Original Article

Occurrence of abortions induced by *Neospora caninum* in dairy cattle from Santa Catarina, southern Brazil

Ocorrência de abortamentos induzidos por *Neospora caninum* em vacas de leite de Santa Catarina, sul do Brasil

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Abstract

The aim of the present study was to investigate the occurrence of *N. caninum* associated with abortions of dairy cattle from Santa Catarina state, southern Brazil by using enzyme-linked immunosorbent assay (ELISA), immunohistochemistry (IHC), and polymerase chain reaction (PCR). Blood from dairy cows that aborted along with intrathoracic fluid and tissue samples (brain, heart, liver, and lung) from their fetuses were collected and used for serology; PCR, histopathological, and immunohistochemistry (IHC) evaluations were also conducted. Twenty-one cows (51.2%) out of 41, and eight fetuses (26.7%) out of 30 were ELISA (HerdCheck, IDEXX) positive for *N. caninum*. Dams > 36 months of age had a higher risk of being serum positive than younger animals. PCR and IHC revealed that 38.8% (14/36) and 25.0% (9/36) of the fetuses were positive for *N. caninum,* respectively for each of the tests. Seropositive cows had a higher frequency of fetuses that were also positive by either intrathoracic fluid, PCR, or IHC. In summary, the present study observed a high frequency of *N. caninum* in abortions from dairy cows from southern Brazil, with a higher *N. caninum* prevalence found in cows that were older than 36 months. In addition, serology, PCR, and IHC should be used all together for better diagnosis of neosporosis in cattle.

Keywords: Neosporosis, fetuses, lesion, serology, cattle.

Resumo

O objetivo deste estudo foi avaliar a ocorrência de *N. caninum* associado a abortamentos em vacas de leite do estado de Santa Catarina, sul do Brasil pelo uso das técnicas de ELISA (HerdCheck, IDEXX), reação em cadeia pela polimerase (PCR) e imunohistoquímica (IHC). O sangue das vacas leiteiras que abortaram, bem como, o líquido intratorácico e amostras de tecidos (cérebro, coração, fígado e pulmão) de seus fetos foram coletados e usados para sorologia, PCR (Np21+ e Np6+), e IHC. Vinte e uma vacas (51,2%) de um total de 41, bem como, oito fetos (26,7%) de um total de 30 foram positivos no ELISA (IDEXX) para *N. caninum*. As vacas > 36 meses de idade tiveram um maior risco de serem soropositivas do que os animais mais jovens. PCR e IHC revelaram que 38,8% e 25,0% dos fetos foram positivos para *N. caninum*, respectivamente para cada um dos testes. As vacas soropositivas tiveram uma maior frequência de fetos que também foram positivos no fluído intratorácico, na PCR ou na IHC. Em resumo, o presente estudo observou uma alta frequência de *N. caninum* em abortos de vacas leiteiras na região estudada, com maior prevalência de *N. caninum* em vacas com mais de 36 meses de idade. Além disso, sorologia, PCR e IHC deveriam ser utilizadas conjuntamente para melhor diagnóstico de neosporose em bovinos.

Palavras-chaves: Neosporose, bovinos, sorologia, vacas, diagnóstico.

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Introduction

Neospora caninum is a protozoan parasite of animals that may have been misdiagnosed as *Toxoplasma gondii* until 1988 when it was recognized as a distinct Species (DUBEY et al., 2007, 2011). Dogs (*Canis lupus familiaris*), coyotes (*Canis latrans*), dingoes (*Canis lupus dingo*), and gray wolfs (*C. lupus*) are considered as definitive hosts for this parasite (DUBEY et al., 2011; GONDIM et al., 2004; KING et al., 2010; McALLISTER et al., 1998). Additionally, several livestock and wild animal species have been identified as intermediate hosts of *N. caninum* (DUBEY & LINDSAY, 1996). Moreover, surveys conducted in several countries have identified *N. caninum* infections as the major cause of bovine abortions (ANDERSON et al., 2000), particularly among dairy cattle (DUBEY & LINDSAY, 1996).

N. caninum can be either transmitted postnatally (horizontal) by the ingestion of food and/or drinking water contaminated with sporulated oocysts, or transplacentally (vertical) (DUBEY, 2005; DUBEY et al., 2007). Vertical transmission of *N. caninum* from one generation to the next generation of cattle appears to be the major route by which the infection is maintained within herds (BILDFELL et al., 1994; HALL et al., 2005; WOUDA, 2000). Vertical transmission may result in fetuses that may die *in utero*, be reabsorbed, mummified, autolyzed, stillborn, born alive with clinical signs, or born clinically normal but persistently infected (DUBEY, 2003; HADDAD et al., 2005).

