



Cholinesterase as an inflammatory marker of subclinical infection of dairy cows infected by *Neospora caninum* and risk factors for disease

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ABSTRACT

The aim of this study was to evaluate the seroprevalence of *Neospora caninum* in dairy cows, as well as to verify the risk factors for *N. caninum* infection and inflammatory response in dairy cows. Using the indirect immunofluorescence reaction, the seroprevalence of neosporosis was determined to be 32.82%. Based on regression analysis, the presence of dogs at a farm increased the probability of a cow testing positive for *N. caninum* (OR = 20.01 [5.21–123.12]). These data suggest that *N. caninum* has a relevant prevalence in dairy cows of the Microregion of Rio do Sul (Brazil), with elevated frequencies of anti-*N. caninum* IgG. The data also suggest that the parasite is widely distributed in dairy herds of the micro-region, because 94.4% of properties screened had at least one seropositive animal. The principal risk factors for disease maintenance in herds may be considered the presence of dogs and absence of a diagnostic test when introducing new animals. Blood from these cows was used to measure variables related to the inflammatory response. Serum cholinesterase activity, as well as serum levels of globulins and C-reactive protein were higher in seropositive to *N. caninum* than in seronegative cows. Furthermore, the infection by parasite causes an intense inflammatory process, contributing to disease pathophysiology.

1. Introduction

Neosporosis is an emergent disease that affects several animal species, and is important because of its high prevalence in dairy farms worldwide [1]. The disease is caused by the intracellular parasite *Neospora caninum*, considered one of the most important causes of abortion in dairy cows [2–4], as well as repetitions of estrus, successive abortions and temporary anestrus associated with *N. caninum* infection [5].

Bovines are considered the main intermediary hosts of *N. caninum*. Its economic importance is associated with costs related to abortion, decreased milk production, and increased discard and replenishment of animals. Several studies report *N. caninum* infection in Brazil; however,

its occurrence in bovines in the Santa Catarina (SC) state, including the Microregion of Rio do Sul (Fig. 1), remains unknown. The introduction of infected animals, without known diagnosis, to a herd can increase transmission of infection, demonstrating the importance of knowing the health status of animals [1].

Cholinesterase is a low-grade inflammatory marker that identifies cell lesions [6]. The cholinergic system is a major modulatory pathway in the central nervous system, serving essential vital functions, including anti-inflammatory and immunomodulatory effects [7]. This system is involved in anti-inflammatory reactions via two enzymes, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), both of which have important roles during immune and inflammatory responses [8,9]. AChE hydrolyzes acetylcholine (ACh), a

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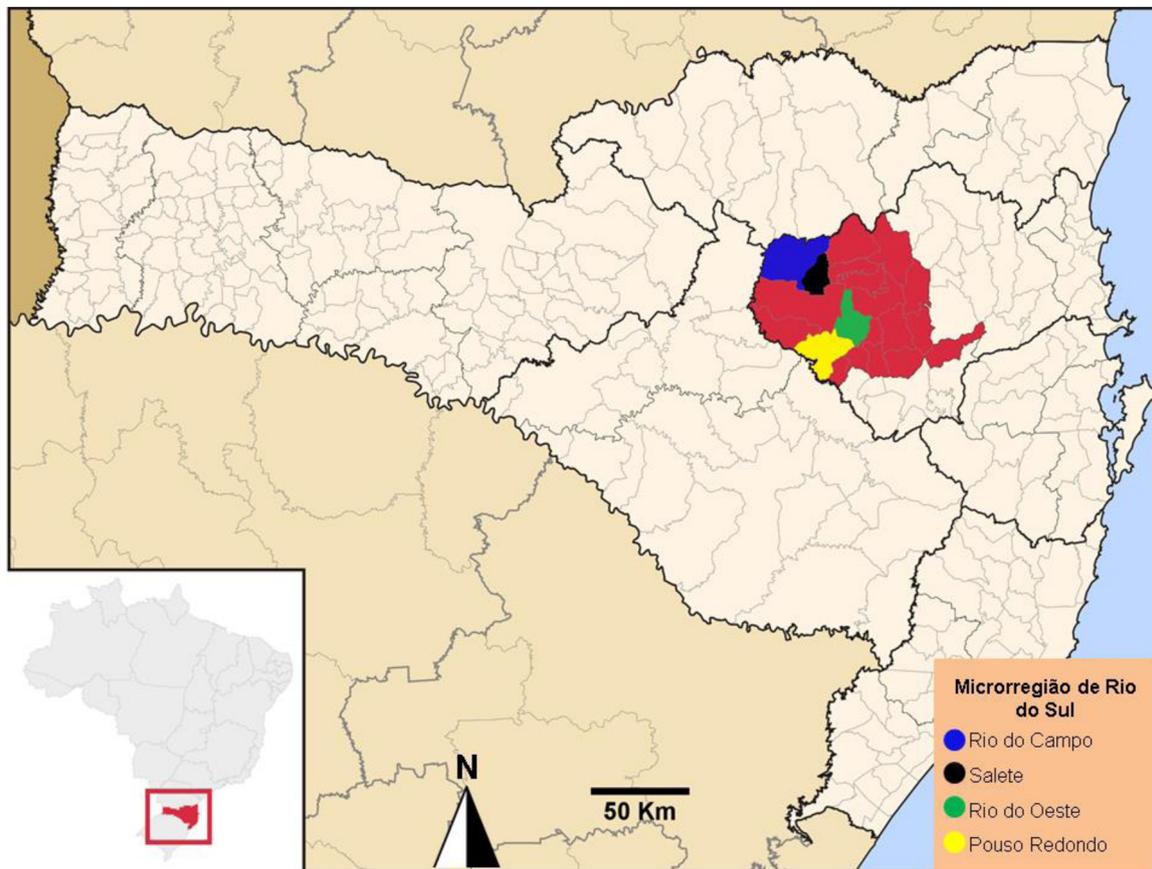


