FREQUENCY AND RISK FACTORS FOR INFECTION BY *Neospora caninum* IN DAIRY FARMS OF UMUARAMA, PR, BRAZIL

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ABSTRACT: Serum samples collected from dairy cattle and dogs in 15 farms in Umuarama, PR, were analyzed for the presence of anti-*Neospora caninum* antibodies using the indirect fluorescent antibody test. From 309 cows examined, 63 (20.4%) were positive, with titers ranging from 100 (23.8%) to 1,600 (11.1%). Three (6.1%) of 49 dogs analyzed were positive. Multivariate analysis of epidemiological factors revealed that production of food on the farm, absence of artificial insemination and access of domestic and wild animals to the facilities or to cattle feed showed significant association with positive serology in cows.
KEYWORDS: Dairy cattle; *Neospora caninum*; Logistic regression; Risk factors.

FREQUÊNCIA E FATORES DE RISCO PARA A INFECÇÃO POR *Neospora caninum* EM PROPRIEDADES LEITEIRAS DE UMUARAMA, PR, BRASIL

RESUMO: Amostras de soro coletadas de vacas leiteiras e cães em 15 fazendas de Umuarama, PR, foram analisadas para a presence de anticorpos contra *Neospora caninum* pela reação de imunofluorescência indireta. Das 309 vacas examinadas, 63 (20,4%) foram positivas, com títulos variando de 100 (23,8%) a 1.600 (11,1%). Três (6,1%) dos 49 cães avaliados foram positivos. A análise multivariada dos fatores epidemiológicos revelou que a produção de alimentos na fazenda, a ausência de programa de inseminação artificial e o acesso de animais domésticos e selvagens às instalações das vacas leiteiras ou ao alimento das vacas demonstraram associação significativa com a presence de anticorpos contra *Neospora caninum* nas vacas.
PALAVRAS-CHAVE: Gado de leite; *Neospora caninum*; Regressão logística; Fatores de risco.

FRECUENCIA Y FACTORES DE RIESGO PARA LA INFECCIÓN POR *Neospora caninum* EN PROPIEDADES LECHERAS DE UMUARAMA, PARANÁ, BRASIL

RESUMEN: Muestras de suero colectadas de vacas lecheras y perros en 15 fincas de Umuarama, Paraná, fueron analizadas para detectar la presencia de anticuerpos contra *Neospora caninum* por reacción de inmunofluorescencia indirecta. De las 309 vacas examinadas, 63 (20,4%) eran positivas, con títulos variando de 100 (23,8%) a 1.600 (11,1%). Tres (6.1%) de los 49 perros evaluados fueron positivos. El análisis multivariado de los factores epidemiológicos reveló que la producción de alimentos en la granja, la ausencia de programa de inseminación artificial, el acceso de animales domésticos y silvestres en las instalaciones de las vacas lecheras o a los alimentos de las vacas, mostraron asociación significativa con presencia de anticuerpos a *Neospora caninum* en las vacas.
PALABRAS CLAVE: Vacas lecheras; *Neospora caninum*; Regresión logística; Factores de riesgo.

Introduction

Since it was initially recognized in Norwegian dogs in 1984 (BJERKÅS; MOHN; PRESTHUS, 1984) and since the description of the new genus and species *Neospora caninum* (DUBEY, et al., 1988), neosporosis has been described in dogs and cattle all over the world. Dogs are both intermediate and definitive hosts of *N. caninum* (McALLISTER, et al., 1998). There are three infecting stages in the life cycle of the parasite: tachyzoites, tissue cysts and oocysts. Dogs, coyotes, and probably other wild canids are definitive hosts of the parasite, and shed nonsporulated oocysts in feces (DUBEY, 2003). The frequency of oocyst shedding in the environment and the survival of these oocysts in environmental conditions is still unknown (DUBEY, 2003).

Little is known about the dissemination and tissue distribution of *N. caninum* in animals, considering normal routes of infection. The parasite may be transmitted by trans-

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placentary route in several hosts, and vertical infection is the main form of transmission in cattle. Carnivores may acquire the infection by the ingestion of contaminated tissues (McALLISTER, et al., 1998).

