

Neospora caninum infection in cattle in the state of Amazonas, Brazil: seroprevalence, spatial distribution and risk factors

Infecção por *Neospora caninum* em bovinos no estado do Amazonas, Brasil: soroprevalência, distribuição espacial e fatores de risco

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Abstract

Livestock in the Amazon has grown significantly and, although neosporosis in cattle has been reported worldwide, there is no information about *N. caninum* in production systems in the state of Amazonas. The objective of this study was to determine the prevalence of anti-*Neospora caninum* antibodies in cattle, their spatial distribution and the risk factors associated with *N. caninum* infection in the state of Amazonas. Questionnaires were applied to farmers to assess risk factors associated with *N. caninum* infection. Blood samples were collected from 1,073 animals on 47 farms in 33 municipalities in the four Amazonian subpopulations. IgG anti-*N. caninum* antibodies were detected by the indirect fluorescence test, with a general prevalence of 30.2%, being seropositive in 43 farms (91.5%), with prevalence ranging from 2.2% to 69.2%. The highest number of high density points was found in subpopulation 3 (municipality of Apuí and other municipalities on the Madeira River and affluent). It was concluded that *N. caninum* is present with high seroprevalence values, when compared to other cattle producing states in the Amazon region of Brazil. The identified factors can be used as risk indicators so that control measures can be implemented to prevent infection by *N. caninum* in these herds.

Keywords: Amazon rainforest, *Neospora caninum*, prevalence, IFAT, cattle, Brazil.

Resumo

A pecuária na Amazônia tem crescido significativamente e, embora a neosporose em bovinos tenha sido relatada em todo o mundo, não há informações sobre *N. caninum* nos sistemas de produção no estado do Amazonas. Objetivou-se determinar a prevalência de anticorpos anti-*Neospora caninum* em bovinos, sua distribuição espacial e os fatores de risco associados à infecção por *N. caninum* no estado do Amazonas. Questionários foram aplicados aos fazendeiros, para avaliar fatores de risco associados à infecção por *N. caninum*. Amostras de sangue foram coletadas de 1.073 animais em 47 fazendas, em 33 municípios das quatro subpopulações amazonenses. Anticorpos IgG anti-*N. caninum* foram detectados pelo teste de imunofluorescência indireta, com prevalência geral de 30,2%, com soropositividade em 43 fazendas (91,5%), com prevalência variando de 2,2% a 69,2%. O maior número de pontos de alta densidade foi encontrado na subpopulação 3 (município de Apuí e demais municípios do rio Madeira e afluentes). Concluiu-se que *N. caninum* está presente com altos valores de soroprevalência, quando comparado a outros estados produtores de gado na região amazônica do Brasil. Os fatores identificados podem ser usados como indicadores de risco, para que medidas de controle possam ser implementadas para prevenir a infecção por *N. caninum* nesses rebanhos.

Palavras-chave: Floresta Amazônica, *Neospora caninum*, prevalência, IFI, gado, Brasil.

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Introduction

In Brazil's Amazon region, composed of municipalities in the states of Rondônia, Acre, Amazonas, Roraima, Pará, Amapá, Tocantins, Mato Grosso, Maranhão, the herds of the states of the Amapá, Roraima, Amazonas and Acre, together, represented only 5.9% of the total for the region (Valentim & Andrade, 2009). Amazonas is estimated to have 1.48 million head of cattle, which corresponds to only 0.69% of the national herd (ADAF, 2019). Several obstacles hinder the development of beef cattle in this state. Factors such as deforestation, pasture degradation, high rainfall indices, and lack of information about diseases that affect zootechnical indices all contribute to meager production and economic rates (Arima et al., 2005). Infectious diseases are considered an important etiological cause of losses in cattle herds (Pinheiro et al., 2000; Reichel et al., 2013), above all neosporosis, which is considered the main disease associated with reproductive impairment in cows in different regions around the world (Dubey et al., 2007; Dubey & Schares, 2011; McAllister, 2016). In Brazil, this infection is considered prevalent throughout the territory (Cerqueira-Cézar et al., 2017).