Fig. 1. Map of Santa Catarina state. The municipalities where blood collections were performed are highlighted (blue, dark, green and yellow). The red area represents the Microregion of Rio do Sul.

neurotransmitter with anti-inflammatory effects, into choline and acetate, while the BChE hydrolyzes butyrylcholine (BCh) and other varieties of esters, including ACh [10]. Studies have demonstrated that increased AChE and BChE activities during parasitic infections create pro-inflammatory profiles due to decreases in ACh levels, because ACh is a cholinergic molecule with anti-inflammatory properties [9]. Tonin et al. [11] reported an augmentation of AChE activity in total blood as well as BChE activity in the plasma of gerbils experimentally infected with *N. caninum* during the chronic phase of disease; however, the effects during natural infections remain unknown.

Therefore, the aim of this study was to evaluate the prevalence of *N. caninum* in dairy cows, as well as verify the risk factors for *N. caninum* infection and inflammatory response in dairy cows.

2. Material and methods

2.1. Area of experiment and samples

The study was performed in dairy cows (2 to 8 years of age) in various phases of lactation or dry period from 36 rural properties in 4 counties (Rio do Campo, Saleté, Pouso Redondo and Rio do Oeste), all localized in Microregion of Rio do Sul, Alto Vale do Itajaí, Santa Catarina state (Brazil) (Fig. 1).

The formula $n = Z^2 \times p(100-p) / e^2$ was used to determine the minimum number of samples to be collected (n), with a level of confidence (z) according to the standard normal distribution (1.96), a tolerated margin of error (e) of 95% [12] and a prevalence (p) of 15%. We used the arithmetic mean of two similar published studies undertaken in SC state, which estimated prevalence of 7.7% [13] and 23.1% [14] considering a standard error of 5%. Therefore, a minimum of 195 animals were required to be sampled; however, we collected 262

samples because of possible losses during collection or transport.

The blood was collected by jugular puncture in sterile tubes without anticoagulant. At this time, a questionnaire was applied to properties to obtain data linked to management, production system, history of reproductive problems and the presence of dogs at the property. After blood collection, the samples were maintained at room temperature for up to 2 h to allow clotting. The serum was obtained after centrifugation (1700 g, 15 min), then stored at -20°C until used in the indirect immunofluorescence reaction at the Laboratory of Veterinary Parasitology (UDESC).

2.2. Epidemiological survey

A questionnaire was administered at the time of sample collection in order to investigate risk factors for *N. caninum*, as well as to verify possible causes and effects related to neosporosis. The following variables were included in the epidemiological survey: type of diet (pasture, concentrate or both), storage of concentrated food (silo or not), water source (natural, river, lake or potable water), presence of dogs on the property, dogs with access to pasture, origin of the animals (born on the farm or acquired), age (in years), completion of tests for neosporosis before purchasing an animal, number of gestations, reproductive problems (abortion, estrus repetition, mummified fetus), neurological problems, and use of natural breeding or artificial insemination.