The diagnosis of *N. caninum*-induced abortions is often expensive and sometimes difficult, either because of the absence of fetus and placenta, especially for beef cattle, or the use of just serology, what may be inconclusive. Serological examination of the dam and fetus (DUBEY & LINDSAY, 1996), observation of histopathological lesions, immunohistochemistry (ANDERSON et al., 2000), and parasite detection by PCR techniques (COLLANTES-FERNANDEZ et al., 2006) are tools that may contribute to a successful diagnosis of bovine *Neospora* abortion.

In Brazil, *N. caninum* was considered an important abortifacient agent in cattle (CORBELLINI et al., 2002; SANTOS et al., 2005b; CORBELLINI et al., 2006; ORLANDO et al., 2013; PESCADOR et al., 2007; CABRAL et al., 2009). Corbellini et al. (2002) showed in Rio Grande do Sul state, southern Brazil, 81% of the aborted fetuses with brain lesions suggestive of *N. caninum*. Santos et al. (2005b) showed by immunohistochemistry this parasite in eight out of 30 fetuses coming from 22 dairy herds from Paraná state, those fetuses were between 3 to 7 months of age. Additionally, in Sao Paulo state, 24.8% of fetuses were found infected with *N. caninum* (CABRAL et al., 2009).

Those previous studies, characterized *N. caninum* by either histopathology or immunohistochemistry (IHC) of aborted fetuses. Thus, the aim of the present study was to investigate the occurrence of *N. caninum* associated with abortions of dairy cattle from Santa Catarina state, southern Brazil by using enzyme-linked immunosorbent assay (ELISA), immunohistochemistry (IHC), and polymerase chain reaction (PCR).

Material and Methods

Study area and sampling

The survey was done in the city of Presidente Getulio (27° 03' 03" S, 49° 37' 22" N), located at an altitude of 255 m in the Itajaí river valley of the state of Santa Catarina, southern Brazil. Forty-one dairy cows (*Bos taurus)* from Holstein and Jersey breed and their aborted fetuses derived from thirty-five dairy farms, were analyzed. At first sampling blood of cows and tissues and fluids of aborted fetuses were collected. Further, all farms were revisited to obtain a second blood sample from the cows 30 days after abortion. This project was approved by Animal Ethical Committee from Universidade Estadual de Londrina (number 18/09).

Blood, intrathoracic fluid and tissue samples

Blood samples, with and without EDTA, were collected from the dairy cows that aborted, while intrathoracic fluid was obtained from their fetuses. The white blood cells (WBC) were separated by centrifugation (550xg – 10min), and used for DNA extraction and in PCR assays. Additionally, a pool of tissue fragments (brain, heart, liver, and lung) from each fetus was obtained and used in PCR assays. All tissue samples were homogenized in 10 ml of saline solution (0.14M NaCl), the homogenate filtered through two layers of gauze, after which 2 ml were used for DNA extraction.

Anti-Neospora caninum antibody detection

Antibodies against *N. caninum* were detected using a commercial indirect ELISA test (HerdCheck, IDEXX) that used *N. caninum* tachyzoite-soluble proteins as the antigen. Sera from cows and fluid from fetuses were diluted 1:100 and 1:25 (WOUDA et al., 1997), respectively. The presence or absence of antibodies to *N. caninum* was determined by calculating the ratio of the result for the sample to the result for the positive control (S/P) for each sample. The ratio (S/P) was calculated as follows: (sample optical density – mean negative control)/(mean positive control – mean negative control). Serum samples with a ratio (S/P) < 0.50 were classified as negative and ratio (S/P) ≥ 0.50 as positive. The sensitivity (Se) and specificity (Sp) of the test were 99% and 97.5%, respectively (VON BLUMRODER et al., 2004). A cow with a single or two positive ELISA results was considered as *N. caninum* infected.