Neosporosis is caused by the coccidium *Neospora caninum*. The main pathways of transmission of this disease are transplacental and the ingestion of sporulated oocysts excreted in the environment by the definitive hosts, especially domestic dogs (McAllister et al., 1998; Donahoe et al., 2015). The pathogenicity of *N. caninum* varies according to its host species. Cattle are the most susceptible hosts, and the disease causes abortions, stillbirths and the birth of weak calves. *N. caninum* infection in cattle is reported in every continent where cattle are raised (Dubey & Schares, 2011). However, prevalence rates of the infection in cattle herds vary significantly, depending on factors such as production systems, the presence of definitive hosts, herd health management (Ghalmi et al., 2012).

Several studies have been conducted in different regions of Brazil to detect anti-*N. caninum* IgG antibodies in cattle, whose prevalence ranges from 9.1% to 97.2% (Hasegawa et al., 2004; Vianna et al., 2008; Andreotti et al., 2010; Amaral et al., 2012; Silva et al., 2017). In the Amazon region, *N. caninum* infection has been described in the states of Pará (Minervino et al., 2008; Silva et al., 2017), Rondônia (Aguiar et al., 2006; Boas et al., 2015) and Tocantins (Martins et al., 2011), with prevalence rates varying from 10.4% to 52%. However, there are no seroepidemiological studies of *N. caninum* infection in the state of Amazonas, so there is a gap in information about herd health and factors associated with infection in this region of the country. Thus, our aim was to determine the seroprevalence, spatial distribution and risk factors associated with *Neospora caninum* infection in cattle herds in the state of Amazonas.

Material and Methods

Farms and sampling

This study was approved by the Ethics Committee on Animal Use (CEUA) of the Federal Rural University of Pernambuco (UFRPE), under protocol no. 80/2018.

Farms were selected in the four subpopulations in the state of Amazonas, defined by the Ministry of Agriculture, Livestock and Food Supply – MAPA (Brasil, 2019) (see Figure 1). These subpopulations are as follows: Subpopulation 1 – comprising the municipalities of Rio Negro and its tributaries; Subpopulation 2 – composed of the municipalities of Rio Solimões and its tributaries; Subpopulation 3 – comprising the municipality of Apuí and the other municipalities of the Madeira River and its tributaries, and Subpopulation 4 – comprising the other municipalities of the state of Amazonas on its border with the state of Pará.

Blood samples were collected from 1073 reproductively mature cows (older than 24 months) on farms with more than 10 cattle (primary sampling units) in 33 municipalities in the state of Amazonas. The animals were identified individually by an ear tag usually attached to the left ear. This tag number was recorded on the label of the vacutainer tube and on a sample collection form. For the study sample calculation, an expected prevalence of 50%, 95% confidence and 5% statistical error were considered (Thrusfield, 2004).

Sample collection for serology

Blood samples were collected by venipuncture of the external jugular vein into sterile and tagged vacutainer tubes. Ten mL of blood without anticoagulant were drawn into individual test tubes after prior skin antisepsis with 3.0% iodinated alcohol. The samples were centrifuged at 1000 x g for five minutes to obtain serum. The blood sera were aliquoted and stored at -20°C until serological tests were performed.

