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Neospora caninum: evaluation of vertical transmission in slaughtered dairy cows (*Bos taurus*)

Neospora caninum: avaliação da transmissão vertical em vacas leiteiras (*Bos taurus*)

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Abstract

Neospora caninum is a worldwide parasite recognized as one of the main parasites responsible for abortion in cattle. The objective of this study was to evaluate vertical transmission of *N. caninum* in dairy cows (*Bos taurus*) that were slaughtered at an abattoir in the state of Santa Catarina, southern Brazil. Blood samples (with and without EDTA) from 60 pregnant dairy cows and blood and tissue samples (brain, lung, heart and liver) from their fetuses were collected and used for PCR and serological evaluation. Blood samples from 60 non-pregnant cows were collected and used to detect antibodies. Anti-*N. caninum* antibodies were detected by indirect ELISA. Antibodies against *N. caninum* were observed in 41.6% (25/60) of the pregnant cows and in 43.3% (26/60) of the non-pregnant cows. Antibodies against the parasite were detected in sera from three fetuses (5.5%). PCR analysis revealed that 3.3% (2/60) of the cows and 6.6% (4/60) of the fetuses evaluated were positive for specific *N. caninum* primers. These positive fetuses were between 4-6 months of age. Therefore, considering PCR and serological tests to be indicative of vertical transmission in fetuses, 11.6% (7/60) of the fetuses were infected by *N. caninum* during gestation.

Keywords: *Neospora caninum*, dairy cattle, vertical transmission, *Bos taurus*.

Resumo

Neospora caninum é um parasita de distribuição mundial reconhecido como um dentre os principais parasitas, responsável por abortamento em bovinos. O objetivo deste estudo foi avaliar a transmissão vertical de *N. caninum* em vacas leiteiras (*Bos taurus*) que foram submetidas ao abate em matadouro no Estado de Santa Catarina, sul do Brasil. Sangue (com e sem EDTA) de 60 vacas leiteiras prenhas e amostras de sangue e tecidos (cérebro, pulmão, coração e fígado) de seus fetos foram coletados e utilizados para PCR e avaliação sorológica. Amostras de sangue de 60 vacas não-gestantes foram obtidas e utilizadas para detecção de anticorpos. A detecção de anticorpos séricos anti-*N. caninum* foi avaliada pelo ELISA-teste indireto. Anticorpos anti-*N. caninum* foram observados em 41,6% (25/60) das vacas prenhas e em 43,3% (26/60) das vacas não-gestantes. Três fetos (5,5%) foram soros positivos para *N. caninum*. Análise pela PCR revelou que 3,3% (2/60) das vacas e 6,6% (4/60) dos fetos avaliados foram positivos para *N. caninum*. As idades dos fetos positivos eram de 4 a 6 meses. Portanto, considerando a PCR e a sorologia como indicativo de transmissão vertical em fetos, 11,6% (7/60) dos fetos foram infectados por *N. caninum* durante a gestação.

Palavras-chaves: *Neospora caninum*, vacas leiteiras, transmissão vertical, *Bos taurus*.

Introduction

Neospora caninum is a protozoan parasite that is distributed worldwide and is a major cause of abortion in cattle (DUBEY; SCHARES, 2006). Animals may be infected via three routes: ingestion of sporulated oocysts; transmission from dams to

fetuses (vertical); and through meat containing tissue cysts. Cattle, as herbivorous animals, are infected via the first and second routes. In dairy herds, transmission principally occurs vertically (SCHARES et al., 1998; BARTELS et al., 2007). Vertical transmission can occur throughout the gestation period; however, abortions are most prevalent at 3 to 6 months of pregnancy (DUBEY; SCHARES, 2006). However, female calves can be born infected without any sign of infection and can keep the parasite

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in their tissue while they are raised, such that when they become pregnant, they may transmit the parasite to their offspring. This type of transmission is known as endogenous transplacental infection (TREES; WILLIAMS, 2005).

Neosporosis has been described as an important cause of abortion in southern Brazil (CORBELLINI et al., 2001, 2002; LOCATELLI-DITTRICH et al., 2001). Locatelli-Dittrich et al. (2001) reported that the abortion risk in *N. caninum*-seropositive dairy cows was four times higher than in seronegative dairy cows. Corbellini et al. (2002) used both histopathological and serum sample examination from bovine aborted fetuses, and described a high rate of occurrence of *N. caninum* as the cause of abortion throughout the state of Rio Grande do Sul.

Although several studies relating to neosporosis among dairy cattle have been conducted, the documentation of vertical transmission in Brazilian herds remains inadequate. Thus, the aim of the present study was to evaluate anti-*N. caninum* antibody occurrence in serum samples, and vertical transmission of *N. caninum* from dairy cows slaughtered at an abattoir located in the southern region of Brazil.