DNA extraction and PCR

DNA extractions from WBC, and intrathoracic fluid, and tissues were performed by using the *PureLink*™ Genomic DNA Purification kit (*Invitrogen*) following the manufacture's recommendations, and a negative control (water) was included in each DNA extraction. All PCR assays were done as previously described (MARQUES et al., 2011). *N. caninum* DNA was amplified by using the Np21+ and Np6+ primers (MULLER et al., 2001) in a PCR assay performed in a mixture containing 5 µl of extracted DNA with 20 µl (final volume of 25 µl) of mixture of 1.0 mM of

each primer, 100mM dNTP (Invitrogen, Life Technologies. USA), 60mM Tris–HCl (pH 9.0), 15mM (NH4)2SO4, 1.5 mM $MgCl_2$ and 0.5U Taq DNA polymerase (Invitrogen, Life Technologies. USA). Amplification of protozoan DNA was done over 35 cycles in a PTC-100 thermocycler (MJ-Research), using the following cycling conditions for PCR: 5 min at 94 °C, followed by 35 cycles for 30 s at 94 °C, 30 s at 65 °C and 60 s at 72 °C. Cycle 35 was followed by a final extension of 7 min at 72 °C. Aliquots of each PCR product were electrophoresed on 2% agarose gels. Tachyzoites of the NC-1 strain (10^6/ml) were diluted in TE buffer, the DNA extracted, and used as positive control. The negative control consisted of water samples without *N. caninum*; positive and negative controls were included in each assay.

Pathological evaluation

Cerebrum tissue fragments were collected from all fetuses, and fixed by immersion in 10% buffered formalin solution, routinely processed for histopathological evaluation, and then evaluated for the presence of pathological alterations.

Immunohistochemistry

Cerebrum, myocardium, lung and liver tissue fragments from fetuses were placed into 10% formal saline solution and fixed for five days. The fixed tissues were then processed by standard procedures to paraffin-wax blocks, after which slides were produced for hematoxylin and eosin staining (MALEY et al., 2003). The immunohistochemistry technique was done as previously described (MALEY et al., 2003). Briefly, paraffin-wax sections were cut and mounted on polylysinated glass slides (Superfrost® Plus; Menzel-Gläser) and dried overnight at 37°C. Glass slides were dewaxed and rehydrated. Endogenous peroxidase was blocked by incubating with 4% hydrogen peroxide in methanol for 30 min at room temperature (RT). Non-specific labeling was reduced by incubation with 25% normal goat serum in TBS for 30 min at RT. The tissue sections were then incubated overnight at 4°C with polyclonal primary antibodies produced against *N. caninum* tachyzoites (VMRD Inc, Washington, USA). After washes with TBS, anti-rabbit IgG polymers (Dako EnVision®) were employed as secondary antibodies. The reaction was revealed after incubation with DAB chromogen (Dako EnVision®) for 8 min. Slides were then washed with distilled water and counterstained using Mayer's Hematoxylin for 30 seconds before being dehydrated and mounted.

Statistical analysis

Variables were analyzed by the Chi-square test (χ^2) corrected by the Yates, Fisher Exact Test using the Epi 6 program (CDC. 6.04b version). The association among variables and occurrence of seropositivity was estimated from values obtained by the odds ratio (OR) with a confidence interval of 95%. A *p*-value of ≤ 0.05 was considered as significant in both tests.

Results

The results for *N. caninum* from cows and aborted fetuses are shown in Table 1. From the 41 dairy cows that aborted 21 (51.2%) were seropositive for *N. caninum*. The average S/P value of cows by ELISA, in the first bleeding, was 1.12±0.98. The average gestational age of abortions was 6.77±1.16 months; 13 (34.2%) were aborted at 4-6 months of pregnancy and 25 (65.8%) at 7-9 months. At second revisited six animals were slaughtered and consequently could not be sampled, thus, 35 paired serum samples were obtained. The positivity at the second collection was 48.5% (17/35) with an S/P average =1.01±0.91. The sample of one cow (N.10) was seropositive during the first reaction but negative at the second round of sampling, and thus considered as *N. caninum* infected. No blood samples derived from cows were positive by PCR.

Three fetuses were mummified, preventing ELISA or PCR evaluations. Eight (26.7%) out of 30 fetal intrathoracic fluids were positive by ELISA, the average of fetal gestational age was 7.22±0.66 months, and the average of S/P for fetuses was S/P=0.56±1.04; six (35.3%) and four (23.5%) of these animals were positive by PCR and IHC, respectively (Table 1). A total of 14 (38.8%) and 9 (25%) tissue samples from 36 fetuses were positive by PCR and by IHC, respectively.

The most significant histopathological alterations observed are shown in Table 1. These consisted primarily of vascular alterations, and included hypertrophy of endothelial cells and congestion of blood vessels; intralesional cysts were not observed. The IHC test detected *N. caninum* antigens in four heart and six brain samples. There were no IHC positive results either in lung or liver.