Since it was pointed out as a cause of abortion in cattle in Brazil (GONDIM, 1999), the parasite has been isolated or demonstrated by histopathology in aborted fetuses of different regions of the country (CORBELLINI, et al., 2002), including Paraná (LOCATELLI-DITTRICH, et al., 2004; SANTOS, et al., 2006). Detection of serum antibodies is more indicated to evaluate exposure to the parasite in epidemiological studies of herds. Indirect fluorescent antibody test (IFAT) was the first test used in the serological diagnosis of neosporosis in 1988, and the method became the gold standard for the analysis of antibodies against *N. caninum* (ANDREOTTI, et al., 2003).

Several factors have been associated with positive results in dairy cattle, with a large variation depending on the region, the test and the cutoff values used (DUBEY; LINDSAY, 1996). Seropositive cows are more prone to abortion than seronegative ones. However, more than 95% of the calves born infected remain clinically normal. Lactation and abortion history generally do not affect congenital infection rates, but there is evidence that vertical transmission is more efficient in heifers than in cows (DUBEY, 2003). Larger Brazilian studies, involving many animals and farms, have demonstrated variable results in relation to management factors (CORBELLINI, et al., 2002; CORBELLINI, et al., 2006; SILVA, et al., 2008). However, the occurrence of abortion (CORBELLINI, et al., 2002; CAMPOS, 2005; SILVA, et al., 2008), and the presence and the number of dogs (CORBELLINI, et al., 2002; CAMPOS, 2005; CORBELLINI, et al., 2006) have been frequently associated with infection in cows.

The objective of the present study was to determine the frequency of cows and dogs positive for antibodies anti-*Neospora caninum* in farms in Umuarama, PR, Brazil, and to assess risk factors associated with the presence of positive animals.

Material and Methods

Collection site and sampling plan

The city of Umuarama, PR, Brazil, is located 430 m above sea level, at 23°47'55" South and 53°18'48" West. It has mesothermal humid subtropical climate, annual mean temperature of 22.1°C (BRASIL, 2009) and Red latosol/Red argisol soil type, from Caiuá sandstone (CUNHA; NÓBREGA; CASTRO, 2008). As for dairy production, the city has small farms that supply the local market, in a total of 465 farms producing 8,668,000 liters of milk / year, in 2006 (BRASIL, 2009). In order to compose the sample of prevalence study, a total of 9,016 animals are used (BRASIL, 2009) with an expected prevalence of 20%, since this is the median prevalence in other brazilian studies. This proportion maximizes the sample size, guaranteeing 95% minimum confidence and 5% statistical error. These parameters provided the minimum sample size (*n*) of 239 cows (THRUSFIELD, 2004) to be examined. Samples were collected from 309 cows in 15 farms.

*Collection of the samples and Detection of anti-***Neospora** *antibodies*

Before samples were collected, the project was submitted and approved by the Ethics Committee for Experiments involving Animals [*Comitê de Ética em Pesquisa Envolvendo Experimentação Animal*], and was approved on September 20th, 2007, according to the Brazilian laws in ethical research with animals.

Blood samples were collected by puncture of the coccygeal vein in bovines and cephalic or jugular vein in dogs. After collection, samples were centrifuged at 1,650 g for 15 minutes to separate the serum, and aliquots of 1 ml of serum were placed in adequately identified plastic microtubes, and stored at -20°C until the moment they were analyzed for the presence of antibodies anti-*Neospora*.

Analysis of serum samples for the presence of antibodies anti-*Neospora caninum* was carried out using the indirect fluorescent antibody test (CONRAD, et al., 1993), with bovine samples initially diluted at 1:100; positive samples were tested in twofold serial dilutions from 200 to 3,200. All samples showing titers equal or greater than 100 were considered to be positive. For dogs, samples were initially diluted at 1:50; positive samples were tested in twofold serial dilutions from 50 to 3,200. All samples showing titers equal or greater than 50 were considered to be positive (SILVA, et al., 2007).

