

***Neospora caninum*: seroepidemiology of cows and dogs from milk farms of Cunha, São Paulo, Brazil**

***Neospora caninum*: soroe epidemiologia de vacas e cães de propriedades leiteiras de Cunha, São Paulo, Brasil**

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Abstract

Neosporosis, a parasitic disease prevalent worldwide, is caused by the protozoan *Neospora caninum*, an obligatory intracellular parasite that can cause abortions and infertility in females, resulting in high economic losses. The aim of this study was to determine the seroprevalence of antibodies against *N. caninum* in dairy cattle, and the occurrence of antibodies in dogs from farms in the municipality of Cunha, São Paulo, Brazil. Additionally, eighteen months after the primary study, an analysis to determine the incidence, seroconversion, and occurrence of anti-*N. caninum* antibodies was conducted in cows. Sampling was carried out in two phases: during the first one, 400 dairy cattle and 50 dog blood samples from 40 farms were collected. During the second phase, blood samples were collected from 119 cows belonging to 16 farms visited 18 months before. Serum samples were analyzed by indirect immunofluorescence (IFI) with titers ≥ 100 for cattle and ≥ 50 for dogs considered as positives. The occurrence of antibodies against *N. caninum* was 15.0% (60/400) and 14.0% (7/50) for cattle and dogs, respectively. After 18 months, the observed incidence of antibodies was 7.4% (4/54), the conversion detected was 71.4% (seropositive to seronegative) and 7.4% (seronegative to seropositive animals), and the observed occurrence was 7.56% (9/119). The calculated odds ratio (OR) was significant ($p < 0.05$) for abortions, presence of dogs, and parturition stalls. However, protection factors were identified in 2- to 4-year-old dairy cattle and in the number of lactating cows ≤ 10 animals. Thus, the presence of antibodies against *N. caninum* in the studied suggested both vertical and horizontal transmission are occurring in cattle.

Key words: Antibodies. Milk. Neosporosis. Seroconversion.

Resumo

A neosporose é uma doença parasitária de distribuição mundial, cujo agente etiológico é o protozoário intracelular obrigatório *Neospora caninum*, o qual pode causar abortamentos e infertilidade em fêmeas, promovendo elevados prejuízos econômicos. O objetivo deste estudo foi determinar a soroprevalência

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de anticorpos contra *N. caninum* em bovinos de leite e a ocorrência de anticorpos em cães destas propriedades do município de Cunha, São Paulo, Brasil. Como segunda etapa, 18 meses após o primeiro estudo, foi conduzido outro estudo em vacas para determinar da incidência, soroconversão e ocorrência de anti-*N. caninum*. As colheitas foram realizadas em duas fases. Na primeira, participaram do estudo 400 bovinos de leite e 50 cães pertencentes a 40 propriedades rurais. Na segunda fase, foram efetuadas novas colheitas em 119 vacas de 16 propriedades já anteriormente visitadas na fase 1. Amostras de soro foram analisadas por imunofluorescência indireta (IFI) com títulos ≥ 100 para bovinos e ≥ 50 para cães foram considerados positivos. A ocorrência de anticorpos contra *N. caninum* foi de 15,0% (60/400) e 14,0% (7/50) para bovinos e cães respectivamente. Após 18 meses a incidência de anticorpos observada foi de 7,4% (4/54), a conversão detectada foi de 71,4% (animais soropositivos para soronegativos) e 7,4% (animais soronegativos para soropositivos) e a ocorrência observada foi de 7,56% (9/119). O “odds ratio” (OR) calculado revelou significância estatística ($p < 0,05$) para os seguintes fatores; abortamentos, propriedades com cães, e piquetes de parição. No entanto, fatores associados a proteção da positividade foram identificados entre; vacas entre 2 a 4 anos de idade, e o número de vacas em lactação ≤ 10 animais. Neste trabalho foi revelada a presença do *N. caninum* circulando na região estudada, sugerindo o papel de fêmeas bovinas e cães como transmissores do agente etiológico. A detecção de anticorpos contra *N. caninum* no presente estudo sugerem que tanto a transmissão vertical como a horizontal estão ocorrendo nos bovinos.