Materials and Methods

The 120 samples were obtained from pregnant and non-pregnant dairy cows (*Bos taurus*) and their respective fetuses at an abattoir located in the municipality of Presidente Getúlio, state of Santa Catarina, southern Brazil during 2010 year. The present study was approved by the Animal Ethics Committee of the State University of Londrina (N. 018/2009).

Antibodies against *N. caninum* were detected by means of the HerdCheck *Neospora caninum* Antibody Test Kit (Idexx Laboratories). Serum samples from the cows were diluted at the ratio of 1:100, and serum from fetuses at 1:25.

Blood samples with EDTA were collected individually in sterile tubes from the pregnant cows after they had been bled along the inspection line (during bleeding of the animals) and from their fetuses by means of cardiac puncture. The white blood cells were separated by centrifugation (550 g for 10 minutes), and were used for DNA extraction and subsequent PCR analyses. The uterus of the dams was obtained individually with the fetuses inside. Each uterine horn containing the fetuses was aseptically opened, the fetuses were taken out from the uterus, and organ material was collected on individual sterilized Petri plates. At the laboratory, approximately 10 g of tissue fragments (brain and myocardium) were collected from fetuses ≥ 3 months of age, while similar tissue fragments (10 g) for fetuses ≤ 2 months were pooled for DNA extraction. The tissue samples were homogenized with 10 mL of saline solution (0.14 M of NaCl), after which the homogenate was filtered, and 2 mL were used for DNA extraction.

The DNA extraction was performed using the DNeasy Blood & Tissue Kit (QIAGEN Sample & Assay Technologies, Brazil), following the manufacturer's recommendations. PCR analyses were performed as previously described (MARQUES et al., 2011). Amplification of *N. caninum* DNA was done by using the Np21 and Np6 primers (MULLER et al., 1996). The PCR cycle was performed on a mixture (final volume of 25 mL) containing 5 mL

of extracted DNA admixed with 20 mL of a solution containing 1.0 mM of each primer, 100 mM of dNTP (Invitrogen, Life Technologies, USA), 60 mM of Tris-HCl (pH 9.0), 15 mM of $(\text{NH}_4)_2\text{SO}_4$, 1.5 mM of MgCl_2 , and 0.5 U of Taq DNA polymerase (Invitrogen Life Technologies, USA). Amplification of parasitic genomic DNA was done over 35 cycles in a PTC-100 thermocycler (MJ-Research), using the following cycling conditions: 5 minutes at 94 °C, followed by 35 cycles of 30 seconds at 94 °C, 30 seconds at 65 °C and 60 seconds at 72 °C; the 35th cycle was followed by a final extension of 7 minutes at 72 °C. Aliquots from each PCR were viewed by means of electrophoresis on 2% agarose gel. For positive controls, DNA extracted from tachyzoites of the NC-1 strain ($10^6/\text{mL}$) of *N. caninum*, diluted in TE buffer, were used. The negative control consisted of commercially prepared water samples without *N. caninum*. One positive and one negative control was included in each assay.

All variables were analyzed by means of the chi-square test (χ^2), with Yates correction, using the Epi Info software (CDC, version 6.04b). *P*-values ≤ 0.05 were taken to be significant.

Results

The serological results for *N. caninum* are presented in Table 1. Out of a total of 120 cows, samples from 51 (42.5%) reacted positively to *N. caninum*. Positive seroreactivity to *N. caninum* occurred in samples from 41.6% (25/60) of the pregnant cows, while the positivity rate among non-pregnant animals was 43.3% (26/60); these values were not statistically different ($p = 1.0$). There was no statistical difference in relation to the breed of cattle (Table 1, $p = 1.0$). When the period of gestation was evaluated, there were no differences in the prevalence of *N. caninum* relative to the first (50%), second (35.7%) or third trimester (44.4%). Considering that almost all of the cows were more than four years old (116 animals), no comparisons regarding prevalence and proportionality with age were calculated.

The average optical density (OD) of the positive samples obtained from the pregnant animals was 1.64 ± 0.92 , compared with 1.82 ± 0.105 among their non-pregnant counterparts (Table 2). In relation to the period of gestation, the mean OD was 1.18 ± 0.89 for the first, 2.16 ± 0.90 for the second and 1.28 ± 0.83 for the

Table 1. Demonstration of the association between the variables studied and the presence of antibodies for *Neospora caninum* in slaughtered pregnant dairy cows (*Bos taurus*), in state of Santa Catarina, 2010.