Questionnaire and statistical analysis

Together with the collection of samples, epidemiological and animal management aspects of the farms were also analyzed. Type of management, frequency of reproductive problems, feeding regimen, contact with dogs and other animals were aspects approached in a questionnaire based on the reports by Corbellini et al. (2006) and Castro (2006). To identify risk factors associated with the infection by *N. caninum*, we conducted a univariate analysis of the interest variables by using the Pearson's chi-square test or Fisher's exact test, when necessary. Further, a multivariate analysis was performed by the model of logistic regression considering the serological status of the animal (positive or negative) as a dependent variable for *N. caninum*. The independent or explanatory variables considered in the model were those that showed a statistical significance of <0.25. This probability was stipulated so that possible risk factors of the event were not excluded from the analysis. The program EpiInfo for Windows, version 3.5.1 (Centers for Disease Control, Atlanta), was used to execute statistical calculations.

Results and Discussion

Blood samples and questionnaire responses were collected in 15 farms in Umuarama. Samples were collected from 309 bovines and from all 49 dogs in the farms. From the 309 cows analyzed for the presence of antibodies anti-*Neospora caninum*, 63 (20.4%; CI95%: 16.3-25.2) were positive, with titers ranging from 100 to 1,600 (Table 1). In the analysis of the results per farm, 93.3% showed at least one positive animal, with values ranging from 5 to 45% positive cows / farm.

Table 1: Prevalence and titers of antibodies anti-*Neospora*, determined by indirect fluorescent antibody test in dairy cattle of 15 farms in Umuarama, PR. 2008.

Farm	Number of cows	Positive sera in indirect fluorescent antibody test		Number of positive samples, according to antibody titers				
		Number	%	100	200	400	800	1,600
1	23	2	8.7	2	0	0	0	0
2	18	0	0.0	0	0	0	0	0
3	23	4	17.4	2	0	1	1	0
4	20	7	35.0	2	3	0	1	1
5	19	7	36.8	2	0	2	3	0
6	23	6	26.1	0	3	2	0	1
7	19	6	31.6	0	3	1	2	0
8	20	3	15.0	1	0	0	2	0
9	20	2	10.0	0	0	0	2	0
10	20	1	5.0	0	0	1	0	0
11	24	4	16.7	2	2	0	0	0
12	20	4	20.0	2	1	1	0	0
13	20	3	15.0	0	0	0	0	3
14	20	5	25.0	0	3	2	0	0
15	20	9	45.0	2	2	1	2	2
Total	**309**	**63**	**20.4**	**15 (23.8%)**	**17 (27.0%)**	**11 (17.5%)**	**13 (20.6%)**	**7 (11.1%)**

The comparison of positive IFAT results in cattle observed in this study and in other reports in Brazil showed that cutoff values used to determine positive results were not the same. Sartor et al. (2003), using 200 as the cutoff titer, observed 15.9% positive cows among 521 animals analyzed in Avaré, SP, similar to the values reported by Hasegawa et al. (2004), in the same region: from 777 samples, 15.3% were positive. If only animals showing titers greater than 200 were considered in the present study, results would have been similar: 15.5% positive animals. On the other hand, Ragozzo et al. (2003) analyzed 802 animals from 55 farms of six Brazilian states, and reported 189 (23.6%) positive cows, using cutoff titer of 25. If these authors considered 100 as the cutoff value, they would have observed 13.7% positive animals. Ogawa et al. (2005) in dairy farms in northern Paraná, observed 12% of 385 samples positive for *Neospora,* values below that observed in the present study. Differently, Campos (2005) observed 23.3% positive cows among 296 samples collected in 46 farms in the cities of Viçosa and Mercês, value similar to the results of the present study.

There were also variations in the titers of antibodies reported in the different studies. Andreotti et al. (2003) reported that cows showing titers over 640 have aborted, and that these titers indicated association with neosporosis. In this study, 31.7% seropositive animals showed titers equal or greater than 800. Guimarães Júnior (2002) and Ragozzo et al. (2003) showed lower results: 12.3% and 15.3% of the cows with titers equal or greater than 800, respectively, whereas Gondim et al. (2004) and Ogawa et al. (2005) observed these titers in 33.3% and 35.5% animals, respectively. Herds from different regions may be submitted to different risk factors and infection pressures, leading to different infection rates, and to diverse titer profiles.

