44'35.86"W) is located in the western region of the state of Paraná, at an altitude of 560 m and encompassing an area of 1196,999 km^2. The climate is subtropical, with an annual average temperature above 16ºC, and with rainfall distributed throughout the year, especially in summer. The estimated human population is 133,824 inhabitants (Instituto Brasileiro de Geografia e Estatística [IBGE], 2016).

Sampling was obtained through the OpenEpi program. 3.01, where a prevalence of 50%, an expected error of 5%, and a confidence interval of 95% were used, resulting in a minimum sampling of 377 animals. The number of properties was obtained from Emater of the municipality. Blood was collected from 10 animals at random from each property, except when the herd size was smaller than 10, in which case blood was collected from five animals at random.

Serum samples from 390 lactating cows over two years old from 58 farms were analyzed. Samples were taken from 2013 to 2014. For all the sampled animals, serum was obtained from whole blood, collected through coccygeal, mammary, or jugular venipuncture; the collected blood underwent centrifugation procedure at the Laboratory of Veterinary Parasitology. The serum samples were placed in microtubes and kept frozen (negative 18°C) until the serological test.

Epidemiological questionnaire

For the study of factors associated with *N. caninum* and *T. gondii*, the epidemiological questionnaire was completed by the researcher at the time of the visit to the properties, with information from the observation of the characteristics and the property database. The questionnaire was elaborated according to Moura, Osaki, Zulpo, Garcia and Teixeira (2012).

Environmental variables were evaluated (presence of dogs; presence of cats; presence of wild canines and felines; presence of rodents; feeding of dogs and cats; water supply); sanitary and reproductive (purchase of animals for breeding; quarantine; childbirth picket; abortion; estrus reoccurrence; veterinary care; artificial insemination; debilitated newborn; reproductive management); and productive (facility characteristics; roughage/ concentrate supplementation; other species; presence of pastures in common with other properties; herd management; breed; number of cattle on the property; number of lactating animals; daily milk production; age).

Serological test

Determinations of anti-IgG antibody titers against *N. caninum* and *T. gondii* were performed using the indirect immunofluorescence (IFI) technique according to Conrad et al. (1993) and Camargo (1974), respectively, using as antigens the strains NC-1 for *N. caninum* and RH for *T. gondii*. Anti-bovine IgG conjugate (FITC - Sigma®) was used. The cutoff points were 100 for *N. caninum* and 64 for *T. gondii*. Positive and negative control samples were included in all tests.

Statistical analysis

The association between *N. caninum* and *T. gondii* seropositive animals and risk or protection variables was investigated by the chi-square (x^2) test (Mantel-Haenszel) and odds ratio with a significance level of 5% and 95% confidence interval (CI), using Epi Info 7 software.

Results and Discussion

The seroprevalence was found to be 15.4% for *Neospora caninum* (60/390; CI 95% 12.4-19.3) and 38.7% for *Toxoplasma gondii* (151/390; CI 95% 34.0-43.6). Antibody titers against *N. caninum* ranged from 100 to 6400 and for *T. gondii* the range was 64 to 1024 (Table 1). In the herd studied the co-infection found between *N. caninum* and *T. gondii* was 8.72% (34/390; CI 95% 6.31-11.94).

Table 1
Results and antibody titers against *Neospora caninum* and *Toxoplasma gondii* in dairy cattle from Toledo, Paraná, Brazil in the years 2013-14

Results/Titers	Number	%	CI 95%
Neospora caninum			
Negative[1]	330	84.6	80.7-87.7
Positive	60	15.4	12.4-19.3
Titers			
100	21	5.4	-
200	18	4.6	-
400	10	2.6	-
800	7	1.8	-
1600	2	0.5	-
3200	1	0.3	-
6400	1	0.3	-
Total	390	100	-
Toxoplasma gondii			
Negative[2]	239	61.3	56.4-66.0
Positive	151	38.7	34.0-43.6
Titers			
64	111	28.5	-
256	38	9.7	-
1024	2	0.5	-
Total	390	100	-

[1]Titers 0-50; [2]Titers 0-16.