Variables	Positive (%)	Negative (%)	Total (%)	<i>P</i>
<i>Neospora</i>				
Cows	25 (41.6)	35 (58.4)	60 (50)	1.0 ¹
Pregnant	26 (43.3)	34 (56.7)	60 (50)	
Non-pregnant	51 (42.5)	69 (57.5)	120 (100)	
Trimester of gestation				
First	7 (50.0)	7 (50.0)	14 (23.3)	0.87 ¹
Second	10 (35.7)	18 (64.3)	28 (46.7)	
Third	8 (44.4)	10 (55.6)	18 (30.0)	

¹Chi-square with Yates correction. % percentage.

Table 2. Outcomes regarding *Neospora caninum* infection in slaughtered pregnant dairy cows (*Bos taurus*) and their fetuses, in state of Santa Catarina, 2010.

Cow no.	ELISA ¹		Age of fetus (months)	PCR		
	Cow	Fetus		Cows	Fetuses*	
				Blood	Brain	Myocardium
2	2.18	N	4	–	–	–
13	1.83	NO	1	+	–	–
25	2.25	N	7	–	–	–
26	2.17	N	3	–	–	–
28	0.55	N	6	–	–	–
53	3.05	N	6	–	–	–
54	0.6	N	7	–	–	–
58	1.13	3.3	8	–	–	–
62	0.59	NO	1	–	–	–
64	2.48	N	5	–	–	–
74	N	N	6	–	–	+
78	2.7	N	5	–	–	–
79	2.48	N	3	–	–	–
85	2.7	N	5	–	–	–
87	N	N	7	–	+	–
91	0.73	N	8	–	–	–
92	2.54	N	4	–	–	–
95	N	NO	2	+	–	–
97	0.74	NO	2	–	–	–
105	2.02	N	7	–	–	–
111	N	N	3	–	+	–
113	0.62	N	7	–	–	–
116	N	3.12	5	–	–	–
119	N	N	8	–	–	–
121	0.574	N	3	–	–	–
124	0.50	N	4	–	–	–
125	2.7	3.3	7	–	–	–
126	2.16	N	6	–	–	–
130	2.7	N	5	–	–	–
131	0.55	N	7	–	–	+
133	0.55	N	3	–	–	–

¹Optical density from ELISA (Idexx); N, –, negative; +, positive. *fetuses that were less than two months of age were macerated and evaluated using PCR. NO, not obtained.

third trimester ($p = 0.051$). Comparative analysis on the PCR and serological results revealed that one of the two cows that was PCR-positive was also serologically negative, while the other was seropositive (mean OD = 1.83). Only one (cow no. 131) out of the four cows that had positive fetuses in the PCR analysis was positive in the serological test (OD = 0.55). The serological test revealed three positive fetuses; however, one of the dams was negative in ELISA.

Sixty fetuses were collected for evaluation: 14 were in the first, 28 in the second and 18 in the third trimester of gestation. Additionally, six fetuses were less than two months of age, and serum samples were not obtained from these animals. Thus, 54 serum samples from the fetuses were evaluated, and three (5.5%) of these samples were serologically positive with high antibody titers (OD > 3.1).

During this study, vertical transmission was demonstrated in 11.6% (7/60) of the fetuses by means of PCR and serological tests.

Cerebral-derived DNA from the fetuses of cow no. 87 and cow N. 111 was positive in PCR analysis. Myocardial-derived DNA from the fetuses of cow no. 74 and cow no. 131 was positive in PCR reactions (Table 2). The DNA-derived blood samples from two cows (animal nos. 13 and 95) were PCR-positive, but only one animal (no. 26) had a corresponding serum titer (OD = 1.83). However, the fetuses of these animals were PCR-negative.

Discussion

In our study, we investigated vertical transmission of *N. caninum* in dairy cattle slaughtered in southern Brazil. The overall results demonstrated an *N. caninum* vertical transmission rate of 11.6% (7/60). Three fetuses (3/7) had antibodies that were detected by ELISA (high levels of antibodies) and were *N. caninum* DNA negative in their tissue samples. One dam was serologically negative, and two others had higher antibody titers. Conversely, the other

fetuses (4/7) were positive for the presence of *N. caninum* DNA in their tissues and were serologically negative, and their dams had low titers of antibodies (OD = 0.2 to 0.5). Fluctuations in the results similar to these have previously been described (McINNES et al., 2006; DIJKSTRA et al., 2008; MORÉ et al., 2009; YAO et al., 2009; MARQUES et al., 2011). McInnes et al. (2006) reported that there was no correlation between the serological tests on dams and the PCR on their fetuses, and a high percentage of infected fetuses had serologically negative dams. Moré et al. (2009) showed that transplacental infection in dairy cattle may occur when antibody titers are low in adult cows.