Palavras-chave: Anticorpos. Leite. Neosporose. Sorocorversão.

Introduction

Milk cattle chain is an important productive system all over the world, reaching a production of 656 thousand tons of milk in 2015 (FAO, 2016). Brazil has one of the largest herds in the world, with 23 million dairy cattle; it produced 34.5 billion liters of milk in 2017, which is estimated to increase annually by 2.1-3.0% (MAPA, 2017).

Neosporosis, a disease caused by the protozoan *Neospora caninum*, is a widespread problem in cattle and dogs (DUBEY, 2003) and is considered the main cause of abortions in cattle herds (ALMERÍA; LÓPEZ-GATIUS, 2015; LINDSAY et al., 1996). Vertical is the most frequent transmission route, where the pregnant cows transmit the protozoan to their fetuses, causing abortion or birth of congenitally infected offspring (RAMOS et al., 2016). In a study on aborted dairy cows in the state of Santa Catarina, 51.2% of the cows and 26.7% of the fetuses were positive for *N. caninum* (MACEDO et al., 2017). This parasite can also be acquired after birth by eating food or water contaminated by sporulated oocysts or by ingesting tissues infected with tachyzoites or tissue cysts, contributing to horizontal transmission (PARÉ et al., 1997).

Neosporosis is responsible for high economic losses in cattle, especially in dairy farming (DUBEY; LINDSAY, 1996). Investment in resources and measure such as professionals to perform diagnoses, control measures for infected animals that have undergone abortion, establishment of new pregnancies, prevention of premature death of adult females following abortion, replacement of slaughtered animals, and compensation for possible losses in milk production is urgently needed (PARÉ et al., 1997; DUBEY, 1999; CERQUEIRA-CEZAR et al., 2017). In several studies carried out on dairy cows in Brazil, the occurrence of antibodies against *N. caninum* varied from 12.2 to 92.1% (CORBELLINI et al, 2002; GUEDES et al., 2008).

Because of the scarcity of information on the presence of *N. caninum* in the herd from the municipality of Cunha, São Paulo, Brazil, the aim of this study was to investigate seroepidemiology and factors associated with *N. caninum* infection in cows and dogs from dairy farms. Additionally, we performed a second sampling from some farms visited 18 months before.