Of the 58 farms where samples were collected, 35 (60.3%) presented seropositive animals only for *N. caninum*; 53 (91.4%) properties presented seropositive animals only for *T. gondii*, and 34 (58.62%) had seropositive animals for both studied protozoa. Four properties (6.90%) had all seronegative animals for both agents.

The results of the associations between seropositivity for *N. caninum* and *T. gondii* and the variables studied are shown in Table 2. As a result of the analysis of the odds ratio (OR), with OR>1 indicating association, only the presence of abortion was found to be a risk factor (OR=5.32) associated with *N. caninum.* For *T. gondii* positive animals, only the presence of abortion (OR= 2.09) was found to be a risk-associated factor and the protection factor was herd size of 10 or more lactating animals (OR= 0.56).

Table 2
Associated factors with infection by *Neospora caninum*, *Toxoplasma gondii* and both protozoa (co-infection) in dairy cattle from Toledo, Paraná, Brazil in the years 2013-14

Protozoan/Variable	Total	Positives (%)	OR[1]	CI 95%[2]	P[3]
N. caninum					
Abortion					
No	95	4 (4.21)	5.32	1.88-20.76	< 0.001
Yes	295	56 (18.98)			
T. gondii					
Abortion					
No	95	25 (26.32)	2.09	1.25-3.48	0.004
Yes	295	126 (42.71)			
Lactating animals by property					
< 10	190	87 (45.79)	0.56	0.36-0.86	0.007
≥ 10	200	64 (32.00)			
N. caninum e T. gondii					
Co-infection					
No	239	26 (10.88)	2.38	1.36-4.16	0.002
Yes	151	34 (22.52)			
Co-infection*					
Abortion					
No	95	1 (1.05)	11.80	2.21-245.94	0.002
Yes	295	33 (11.19)			

[1]Odds Ratio; [2]Confidence interval; [3]P value [chi-square (x^2) test Mantel-Haenszel)]; *Reference.

In the present study, it was observed that 32.35% (11/34; CI 95% 7.39-50.53) of the co-infected animals had antibody titers indicating chronic infection for both diseases, and 20.59% (7/34; CI 95% 8.70-37.90) indicated acute infection titers for the two protozoans studied. Of these co-infected animals, 70.59% (24/34; CI 95% 52.52-84.90) and 41.18% (14/34; CI 95% 24.65-59.30) were determined to have chronic infection for *T. gondii* and *N. caninum*, respectively, and 29.41% (10/34; CI 95% 15.10-47.48) and 58.82% (20/34; CI 95% 40.70-75.35) presented acute infection titers for *T. gondii* and *N. caninum*, respectively.

The municipality of Toledo has a herd of approximately 19,415 lactating animals, distributed in 1,630 properties and producing approximately 100,667,000 liters per year, making the region the third largest milk producer in Paraná (IBGE, 2016). Extrapolating the percentages of the study against the two agents surveyed to an absolute value, we find that infection from these agents is highly relevant in the Toledo cattle population. This finding underscores the importance of control and prevention measures.

Epidemiological studies indicate that there are considerable differences in the prevalence of neosporosis between countries, regions, dairy and beef cattle, and management systems (Dubey & Schares, 2011). Even with the economic losses generated by the infection, few studies of dairy cattle have been performed in our state. Locatelli-Dittrich et al. (2008) detected an occurrence of antibodies of 33% in dairy cattle in the broad Paraná. Camillo et al. (2010) detected 24.2% seropositivity in dairy cattle from the southwest of the state. Also in this same region, Rocha et al. (2015) detected an antibody frequency in 35.1% of the Dutch cows studied. Snak, Garcia, Lara, Pena and Osaki (2018) studying the agent detected a prevalence of IgG immunoglobulin of 23.7% in dairy cattle in the western region of the state. When analyzing occurrence by property, Locatelli-Dittrich et al. (2008) found 77% positivity and Snak et al. (2018) reported 80% of farms with at least one positive animal. The authors mentioned above showed high rates when compared with the present study in both animals and properties; this reveals the diversity of the level of protozoan infection in our state.