Cows between 24-36 months of age had a protector factor of being serum positive [OR=0.00; (0.00<OR<0.31); p=0.0001] than animals > 36 months of age (Table 2). All cows aborted at more than four months of gestation, and there were no statistical differences (p=0.84). No significant differences (p=0.97) were observed when the breed (Holstein and Jersey) of the cows was evaluated.

When we compared the positive maternal ELISA results with the results obtained by ELISA, PCR, and IHC from their fetuses we observed higher frequencies of having either serum (p=0.0002), PCR (OR=9.78; 1.63<OR<67.5; p=0.007) or IHC (OR=5.9; 0.85<OR<52; p=0.05) being positive (Table 3). All positive ELISA fetuses had positive ELISA cows.

Discussion

In the present study, from the 41 dairy cows that aborted, 51.2% were seropositive for *N. caninum*; comparatively lower seropositivity were reported for *N. caninum* in other studies, varying between 6.3 to 28.6% (MEERSCHMAN et al., 2002; KOIWAI et al., 2005; YILDIZ et al., 2009). In this study, dams that were more than 36 months of age had a higher risk of being seropositive; similar findings were previously described

Table 1. The outcome of *Neospora caninum*-induced infections in dairy cows (*Bos taurus*) and their aborted fetuses.

Cow Nº	Breed	Age of cattle (months)	ELISA[1]		Age of fetus (months)	Fetuses PCR	Principal histopathological findings of the brain		Fetuses IHC
			Cows	Aborted fetuses		Pool of organs	Endothelial hypertrophy	Congestion	
1	JER	> 48	2.23 / 1.53	ND	Mum[2]	ND	ND		ND
2	HOL	> 48	0.4	ND	7	-	0	-	-
3	JER	36	0.27	ND	8	ND	ND	ND	ND
4	HOL	33	0.12 / 0.09	ND	9	-	0	0	-
5	HOL	> 48	2.52 / 2.01	ND	5	+	0	+1	+1
6	JER	> 48	0.97 / 1.05	ND	6	-	0	+2	+2
7	HOL	24	0.0 / 0.07	0.01	8	+	+	N	-
8	HOL	25	0.13 / 0.23	0.01	8	+	+	+1	+1
9	JER	36	0.22 / 0.14	0.0	7	-	++	0	-
10	HOL	> 48	2.07 / 0.18	ND	mum	ND	ND		ND
11	JER	24	0.01	0.0	7	-	++	0	-
12	HOL	> 48	2.11 / 2.07	0.31	8	+	+	0	-
13	JER	> 48	1.9 / 2.15	3.11	7	-	++	+1	+1
14	HOL	> 48	2.27 / 2.2	0.0	7	+	+	0	-
15	JER	> 48	2.3 / 1.96	1.91	7	+	+	0	-
16	JER	> 48	2.05 / 1.09	0.0	6	-	0	+2	+2
17	JER	> 48	0.2 / 0.3	0.0	7	-	+	0	-
18	HOL	24	0.1 / 0.12	0.0	7	-	0	+2	+2
19	JER	> 48	2.01 / 1.95	3.02	7	+	+++	+2	+2
20	JER	> 48	0.29	0.0	8	-	+	0	-
21	JER	48	0.2 / 0.15	0.0	9	-	++	0	-
22	JER	>48	1.92	ND	4	ND	ND		ND
23	JER	>48	0.36 / 0.19	0.0	6	+	0	0	-
24	JER	>48	1.98 / 1.73	ND	6	-	0	0	-
25	HOL	>48	1.79 / 2.07	ND	4	+	0	0	-
26	HOL	36	0.14 / 0.05	0.0	6	-	+	0	-
27	JER	>48	0.17 / 0.05	0.0	6	-	+	0	-
28	HOL	>48	2.6 / 2.33	0.04	6	+	+	0	-
29	HOL	>48	2.26 /2.0	0.51	7	+	+	+2	+2
30	HOL	28	0.32 / 0.4	0.06	8	-	+	0	-
31	J/H	>48	0.11 / 0.21	0.06	5	-	0	0	-
32	HOL	>48	1.98 / 1.92	3.08	8	+	+	0	-
33	HOL	>48	0.21 / 0.16	0.0	7	-	+	0	-
34	JER	>48	0.53 / 0.17	1.68	7	+	++	+1	+1
35	JER	>48	1.95	2.35	6	-	+	0	-
36	JER	>48	2.3 / 2.13	0.03	7	-	++	0	-
37	JER	>48	0.16 / 0.18	0.0	6	-	0	0	-
38	HOL	>48	2.3 / 2.12	1.14	8	+	+	0	-
39	JER	>48	0.13 / 0.06	0.0	7	-	+	0	-
40	JER	30	0.05 / 0.13	0.0	7	-	++	0	-
41	HOL	>48	2.07/0.18	ND	mum	ND	ND		ND