Materials and Methods

Study area and population

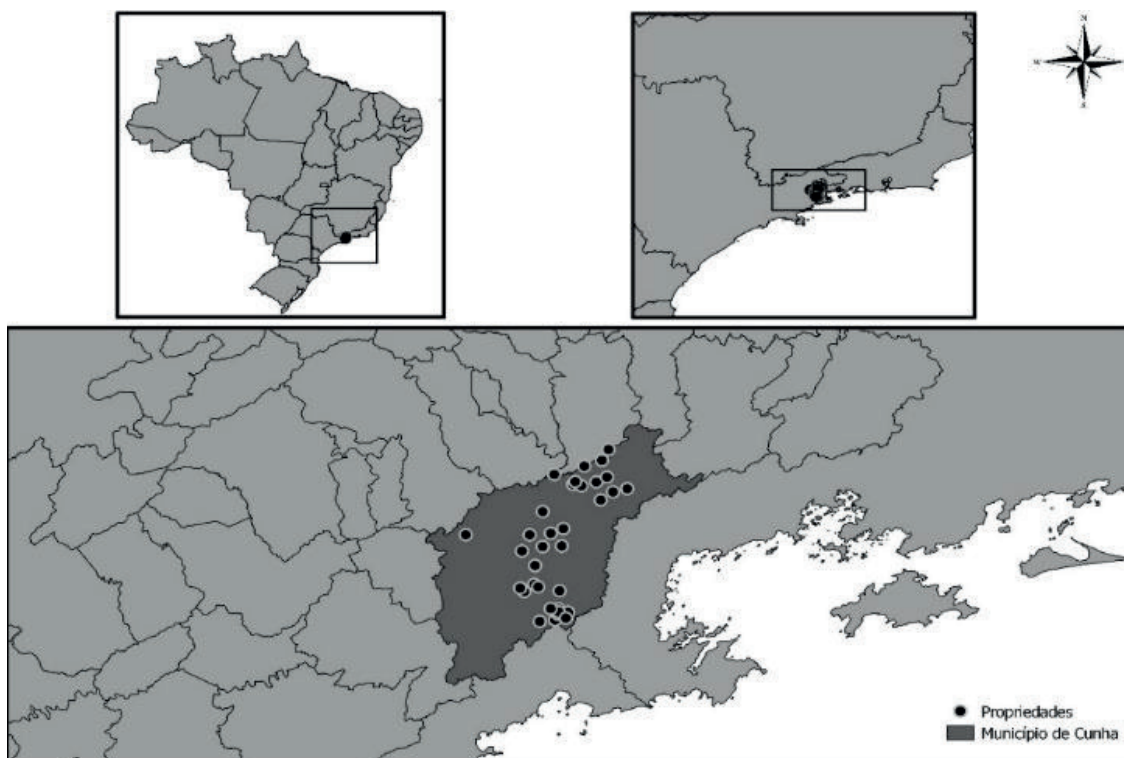
The municipality of Cunha is located at the border between the states of São Paulo and Rio de Janeiro, in the Vale do Paraíba Paulista region, with an area of 1,400 km² and a population of 22 thousand inhabitants (IBGE, 2010). Extensive dairy/beef farms are the most important activity among the Agricultural Production Units, accounting for approximately 50% of the productive areas in the municipality. In 2014, the number of bovine heads registered in the Agricultural Defense Coordination was 90,000, distributed in 1,886 properties (CDA-SP, 2014); the daily milk production was estimated at 48,000 liters (CATI, 2008). A cross-sectional study was designed to document *N. caninum* in dairy cattle from the city of Cunha, São Paulo, Brazil.

Sampling

Phase 1. *N. caninum* prevalence study in dairy cattle and occurrence in dogs was conducted between November and December 2015.

The sample size was obtained using the Open Epi 3.01 program, where a prevalence of 50%, expected error of 5%, and 95% confidence interval (CI) were used, resulting in a minimum sample size of 384 cows. Sampling was non-probabilistic for convenience, and properties were selected according to the availability of the owner, so that there was representativeness in the municipality (Figure 1).

Figure 1. The Cunha municipality and milk farms location. Samples were obtained from 40 farms as indicated on dots.



In total, 400 blood samples were collected from lactating cows in 40 small, medium, and large-size properties. Number of collected samples depended on the number of lactating cows in each farm: 5 samples from farms with up to 10 lactating animals, 10 samples from farms with up to 20 lactating animals, and 20 samples from farms with more than 20 lactating animals. In total, blood samples were collected from 50 dogs, belonging to 26 properties; 14 properties had no dogs.

Phase 2. Study of *N. caninum* incidence, seroconversion, and occurrence in cattle conducted between May and June 2017

Eighteen months after phase 1, new blood samples were collected in bovine females in 13 previously evaluated properties, obtaining 187 samples. Of these, 68 samples were collected from cows already studied in phase 1 in order to evaluate *N. caninum* incidence and seroconversion.

The present study was approved by the Ethics Committee on the Use of Animals of the Pontifical Catholic University of Paraná (protocol no. 0991/2015).

Serological testing

The determination of anti-*N. caninum* IgG antibody titers was performed using the indirect immunofluorescence (IFI) technique according to Conrad et al. (1993). The slides for antibody detection were prepared with *N. caninum* tachyzoites (strain NC-1), and samples with titers ≥ 100 were considered positive for bovines (CARDOSO et al., 2012; KATTO et al., 2017), while titers ≥ 50 were considered positive for dogs (MASCOLLI et al., 2015). Positive and negative controls were used at each slide.

Epidemiological questionnaire

For the study of the factors associated with seropositivity for antibodies against *N. caninum*, an epidemiological questionnaire with information on

the characteristics of each farm was prepared. We studied variables consisting of environmental factors (water source and characteristics of the buildings), sanitary and reproductive factors (abortion, infertility, occurrence of debilitated newborn, use of insemination, diagnosis of reproductive diseases, use of vaccine, purchase of cattle for breeding), and productive factors (management of the herd, number of cattle in the property, number of lactating animals, daily milk production, race, age, veterinary assistance, rental of pasture, exchange of animals between third-party properties, and mineral salts).