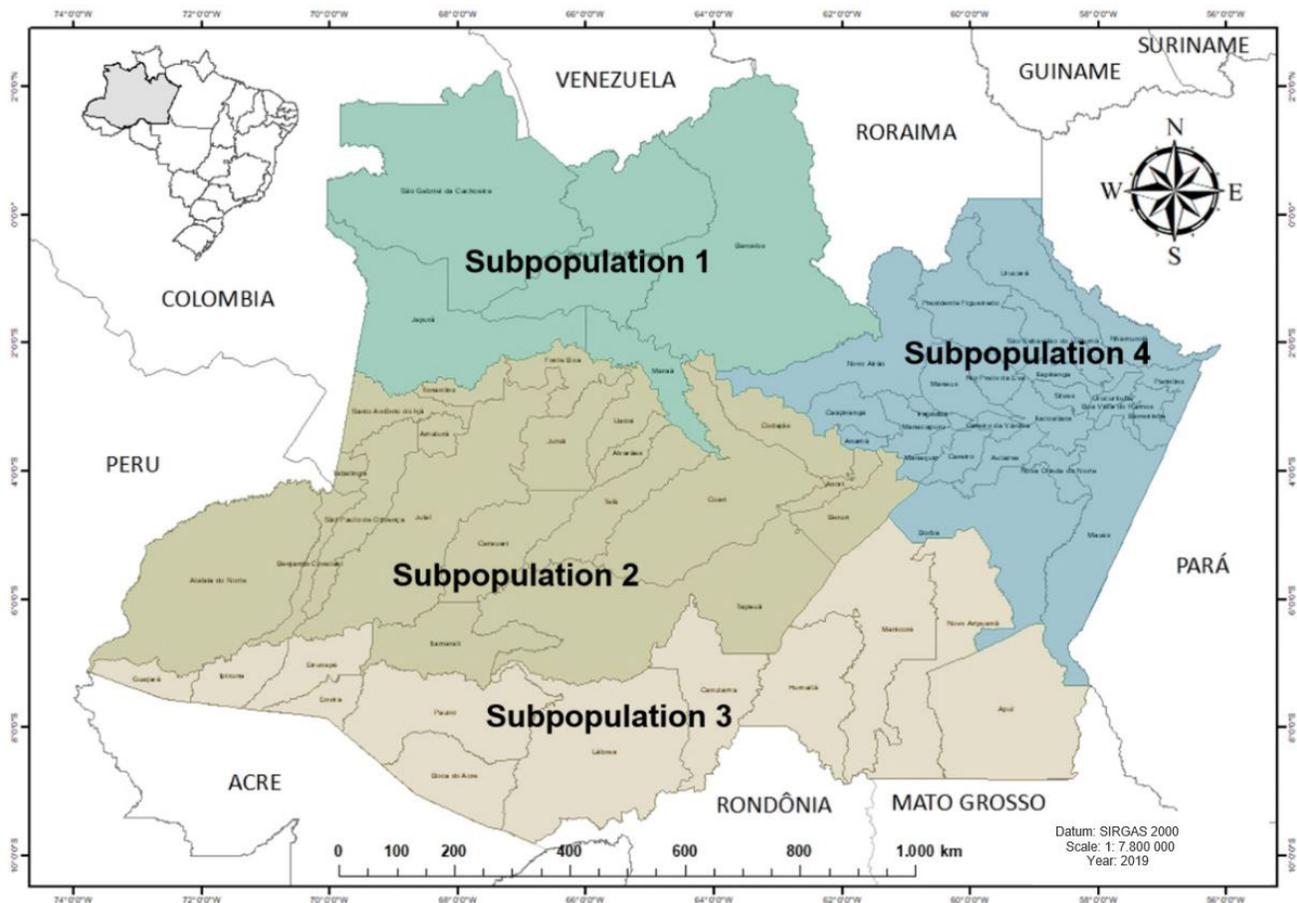


Figure 1. Map of the subpopulations (1, 2, 3, and 4) of the state of Amazonas, according to the Ministry of Agriculture (Brasil, 2019), Livestock and Supply of Brazil.

Preparation of antigen for the IFAT

Neospora caninum antigen stored in a culture of MARC-145 cell monolayers was used, according to the conditions described by Regidor-Cerrillo et al. (2010). The number of viable tachyzoites was determined by counting in a Neubauer chamber using 0.2% trypan blue. The purified tachyzoites were suspended in 0.6% formalin solution in PBS, adjusted to a concentration of 1200-1500 parasites/ μ L, and each well of the slides for the indirect fluorescent antibody test (IFAT) was sensitized with 10 μ L of the tachyzoite suspension. All the microscope slides were fixed in acetone at -20°C and stored under refrigeration until use. All slides were fixed in acetone at -20°C and stored under refrigeration until use.