Forty-nine samples were collected from dogs and analyzed, with 6.1% (CI95%: 2.2-16.5) positive results (3/49). From the farms studied, two did not have dogs at the moment samples were collected. Three (23.1%) farms showed positive dogs, representing 14.3%, 16.7% and 25.0% of the dogs in each farm. The proportion of positive samples from dogs in this study may be considered to be low. In the study by Guimarães Júnior (2002), 21.6% of 134 samples analyzed were positive in IFAT, whereas Campos (2005) found 18.5% seropositive dogs among 54 analyzed, without any reports of previous clinical infection. Silva et al. (2008) observed 33.1% reactors among 169 dogs analyzed in two rural settlements in northern Paraná.

Table 2 shows variables with $p < 0.25$, which were submitted to a logistic regression model.

Brazilian literature presents several risk factors associated with the infection by *N. caninum* in dairy cattle, with regional variations. Corbellini et al. (2002) determined the presence of *Neospora caninum* in 81.8% (18/22) of the samples of fetal brains with encephalitis in Rio Grande do Sul. All cows were seropositive - ranging from 3.1 to 30.7% - in the five farms analyzed, and seroprevalence was greater in cows that aborted (23.3%) compared with the other animals (8.3%). All farms had dogs, and the presence of wild canids was reported twice in one of the farms where abortion outbreaks occurred two times during the same year.

Table 2: Uni and multivariate analysis of the association between epidemiological variables and presence of antibodies anti-*Neospora* using indirect fluorescent antibody test in dairy cattle from 15 farms in Umuarama, PR. 2008.

				Univariate analisis			Multivariate analisis	
Variable	**Negative**	**Positive**	**Total**	**χ2***	**p-value**	**OR (CI95%)**	**Odds Ratio (C. I. 95%)**	**P value**
Total number of bovines								
< 100	123 (72.8)	46 (27.2)	169 (58.5)	3.19	0.0738	0.57 (0.32 – 1.01)	0.87 (0.28 - 2.66)	0.8082
≥ 100	99 (82.5)	21 (17.5)	120 (41.5)					
Breed								
Mixed	181 (74.8)	61 (25.2)	242 (78.3)	4.58	0.0322	2.48 (1.12 – 5.49)	1.04 (0.11 - 9.60)	0.9695
European	59 (88.1)	8 (11.9)	67 (21.7)					
Before milking, animals are kept in facilities that have...								
Dirt floor	74 (73.3)	27 (26.7)	101 (32.7)	1.32	0.1257	0.69 (0.40 – 1.21)	0.59 (0.18 - 1.89)	0.3763
Concrete floor	166 (79.8)	42 (20.2)	208 (67.3)					
Feed is produced in the farm								
Yes	205 (82.3)	44 (17.7)	249 (80.6)	14.70	0.0001	0.30 (0.16 – 0.55)	0.32 (0.16 - 0.65)	0.0017
No	35 (58.3)	25 (41.7)	60 (19.4)					
Areas are shared								
Yes	13 (65.0)	7 (35.0)	20 (6.5)	-	0.1308	1.97 (0.75 – 5.15)	0.19 (0.03 – 1.14)	0.0697
No	227 (78.5)	62 (21.5)	289 (93.5)					
Wild animals have access to the facilities and / or cattle feed								
Yes	41 (65.1)	22 (34.9)	63 (20.4)	6.34	0.0117	2.27 (1.24 – 4.17)	9.33 (1.73 – 50.38)	0.0094
No	199 (80.9)	47 (19.1)	246 (79.6)					
Use of artificial insemination								
Yes	94 (87.0)	14 (13.0)	108 (35.0)	7.59	0.0058	2.53 (1.28 – 5.06)	0.07 (0.01 - 0.51)	0.0083
No	146 (72.6)	55 (27.4)	201 (65.0)					
Occurrence of abortion outbreaks								
Yes	18 (100.0)	0 (0.0)	18 (5.8)	-	0.0091	0.00 (0.00 – 0.60)	0.00 (0.00 - >1012)	0.9734
No	222 (76.3)	69 (23.7)	291 (94.2)					
Use of measures for rodent control								
Yes	203 (76.0)	64 (24.0)	267 (92.1)	2.01	0.1564	3.31 (0.76 – 14.50)	0.61 (0.07 - 5.49)	0.6593
No	21 (91.3)	2 (8.7)	23 (7.9)					
Other animals raised on the farm								
Yes	222 (76.8)	67 (23.2)	289 (93.5)	-	0.1347	2.71 (0.61 – 12.01)	2.04 (0.46 - 9.14)	0.3501
No	18 (90.0)	2 (10.0)	20 (6.5)					
Raises poultry								
Yes	222 (76.8)	67 (23.2)	289 (93.5)	-	0.1347	2.71 (0.61 – 12.01)	1.76 (0.36 - 8.68)	0.4868
No	18 (90.0)	2 (10.0)	20 (6.5)					