Statistical analysis

The association between seropositivity and risks or protection was investigated by chi-square test (X^2) and odds ratio (OR), with significance level of 5% and CI of 95%, using the Epi Info 7 software. Results were considered significant at $p < 0.05$.

Results

The seroprevalence of *N. caninum* detected for bovines was 15% (60/400, 95% CI: 11.83-18.83). Regarding the properties, 70% (28/40, 95% CI: 53.47-83.44) showed at least one animal positive for *N. caninum*. When antibody titers were studied, 46.3% (25/60) of cows presented titers of 100, 27.8% (15/60) of 200, 16.7% (9/60) of 400, 1.8% (1/60) of 800, 3.7% (2/60) of 1,600, and 3.7% (2/60) of 3,200. Regarding the results of the serological tests performed on the dogs present in the properties, 14% (7/50) presented anti-*N. caninum* antibodies.

Epidemiological risks for seropositivity are presented on Table 1, significant results were showed for abortion (OR = 3.77, CI: 1.19-11.95, $p=0.002$), farms with seropositive dogs (OR = 2.45, 1.23-5.00, $p=0.009$), and presence of stalls for calving (OR = 2.32, 1.31-4.10, $p=0.003$). Animals between 2 and 4 years old (OR 0.20, 95% CI: 0.08-0.47, $p=0.0001$) and farms with fewer than 10 lactating cows (OR 0.44, 95% CI: 0.20-1.01, $p=0.049$) were considered as preventing factors.

Table 1. Studied variables showing statistical association with serological results.

Variables	Total	Positives (%)	OR	IC 95%	p
Abortion					
yes	13	5 (38.5)	3.77	1.19-11.95	0.002
no	387	55 (14.2)			
Farms with dogs					
yes	95	20 (21.0)	2.45	1.23-5.00	0.009
no	175	17 (9.7)			
Offspring stalls					
yes	105	25 (23.8)	2.32	1.31-4.10	0.003
no	295	35 (11.9)			
Age (years)					
≥ 5	274	54 (19.7)	0.20	0.08-0.47	0.0001
2 to 4	126	6 (4.8)			
Number of lactating cows					
≤ 10	85	7 (15.6)	0.44	0.20-1.01	0.049
> 10	315	53 (14.8)			

The second sampling showed an occurrence of anti-*N. caninum* antibodies in cows of 7.56% (9/119) and for farms of 46.2% (6/13). Sixty eight out of 119 cows, with samples collected during phase 1, were used for a comparative study, and the occurrence of anti-*N. caninum* antibodies was 11.8% (8/68). When we compared the serological status of those 68

animals, 20.6% (14/68) had seroconversion either for positive or negative, and 79.4% (54/68) cows maintained the same serological status (Table 2). The majority of animals that seroconverted changed from seropositive to seronegative (71.4% (10/14)), while 7.4% (04/54) changed from seronegative to seropositive.

Table 2. *Neospora caninum* serological status from same dairy cows at first and second sampling.

Results	First Sampling		Second Sampling (18 months apart)		
			=	≠	
+	14 (20.6%)	+	10 (71.4%)	4 (7.4%)	4 (5.9%)
		-			
-	54 (79.4%)	+	14 (20.6%)	54 (79.4%)	68 (100%)
		-			
Total	68 (100%)		14 (20.6%)	54 (79.4%)	68 (100%)

Discussion

The 15% prevalence of anti-*N. caninum* antibodies observed in the present study can be compared to that described in two studies carried out in the Paraíba Valley. Cardoso et al. (2012)

evaluated three farms in Pindamonhangaba-SP and reported occurrence of anti-*N. caninum* antibodies in cows (3.3-22.1%). Munhoz et al. (2006) studied 29 and 28 farms in Rio Claro and Resende-RJ, respectively, and described an antibody occurrence

of 20.4% and 25.7%, respectively. These studies showed the protozoan circulation in the animals of Vale do Paraíba and the variation in the occurrence of antibodies.