Survey of anti-*N. caninum* IgG antibodies

To begin with, serum samples were diluted 1:200 (cutoff) (Gondim et al., 1999) in 1X PBS, distributed in the wells of the sensitized slides and incubated in a humidifying chamber at 37°C for 30 min. The slides were then washed and reincubated with fluorescein isothiocyanate-conjugated anti-bovine IgG serum (Sigma Chemical, USA) containing 0.02% Evans blue (Sigma Chemical, USA). Lastly, the slides were washed again, covered with buffered glycerine and coverslips and examined under an epifluorescence microscope (Nikon Eclipse 40x objective lens). Samples were considered positive when 50% of the tachyzoites in the wells presented total peripheral fluorescence (Kim et al., 2019). Serum samples known to be positive and negative were included as control samples. Positive control sera were obtained from cattle with consistently positive serological results by IFI and negative control sera from healthy cattle with consistently negative serological results by IFI, as mentioned above. The slides were examined by an epifluorescent microscope (Olympus, Mod. BH2, Tokyo, Japan).

Spatial distribution and of *N. caninum* infection

The coordinates of each farm were determined by georeferencing, using a TrackMaker® PRO GPS to characterize the map of the state of Amazonas, Brazil. The georeferenced data were entered into the ArcMap version 12.2.2 program, using the Kernel Density Estimator (KDE), which is a non-parametric technique that allows the variability of a data set to be filtered while preserving the essential local characteristics of the data (Bailey & Gatrell, 1995). The parameter used for elaboration of the maps using the KDE was the frequency of *N. caninum*-positive cattle per farm studied. This frequency was classified in ranges and the density expressed by color: very high (red color), high (dark orange color), medium (yellow color), low (green), very low (yellow color) and negative (blue). The number of farms located in the different areas was counted on the maps resulting from this operation.

Risk factors associated with *N. caninum* infection

Pre-structured questionnaires were applied the farmers to assess the risk factors associated with *N. caninum* infection, variables included in the analysis final model were: type of farming system (type of herd; livestock farming system; farm size; herd size) and management-related (water source; presence of other domestic and wild animals; occurrence of abortion; birth of weak calves).

The absolute and relative frequencies were subjected to dispersion. The risk factors associated with *Neospora caninum* infection were studied based on an analysis of the variables of interest using Pearson's chi-square test or Fisher's exact test, when necessary. A logistic regression analysis was then performed, considering the IFAT results (positive or negative) as the dependent variable. The independent or explanatory variables considered in the final model were those with a statistical significance of <0.05. The statistical calculations were made using Epi Info version 3.5.1 software.

Results

The prevalence of anti-*Neospora caninum* antibodies in cattle in the state of Amazonas was 30.2% (324/1073; CI 95%: 27.5% – 33.0). The frequencies of each the subpopulations are shown in Table 1. No significant differences (p>0.05) were observed between the prevalence of subpopulations.

Table 1. Prevalence of *Neospora caninum* infection in cattle per subpopulations (1,2,3 and 4) in the state of Amazonas, Brazil.

Subpopulation	Total No. of animals	No. positive animals	<i>Neospora caninum</i> infection (%)
1	64	17	26.6%
2	174	51	29.3%
3	467	136	29.1%
4	368	133	36.1%
Subtotal	1073	324	30.2%

The analysis of the results per farm indicated that 91.5% (43/47) of farms had at least one positive animal. The KDE of the *N. caninum* infection per farms in the state of Amazonas revealed the presence of seropositive animals distributed throughout the all subpopulation of state, indicated rates varied from 2.2 to 69.2%, with the more points of high density found in subpopulation 3 (Figure 2).