Cabral et al. (2009) used histology, immunohistochemistry and nested-PCR to evaluate 105 aborted fetuses that were sent to the Biological Institute of São Paulo, Brazil, and found a neosporosis rate of 24.8%. In addition, Corbellini et al. (2002) found that 23% of the aborted fetuses that they collected in Rio Grande do Sul, Brazil, were infected by *N. caninum*. The rate of vertical transmission of neosporosis may vary between cattle herds, and it can range from 4.0 to 100% (DUBEY; SCHARES, 2011).

Just three cows in the present study had positive blood samples in PCR, in spite of the relatively high prevalence of IgG anti-*N. caninum* in serum samples. Yao et al. (2009) reported that negative PCR results of blood samples did not exclude *N. caninum* infection, which corroborates the observation that few *N. caninum* are present in the blood of intermediate hosts. Additionally, Okeoma et al. (2005) observed greater variation in the concentration of *N. caninum* DNA in blood samples than in heifer brain samples, which indicated that this organ should be chosen for isolating DNA from the parasite.

Therefore, since seronegative dams generate congenitally infected fetuses, the presence or absence of antibodies for *N. caninum* possibly neither supports nor rules out vertical transmission. Yao et al. (2009) stated that seronegative abortion cases should be confirmed by means of PCR analysis on the fetus tissues. The mechanism for *Neospora* infection in seronegative dams might be explained in terms of: a) early-stage infection; b) the sensitivity of the serological assay (low antibody levels); or c) seronegative conversion in infected cows and immunotolerance of early in-utero infection (McINNES et al., 2006; WILLIAMS; TREES, 2006; YAO et al., 2009). Under natural conditions, most infections caused by *N. caninum* are “in utero”, and parasite-induced reactions can be classified as self-infections (as are observed in some viral infections) without producing an immune response.

Previously, Marques et al. (2011) studied vertical transmission in zebu cattle in southern Brazil and found a rate of 4.8%, which is a lower rate than what was observed in the present study (11.6%). This phenomenon might represent the manifestation associated with each management system, i.e. the difference between having or not having access to infectious sources, nutritional differences and emphasis on production activity (such as milking or weaning), or the variability in the duration of time for which the animals are kept within breeding programs (MARQUES et al., 2011).

The prevalence of *N. caninum* among the cows in the present study was similar to what was described in some previous reports (RAGOZO et al., 2003; SARTOR et al., 2003; LOCATELLI-DITTRICH et al., 2008; BENETTI et al., 2009), but was lower than in other studies (COSTA et al., 2001;

GUIMARÃES JUNIOR et al., 2004; MINERVINO et al., 2008; MUNHOZ et al., 2009; EIRAS et al., 2011). The prevalence of *N. caninum* in dairy cattle in Brazil was previously described as ranging from 15.1 to 91.2% (LOCATELLI-DITTRICH et al., 2001, 2008; RAGOZO et al., 2003; GUIMARÃES JUNIOR et al., 2004;). Therefore, caution is needed in evaluating the results from prevalence studies, since the differences in results might be directly related to the serological techniques used, the cutoff values, the sample size or the type (breed and/or species) of animal that is being investigated.

Locatelli-Dittrich et al. (2008) and Munhoz et al. (2009) in Brazil, Wang et al. (2010) and Xia et al. (2011) in China and Eiras et al. (2011) in Spain evaluated anti-*N. caninum* antibodies in dairy cattle by using ELISA (Idexx). These authors observed a seroprevalence range from 13.3 to 38.2%. Thus, the results obtained from these previous studies might be easier to compare with those from the present study.

In our study, it was difficult to precisely determine whether endogenous or exogenous transmission had higher or lower importance. We could speculate, considering that 5/6 of the *N. caninum*-positive fetuses had ≥ 5 months of gestation and were almost ready to be born, that endogenous transplacental infection could be considered to be a major infection source for fetuses, but further studies need to be performed to confirm this hypothesis. Bartels et al. (2007) described rates of 44.9% and 4.5%, respectively, for vertical and horizontal transmission in Dutch dairy herds.

We did not observe any statistical difference between seropositive pregnant (41.6%) and non-pregnant cows (43.3%) during this study. This finding could mean that natural immune suppression during the gestational period does not change the prevalence of antibodies against *N. caninum*. The events that occur during pregnancy and the influences of prenatal exposure to the parasite on the development of an immune response are critical issues (INNES et al., 2002) that have to be addressed if the associated mechanism is to be completely understood (WILLIAMS; TREES, 2006).

In conclusion, this study demonstrated a vertical transmission rate for *N. caninum* of 11.6% in naturally infected pregnant dairy cows in southern Brazil. Additionally, this study was the first description of the occurrence of anti-*N. caninum* antibodies in dairy cattle (42.5%) in the state of Santa Catarina.

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