Studies on dairy cattle in Brazil using IFI and the same cut-off we used in our study showed a similar occurrence of antibodies against *N. caninum* of 10.9% to 13.8% (AGUIAR et al., 2011; AMARAL et al., 2012; MOURA et al., 2012; SILVA et al., 2015; PADILHA et al., 2017). However, other studies showed higher occurrence of antibodies compared to our data ranging from 23.7 to 50.7% (TEIXEIRA et al., 2010; MELO et al., 2006; SNAK et al., 2018). These data and discrepant results may be explained by the different management practices in each property, and environmental and climatic conditions in each region.

Snak et al. (2018) observed 80% of farms positive for *N. caninum*, similar to 85% (SILVA et al., 2008) and 85.7-89.7% (MUNHOZ et al., 2006) described previously. However, Moura et al. (2012) detected animals positive for *N. caninum* in only 40% of the evaluated properties. We detected 70% of farms with at least one cow positive for *N. caninum*. These results show a considerable spread of the parasite, indicating the importance of detecting the occurrence of vertical and horizontal transmission.

Dogs as definitive hosts of *N. caninum* can be associated to infection in cows, as the results of risk association tests confirmed, where cows from farms with dogs had 2.45 times more chances to be infected by *N. caninum* than cows from farms without dogs. Ribeiro et al. (2019) showed that cattle from farms with dogs were 1.84 times more susceptible to infection by *N. caninum* than those from farms without dogs. In our study, the occurrence of 14% antibodies anti-*N. caninum* detected in the dogs was higher than that detected by Masculli et al. (2015) (7%) and Martins et al. (2012) (6.1%).

The offspring stall was considered in the present study as a risk of association for neosporose, what could be associated to greater exposure to excretions

from uterus and consequently attraction for dogs (DUBEY et al., 2007). Dogs could be infected by placental remnants and abortion products, and cows by ingestion of oocysts present at the site, increasing the frequency of horizontal transmission (DUBEY; SCHARES, 2011). Thus, properties with dogs and with offspring stall may present favorable conditions for the maintenance of the parasite cycle.

The number of lactating cattle per property ≤ 10 was considered a protective factor against cow seropositivity for *N. caninum*. Hasegawa et al. (2004) reported that the lower the number of cattle per hectare, the lower the rate of infected animals. Eiras et al. (2011) also observed that seropositivity was significantly associated with the large size of the herd.

Another protection factor detected in the study was the age of the cows (2-4 years old), because the younger the animal, the lower the risk of contact with the protozoan in the environment. Fávero et al. (2017) reported that each year of life of cattle increases the chance of becoming infected by 1.25 times.

Eighteen months after the first study, we performed a second sampling from 68 cows that were sampled before, and it was observed an incidence of antibodies of 7.4%, corroborating the data showed by Katto et al. (2017) who reported an incidence of 7.7%.

We also showed that 71.4% (10/14) of seropositive females in 2015 became seronegative in 2017, indicating a high rate of seroconversion. This may be related to the immunological factors against the protozoan and shows the importance for the understanding of the pathogenesis and definition of diagnostic criteria (ANTONELLO et al., 2015). In addition, the fluctuation of antibodies may influence the seropositivity for *N. caninum* of the herds over time. Katto et al. (2017) detected 23.1% of seropositivity in cattle that changed to 15.5% after 2 years.

Conclusion

The present study demonstrated that *N. caninum* circulates in the dairy herd and among the dogs in the properties of the studied region. Abortion, seropositive dogs, and offspring stalls were factors associated with the risk of infection in cows; 2 to 4-year-old dairy cattle and properties with fewer than 10 cows seemed to be protected against infection. The study of seroconversion showed 7.4% horizontal transmission and fluctuation of antibodies against *N. caninum*. Therefore, monitoring the circulation of the agent in the environment via assessments of prevalence, occurrence, antibody fluctuation, and seroconversion should be promoted and carried out frequently.

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