According to Thurmond and Hietala (1996), occurrence of anti-*N. caninum* greater than 10% on the property may indicate frequent miscarriages throughout the year. Also, Andreotti et al. (2010) found a 15% loss of pregnancy (resorption and abortion) in seropositive cows relative to seronegative cows. Corbellini, Driemeier, Cruz, Gondim and Wald (2002) observed that *N. caninum* seropositive cows had 3.3 times higher risk of abortion, which may account for the high number of properties reporting reproductive disorders in their herds. These data indicate that the presence of *N. caninum* in cattle herds is a relevant factor in the reproductive efficiency of cows.

Dubey (1986), reported that titers for *T. gondii* in cattle are low, most often 64 and rarely ≥256, which may be due to the fact that bovines are more resistant to the protozoan. Perhaps this episode is related to the few existing studies in cattle on *T. gondii*. Ogawa, Freire, Vidotto, Gondim and Navarro, (2005) studied the occurrence of *T. gondii* in dairy cattle in the northern region of Paraná state and detected 26% seropositivity. In the case of beef cattle, Moura, Osaki, Zulpo, Garcia and Teixeira (2010) reported 30.8% seropositivity occurrence in cattle from a slaughterhouse in Guarapuava-PR. Analyzing dairy cattle samples in Toledo-PR, Meirelles, Locatelli-Dittrich, Castilhos and Busch (2014) detected 32.2% seropositivity. In comparison with these published studies, our study presented a higher seroprevalence; this may be related to the fact that it is a prevalence study, different from the above-mentioned occurrence studies, and may also be due to differences in management systems and across regions.

Presence of reproductive problems, such as abortion, reported in the results of the epidemiological questionnaire, was a risk association detected in this study for both *N. caninum* and *T. gondii*. Cardoso, Amaku, Araújo and Gennari (2012) observed a relative risk of abortion in *N. caninum* seropositive cows, and among the evaluated reproductive indexes only this was significant. Mazuz et al. (2014) in a study of pregnant cows, found that the percentage of abortions in *N. caninum* seropositive cows was three times higher than in seronegative cows. Frigotto, Genz, Nino, Zulpo and Garcia (2018), studying *N. caninum* in dairy cows, observed an association between seropositive cows and history

of abortion; the authors reported that cows with a history of abortion were 10.6 times more likely to be seroreactive than cows without a history of abortion. Our work further reinforces the relationship between abortion and *N. caninum* seropositivity, but we must not forget that *T. gondii* was also identified as a risk factor associated with abortion, so this etiological agent should also be included as a differential in reproductive cases of this species.

Snak et al. (2018), studying dairy properties in Western Paraná, found as a risk association with *N. caninum* low milk production, which corroborates with our study, as we detected properties with more than 10 animals acting as a protective association with *T. gondii*. We can consider that these farms with more than 10 lactating animals use or seek technologies to increase livestock production, resulting in better herd health, a fact that may explain the protection factor found. Small farms are usually family farms with lower dairy production, where animals are raised mainly on pastures, with a greater chance of contact with the *T. gondii* oocyst that may be present in pasture soil or water.

In the present study, co-infection was statistically a factor associated with the risk for both diseases. This data is possibly not directly related to co-infection or immunosuppression by the studied protozoa, but rather to postnatal infection in association with the type of diet of these animals, since farm dogs and cats can spread the oocysts in the food and environment (Galvão, Vaconcellos, Navarro & Bresciani, 2014).

Infection by *N. caninum* and *T. gondii* may be associated with immunosuppressive diseases that hinder the host immune response. (Such diseases were not included in our analysis). As both researched protozoans are chronic, etiological agents, the bovine leukemia virus, for example, can reactivate neosporosis and latent toxoplasmosis, resulting in disruption of tissue cysts and causing new parasitemia in the bovine (Akhtardanesh, Ziaali, Sharifi, & Rezaei, 2010).

Conclusion

Through a serological survey we concluded that the protozoa *N. caninum* and *T. gondii* are circulating among the dairy herd surveyed and that abortion is a risk factor for both agents surveyed in this region. In this sense, neosporosis and toxoplasmosis should be included as a differential diagnosis for reproductive disorders of cattle in this region. Control and prophylaxis measures for these diseases should be adopted by veterinarians and owners to reduce economic losses.

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