A univariate analysis revealed a significant association between *N. caninum* infection in cattle and the following variables: type of herd (p=0.009); type of livestock farming system (p=0.012); source of water supply (p=0.000); presence of dogs (p=0.000); occurrence of abortion (p=0.000); newborn mortality rate (p=0.000); and birth of weak calves (p=0.000). The following risk factors for cattle in this region were identified by logistic regression: dairy farming (OR=2.71; p=0.021), beef farming (OR=1.61; p=0.008), herds raised on a combination of dry and flooded lands (OR=1.54; p=0.007), source of running water in association with standing water (OR=2.10; p=0.000), presence of dogs (OR=1.80; p=0.000), occurrence of abortion on the farm (OR=2.35; p=0.000) and birth of weak calves (OR=1.88) (see Table 2).

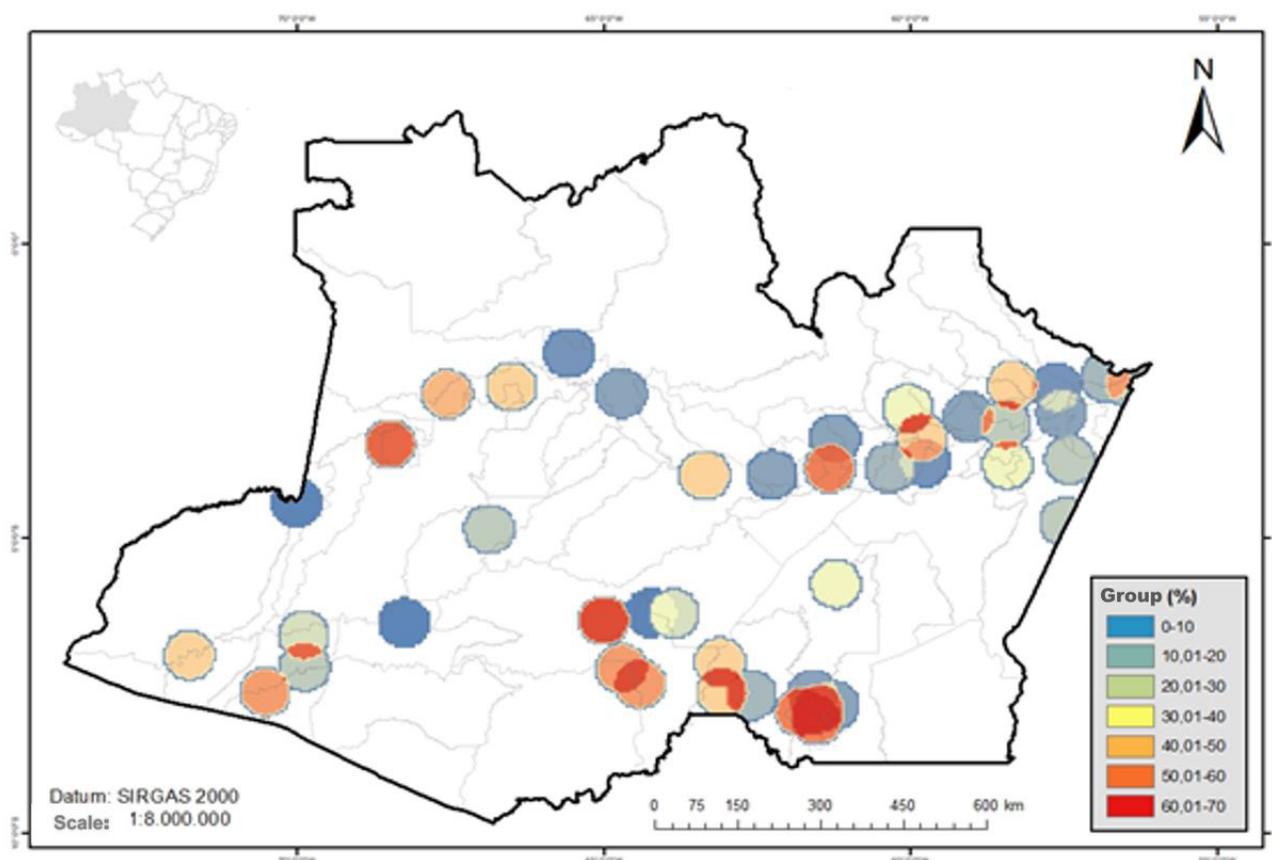


Figure 2. Kernel density estimation of the prevalence of *Neospora caninum* infection in cattle in the state of Amazonas, Brazil.