2.3. Serological testing

Serum samples were subjected to serological testing by indirect immunofluorescence reaction, considered the reference test for neosporosis [15] because of its safety and high specificity for the detection of maternal infections [16]. In this technique, anti-*N. caninum*

Table 1

Analysis of the questionnaire about risk factors linked to *N. caninum* infection in the Microregion de Rio do Sul, Santa Catarina state, Brazil.

Variable	OR	CI _{95%}
Age		
2 – 3 years	–	–
3 – 4 years	0.80	(0.43 –1.53)
4 – 6 years	0.60	(0.29 –1.05)
> 6 years	0.67	(0.35 –1.18)
Food storage		
Silo	–	–
Bag	1.58	(0.94 –2.86)
Presence of dogs		
Yes	20.01	(5.21 –123.12)
No	–	–
Dogs with access to pasture		
Yes	–	–
No	0.81	(0.51 –1.44)
Gestations		
1 gestation	–	–
2 gestations	0.74	(0.37 –1.38)
3 gestations	0.60	(0.33 –1.29)
≥ 4 gestations	0.74	(0.42 –1.43)
Reproductive problems		
Abortion	–	–
Repetition of estrus	0.36	(0.42 –1.56)
Mummification	0.19	(0.24 –2.89)
More than one up	0.37	(0.46 –1.59)
Number of reproductive problems	0.76	(0.78 –1.08)
Reproduction		
Artificial insemination	–	–
Natural mount	0.64	(0.65 –1.65)
Origin of animals		
Property	–	–
Acquired	0.46	(0.55 –1.28)

immunoglobulin G (IgG) was measured in the serum. The samples were applied to glass slides previously sensitized with antigen obtained from *N. caninum* tachyzoites (strain NC-1) maintained in a culture of VERO cells [12]. The serum was diluted (1:100) and rabbit fluorescein isothiocyanate (FITC)-labeled anti-bovine IgG was used as the secondary antibody [17]. Seroreagent samples at 1:100 were tested in higher dilutions (1:200, 1:400 and 1:800) to determine total antibody titer.

Control serum from animals known to be positive and negative were used as internal quality controls, and test samples were considered positive when total peripheral fluorescence of the tachyzoites was observed [12].

2.4. Cholinesterase, total protein, albumin, globulin and C-reactive protein

After the serological test, 25 negative and 25 positive samples were separated and biochemical analyses were performed. The analyzed samples had been frozen for six months and had not been thawed since the collection period. The cows included in this stage of the research were apparently healthy; they had not been tested for other morbidities because there are "n" possibilities of infectious agents in cattle, and this was a field study.

Cholinesterase activity and total protein and albumin levels were evaluated on a semi-automated biochemical analyzer (BioPlus Bio-2000) using commercial kits (Analisa[®]). Globulin values were calculated from the difference between total serum protein and albumin.

The quantification of serum C-reactive protein (CRP) was undertaken using an ultrasensitive CRP commercial kit (BioTécnica, Minas Gerais, Brazil) following the manufacturer's protocol. All assay steps were performed automatically on a Bio-2000 analyzer (BioTécnica, Minas Gerais, Brazil), with results expressed as mg/dL.

2.5. Statistical analysis

The observed occurrence of neosporosis in the study region was calculated from the proportion of positive test results. Information regarding animal health and management characteristics of the sampled farms were used to evaluate possible factors influencing the risk of *N. caninum* infection. A Bayesian logistic regression was performed using Hamiltonian Monte Carlo (HMC), an algorithm particularly good for use with high-dimensional models, as implemented in RStan (Stan Development Team, 2015), a convenience package for Stan. The model was specified using weakly informative priors, reducing overfitting and helping the Markov chain to converge to the posterior distribution more effectively than flat priors. The posterior distribution we present here is based on 5000 samples from three chains (after 1000 adaptation steps). These samples were sufficient to establish convergence to the target posterior distribution. Running the chains longer produced no change in inference. We assessed convergence using R-hat Gelman and Rubin statistics. All R-hat values were less than 1.01 (R-hat values > 1.01 can indicate that the chain did not converge). Finally, the mean and Bayesian 95% credible interval (CI) were estimated and expressed as odds ratios (OR) for risk factor representation. One-way ANOVA was performed for comparison between groups (seropositive and seronegative). Linear correlation analysis was used to evaluate the effect of antibody titration on cholinesterase, total protein, albumin, globulin and C-reactive protein. A p-value of 0.05 was considered statistically significant.