* If there is no value for χ^2, Fisher's exact test was used.

In another large study in Rio Grande do Sul, Corbellini et al. (2006), determined the presence of four variables associated with the presence of seropositive animals. The number of dogs was associated with heavy environmental contamination with oocysts. Small farms, on the other hand, were associated with a greater risk of infection in bovines, because dogs may easily have assess to the whole farm, and therefore, to bovine carcasses, aborted fetuses, placentas and uterine discharges, making it easy for these animals to be contaminated. The use of colostrum from several cows was also significantly associated, demonstrating the possible importance of infection by oral route. The region studied was also an important factor, and may be related to economic, temporal and environmental variables, which were not measured in the study.

Silva et al. (2008) reported a prevalence of 31.7% among the 469 animals analyzed in a farm in the state of Pernambuco, showing that infection was associated with lack of veterinarians, presence of floodable facilities, handling fetuses without adequate protection equipment, nutritional

deficiencies, not culling cows that aborted, and history of abortions.

In the present study, the exclusive use of natural mating was significantly associated with cow infection, both in the univariate and in the multivariate analysis. The semen of infected bulls has a potential impact in *Neospora caninum* infection. Ferre et al. (2005) demonstrated the presence of the parasite in 15% semen samples from naturally infected bulls. Besides, intrauterine infection of cows with tachyzoites led to seroconversion and detection of DNA of the parasite in 66% of the animals studied (SERRANO-MARTINEZ, et al., 2006). On the other hand, the use of semen in which the parasite was frozen by common methods of cryopreservation prevented the infection of cows submitted to artificial insemination (CANADA, et al., 2006).

In this study, there was an association between the use of feed produced away from the farm and the frequency of seropositive animals. Guimarães Júnior (2002) observed that the farms where cattle were fed silage or concentrate produced in site showed lower rates of infection by *Neospora caninum*. These authors considered that the way feed was stored may protect it from contamination by oocysts. Thus, the lack of control of outside feed may be a source of infection for the animals.

Multivariate analysis pointed out that the frequency of positive animals was higher in farms where wild animals had access to cattle feed and/or facilities. Although no particular species was implicated, this result suggests that wider studies are necessary on the role of wild Brazilian fauna as *Neospora* reservoirs. Gondim (2006) reported that, up to now, only the coyote (*Canis latrans*) was observed to shed *Neospora* oocysts, and may function as a definitive host. Although there is only serological evidence of infection among Brazilian wild canids, such as *Cerdocyon thous*, *Lycalopex gymnocercus* and *Chrysocyon brachyurus*, this author concluded that prophylactic measures against neosporosis should involve the possible participation of these animals in the life cycle of the parasite.

None of the variables was significantly associated with positive results in dogs. Campos (2005) reported that the occurrence of positive dogs in the farms was associated with positive results in cows, but not with reproductive problems in cattle.

Conclusion

Low prevalence in dogs and high prevalence in cows may show the importance of vertical transmission in bovine neosporosis. However, it is necessary to investigate the possible role of wild canids in the transmission of the infection. On the other hand, factors associated with animal management and low technification in the farms were associated with high infection rates in bovines, and should be considered in control and prevention programs for this disease.

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