Discussion

To the best of our knowledge, this is the first study on the epidemiology of *N. caninum* infection in cattle herds in the state of Amazonas, where a prevalence rate of 30.2% (324/1073) was found. The territory of Amazonas state is covered entirely by the largest tropical forest in the world, the Amazon Rainforest, is a continental size state with sociocultural, economic and political differences, as well as geographical and ecological characteristics that may partly explain the widely varying prevalence rates among the farms (Vasconcelos, 2015). In fact, the reported prevalence rates of *N. caninum* infection in cattle in Brazil vary widely and in the northern region of Brazil, where the state of Amazonas is located, search data is limited.

This is an important and sensitive region of world for the preservation of fauna and flora. However, there has been a growth in deforestation in the region, especially in the last 30 years (IBGE, 2017), one of the main direct causes of livestock and large-scale agriculture, and among these, the expansion of cattle livestock is the most important (Rivero et al., 2009). In this sense, is necessary to encourage the development of a livestock based on productivity supported by scientific data and animal health knowledge.

In Brazil, the presence of *N. caninum* antibodies in cattle has been studied in several areas (Gennari, 2004), but only two of these reports were related to the Amazon region, under management conditions similar to that carried out in this study. Aguiar et al. (2006) in the state of Rondônia and Minervino et al. (2008) in the state of Pará, reported prevalence rates ranging from 10.4% to 19%. These studies show different seroprevalence values for anti-*N. caninum* antibodies to those found in our study.

This difference may occur due to the cutoff point used for each study, as well as the breeding conditions for each herd studied. In the Rondônia State, there was not the floodplain system (a kind of wet meadow) and the animals are raised only in cultivated pastures in more intensive conditions; also there are some different climatic conditions in special related to the rainy season period. In the states of Pará and Amazonas, the climatic conditions

Table 2. Univariate analysis and logistic regression of risk factors associated with *Neospora caninum* infection in cattle in the state of Amazonas, Brazil.

Variable	N*	Positive (%)	Univariate	Regression	
			p value	OR	p value
Type of herd					
Mixed	214	48 (22.4%)	0.009	-	
Beef	834	265 (31.8%)		1.61 (1.13 – 2.29)	0.008
Dairy	25	11 (44.0%)		2.71 (1.15- 6.37)	0.021
Livestock farming system					
Dry land	810	227 (28.0%)	0.012	-	
Floodplain	10	2 (20.0%)		0.89 (0.18 – 4.35)	0.886
Dry land + Floodplain	253	95 (37.6%)		1.54 (1.12 – 2.11)	0.007
Size of farm (hectare)					
< 50	170	56 (32.9%)	0.213	-	
51 to 500	626	176 (28.1%)			
> 501	277	92 (33.2%)			
No. of cattle					
1 – 10 animals	36	10 (27.8%)	0.595	-	
11 – 50 animals	72	25 (34.7%)			
51 – 100 animals	236	77 (32.6%)			
More than 100 animals	729	221 (29.1%)			
Source of water supply					
Running water	306	64 (20.9%)	0.000		
Still water	230	68 (29.6%)		1.58 (1.06 – 2.35)	0.021
Running + Still water	537	192 (35.8%)		2.10 (1.51 – 2.91)	0.000
Presence of dogs					
Yes	737	254 (34.5%)	0.000	1.80 (1.28 – 2.52)	0.000
No	239	54 (22.6%)			
Occurrence of abortion					
Yes	501	199 (39.7%)	0.000	2.35 (1.80 – 3.07)	0.000
No	572	125 (21.9%)			
Birth of weak calves					
Yes	431	165 (38.3%)	0.000	1.88 (1.44 – 2.45)	0.000
No	642	159 (4.8%)			

*Number of animals tested. P – significance probability. Odds Ratio – is a measure of association between an exposure and an outcome.

are similar, alternating in wetter periods and with greater concentration of rainfall. Condition that can affect the survival of *N. caninum* oocysts (Minervino et al., 2008).