3. Results

3.1. Seroprevalence and risk factors

The seroprevalence to *N. caninum* in the study was 32.82% (86/262). The maximum antibody titers against *N. caninum* were as follows: in 54 cows (62.79%) it was 1:100; in 26 cows it was 1:200 (30.26%); and in 6 cows it was 1:400 (6.97%).

Regression analysis showed that only the presence of dogs at a farm increased the probability of a cow testing positive for *N. caninum* (OR = 20.01 [5.21–123.12]); with a very large confidence interval; therefore, this result should be interpreted with caution. Results of univariate analysis are shown in Table 1.

3.2. Cholinesterase, total protein, albumin, globulin and C-reactive protein

Among the 25 seropositive *N. caninum* samples used for measurement of cholinesterase, total protein, albumin, globulin and C-reactive protein, 9 showed titers of 1:100, 11 were 1:200 and 5 were 1:400. The levels of cholinesterase, total protein, albumin, globulin and CRP are shown in Table 2. Cows seropositive for *N. caninum* exhibited higher total cholinesterase activity, as well as higher globulin and CRP levels ($P < 0.05$), compared to seronegative cows.

There were positive correlations ($P < 0.05$) between antibody concentration in serum and cholinesterase activity ($r = 0.85$), concentrations of total protein ($r = 0.44$), globulin ($r = 0.96$), and CRP ($r = 0.90$). No correlation was found between antibody concentration and albumin levels ($P > 0.05$).

Table 2

Mean levels of cholinesterase, total protein, albumin, globulin and C-reactive protein (CRP) in serum from dairy cows tested for *Neospora caninum*.

Variables	Seronegative cows	Seropositive cows	P value
Cholinesterase (U/L)	2654.4 ± 1624.9	4624.0 ± 2427.1	0.0365
Total protein (g/dL)	7.74 ± 1.56	8.91 ± 1.12	0.0872
Albumin (g/dL)	3.42 ± 0.96	3.06 ± 1.31	0.6478
Globulin (g/dL)	4.32 ± 1.13	5.85 ± 1.03	0.0011
CRP (mg/dL)	6.57 ± 2.40	11.72 ± 2.78	0.0001

4. Discussion

We found that 32.8% of dairy cows tested were seropositive for *N. caninum*, with 94.4% of the properties sampled in the Microregion of Rio do Sul showing at least one seropositive animal. This result suggests that *N. caninum* is an important cause of reproductive problems in this region. Several variables were tested to evaluate relationships with seropositive animals; however, only one significant difference was observed and this was the presence of dogs on properties, suggesting an increased risk for cows to be infected by the protozoan. Dogs were present at the vast majority of properties, suggesting that dogs are an important risk factor associated with disease transmission [13]. Evidence that dogs play an important role in the transmission of *N. caninum* has been presented in numerous studies, and recent evidence demonstrated that dogs can release up to 503,300 oocysts into the environment [18]. It is also possible that animals can eliminate the oocysts intermittently or for long periods of time, possibly increasing the risk of infection. Bovines infected by the parasite can transmit the disease vertically to offspring, further increasing the proportion of cattle contaminated in the herd [18]. According to Mello et al. [19], for each dog at the property, the probability of a cow being seropositive increases 1.13 times. Dogs can also transport aborted fetuses and placental remnants from one place to another, serving as food for other definitive hosts, including wild canids [19].

The relatively high seroprevalence (32.8%) identified in this study suggests that herds in this region are likely to be more prone to abortions, since Fávero et al. [17] reported a 3- to 7-fold increase in likelihood of abortion in cows in which the disease is present. Additionally, Macedo et al. [20] demonstrated that 51.2% of dairy cows in SC state that aborted were seropositive for *N. caninum*.