The analysis of the Kernel density estimation map for *N. caninum* infection in cattle in the state of Amazonas revealed the presence more points density very high in the subpopulation 3, with at least one animal testing positive for the infection at 91.5% of the farms involved in this study (Figure 2). The subpopulation 3 represents almost

80% of the bovine population in the state, this concentration of hot areas may be due to the animals staying for a longer time together on the same property, different from subpopulations 1, 2 and 4 that have high turnover of herds and lower population density.

Dairy farms were identified as a risk factor for *N. caninum* infection and were 2.71-fold more likely to have infected cattle. The animal farming system employed by these farms may increase the risk of infection because it is more intensive, thus facilitating the transmission of the parasite. Almería et al. (2009) reported a similar finding that dairy herds are more susceptible to *N. caninum* infection than beef cattle, because this livestock farming system is more intensive, and the animals remain in this system for longer periods.

Raising herds on a combined system of dry and flooded lands, which is exclusively the case of Subpopulation 4, was also identified as a risk factor (OR=1.54). This mesoregion has two characteristic seasons: the dry season, from July to December, characterized by low water levels of the Amazon River, exposing the naturally fertilized floodplain lands, and the wet season, from January to June, characterized by the rising level of the river, whose waters flood the surrounding lowlands, forcing the riverine population and their livestock, including cattle, to move to higher ground (Filizola et al., 2006). This seasonal flooding causes cattle herds to concentrate on small areas of dry land, where they are in direct contact with dogs and other domestic animals in the surroundings, in livestock sheds and at feed troughs. Conversely, in the dry season, cattle are raised on the floodplains, in collective systems where several species of livestock occupy the same area. Similar findings were reported by Dubey et al. (2007), who demonstrated that husbandry management is a risk factor for environmental contamination by *N. caninum*.

The presence of dogs on the farms was also considered a risk factor in our study. Cattle raised on farms where dogs lived were 1.80-fold more likely to be infected with *N. caninum*. According to Dubey et al. (2007) and McAllister et al. (1998), the presence of dogs is considered a risk factor, since they are the definitive hosts of *N. caninum*. Oocysts, the environmentally resistant stage of the parasite, are excreted in dog feces, contaminating the environment and transmitting the parasite to cattle via horizontal transmission through the ingestion of sporulated *N. caninum*. Ghalmi et al. (2012) found that water sources on a farm are important reservoirs of *N. caninum* oocysts, because they can become contaminated by these infecting forms excreted in the feces of the definitive hosts. The findings of the aforementioned authors corroborate those of our study, which confirmed that animals that drink running water in association with standing water were 2.10-fold more likely to become infected.

A strong correlation was found between abortion and the birth of weak calves on farms where cows tested positive for anti-*N. caninum* antibodies. This finding is similar to that reported by Boas et al. (2015), who stated that the birth of weak calves, abortions and the presence of *N. caninum* are correlated, and that the occurrence of the disease is closely associated with production losses, as we found in our study. Other studies have demonstrated that the probability of abortion occurring among seropositive cows is two to seven times higher than among seronegative animals (Dubey & Schares, 2011), and that this risk is 7.4-fold higher among congenitally infected heifers (Thurmond & Hietala, 1997).

Conclusions

In this first seroepidemiological study of *N. caninum* infection in cattle in the state of Amazonas, it was concluded that *N. caninum* is present with high values of seroprevalence, when compared to other cattle producing states in the Amazon region of Brazil. The factors identified in our study can be used as risk indicators, so that control measures can be implemented to prevent infection by *N. caninum* in herds in this region.

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