The seroprevalence observed in this study was higher than the national average in dairy cows, which is 16.1% [21]. This increased seroprevalence may have occurred because all samples were analyzed from animals older than 24 months, with animals in this age group 3.1 times more likely to be infected than younger animals [4]. The reported prevalence is similar to that reported by Fávero et al. [17], who demonstrated a seropositivity of 30.7% in the west of SC state, and was substantially greater than that found in the Concórdia region (7.7%) [13] as well as other regions of SC state [22], including Lages city (SC), where the prevalence of dairy cows seropositive for *N. caninum* was 23.1% [22]. An experimental study performed in 2010 using 120 blood samples obtained from dairy cows (pregnant and not pregnant) located in Presidente Getúlio city (SC), revealed that 42.5% of animals were seropositive for *N. caninum* [23], a similar result to what we found in this study. The reported prevalence of *N. caninum* in dairy cows in Brazil varies between 15.1% and 91.2% [4,24–26], although differences may be directly linked to the serological techniques used, the adopted cut-off values, the sample size, and the type of breed.

On the questionnaire, all producers responded that they did not perform any tests for neosporosis, and this fact demonstrates an important source for the introduction, dissemination and permanence of the disease in herds. The constant acquisition of seropositive cattle may be another explanation for the prevalence of infection, as well as for maintenance of the parasite [19]. Because the disease can be introduced into properties through the acquisition of seropositive cows, testing of cows prior to their introduction to the herd can help owners to avoid this problem [17]. Despite the fact that the detection of antibodies is insufficient to affirm that *N. caninum* is the unique agent responsible for disease in the herd, its presence is of great use for the epidemiological study of disease [27]. A study conducted by Nicolino et al. [28] reported more than 474 million abortions in dairy cows in Brazil caused by neosporosis, resulting in severe economic losses for dairy farms [28].

In the present study, we observed a significant increase in serum cholinesterase activity, as well as elevated serum levels of globulin and CRP, in agreement with results observed by Tonin et al. [11] in gerbils

experimentally infected by *N. caninum*. Augmentation of AChE activity in total blood and BChE activity in plasma can be considered a pro-inflammatory response due to the resulting reduction of ACh levels, a molecule with anti-inflammatory and immunomodulatory properties [10,11]. The intense inflammatory process can be confirmed by elevated serum CRP levels, an important inflammatory protein that increases up to 1000-fold at sites of infection and/or inflammation [29]. Despite the fact that CRP has been traditionally utilized as a marker of infection, there is now growing evidence that CRP plays important roles in inflammatory processes and host responses to infection, including the complement pathway, apoptosis, and the production of cytokines and globulins [29]. Therefore, the augmentation of serum globulin levels can be thought of as a consequence of increased serum CRP levels, and can be considered an attempt to augment the immune response during infection, as demonstrated for neosporosis by Donahoe et al. [30].

Among the limitations of this study, we emphasize that co-morbidities in the cows were not evaluated; therefore, we do not know the impact of a co-infection on the results concerning inflammatory markers. There are many possibilities with respect to subclinical infection; for example, mastitis, gastrointestinal parasites, ectoparasites, babesiosis, and anaplasmosis, among others, are endemic in Brazil and commonly affect cows. It is important to emphasize that this was a field study; these risks are expected because they are "n" possibility of co-morbidities. We believe our results are reliable because the animals were selected for the presence and absence of antibodies to *N. caninum*.

Our data suggest that *N. caninum* has a relevant prevalence in dairy cows in the Microregion of Rio do Sul, with elevated frequencies of anti-*N. caninum* IgG; furthermore, the parasite is widely distributed in dairy herds, with 94.4% of the properties surveyed having at least one seropositive animal. The data further suggest that the main risk factors for disease maintenance in herds may be the presence of dogs and absence of diagnostic testing when introducing new animals. In our study, we did not evaluate anti-*Neospora* seropositivity in dogs on the farms. However, according to a study carried out in 2011, the seroprevalence of dogs for *N. caninum* ranges from 11.5% to 13% in municipalities of Santa Catarina [31], regions close to our study with cows. The results reinforce the need for new studies to verify the possible participation of this disease agent in bovine reproductive disorders, as well as the need for a vaccine that provides protection against transplacental infection caused by this protozoan. It can also be concluded that seropositive cows, although not symptomatic, present higher levels of inflammatory markers, including cholinesterases, globulins and C-reactive protein. We also concluded that there is a strong positive correlation between the concentration of antibodies against *N. caninum* in cows and the levels of total protein, globulin and C-reactive protein as well as cholinesterase activity in the blood.

Ethical approval

The research was approved by the Committee of Ethics in the Use of Animals (CEUA) of the IFC-Concordia under the number 17/2017.

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