[1]S/P from ELISA (Idexx) S/P ≥ 0.50 (positive) and <0.50 (negative). [2]Mummified fetuses (mum) did not have their tissues and blood tested, and age determined. ND = not done; - (negative); + (positive); JER (Jersey); HOL (Holstein); J/H (Jersey/Holstein); Legend for histopathology: 0, absent; +, discrete; ++, moderate; +++, severe; IHC = Immunohistochemistry; +1= positive in the myocardium; +2=positive in the cerebrum.

(DUBEY et al., 2007; HORNOK et al., 2006). It has been demonstrated that sero-prevalence might increase with the age and the number of gestations in beef and dairy cattle (DUBEY et al., 2007; HORNOK et al., 2006), suggesting that horizontal transmission is of importance in cattle herds.

This study demonstrated that 26.7% (8/30) of the serum evaluated fetuses were positives to *N. caninum* antibodies; this results is more elevated, despite the use of different techniques, than that described in a similar study (SANTOS et al., 2005a), that used the IFA and obtained 11.7% (4/34) of seropositivity.

Table 2. Association between the age of cows, gestational period, and breed with the presence of antibodies against *Neospora caninum* from dairy cows that aborted.

Variables	Positive (%)	Negative (%)	Total	*p-value*[1]	OR	OR95%
Age-cows (months)						
24-36	00(0.0)	10(100)	10(24.4)	0.0001*	0.00	(0.00, 0.31)
>36	21(67.7)	10(32.3)	31 (75.6)			
Total	21(51.2)	20(48.8)	41[2]			
Trimester of gestation						
Second(4-6)	8(61.5)	5(38.5)	13(34.2)	0.84	2.40	(0.50, 11.9)
Third (>7)	10(40.0)	15(60.0)	25(65.8)			
Total	18(47.4)	20(52.6)	38[3]			
Breeds						
Jersey	11(50.0)	11(50.0)	22(55%)	0.97	0.8	(0.19, 3.34)
Holstein	10(55.5%)	8(44.5%)	18 (45%)			
Total	19(47.5%)	21 (52.5)	40[4]			

[1]Chi-square by Yates corrected. [2]One animal (N.15) did not have serum tested because of contaminated serum. [3]Three cows did not have their gestational age determined because fetuses were mummified. [4]Two cows were not used N.15 (contaminated serum) and N.32 (a crossbreed Jersey/Holstein). *Fisher exact test.

Table 3. Results of ELISA from dams compared with ELISA, PCR, and IHC from fetuses.

Dams	Fetuses			*p-value*	OR	OR95%
	ELISA					
	Positives (%)	Negatives (%)	T(%)			
+	8(61.5)	5 (38.5)	13(43.3)	0.0002[1]	UN	Not accurate
-	0(0)	17(100)	17(56.7)			
T(%)	8(26.7)	22(73.3)	30			
	PCR					
+	11(64.7)	6(35.3)	17(47.2)			
-	3(15.8)	16(84.2)	19(52.8)	0.007[2]	9.78	(1.63, 67.5)
T(%)	14(38.8)	22(61.2)	36			
	IHC					
+	7(41.2)	10(58.8)	17(47.2)	0.05[1]	5.9	(0.85, 52)
-	2(10.5)	17(89.5)	19(52.8)			
T(%)	9(25.0)	27(75.0)	36			

[1]Fischer exact. [2]Chi-square by Yates corrected. UN = Undefined.

Additionally, during this study all fetuses that were serum positive originated from dams that were also serum positive for *N. caninum*.

In the present study, there were three cows that had negative serology results for *N. caninum* but the fetuses of 3 were positive by PCR and one of their fetuses tested positive by IHC. Several factors can affect the PCR techniques; sensitivity, specificity, parasite detectability in the presence of host DNA and defective templates, mainly linked to quality of DNA extraction, additionally a false positive result due to contamination at nested-PCR is reported (MÜLLER et al., 2001; MARQUES et al., 2011). Fluctuating of *N. caninum* antibody levels in naturally infected cows was previously described (CARDOSO et al., 2012; CONRAD et al., 1993; DIJKSTRA et al., 2008; KYAW et al., 2005; MARQUES et al., 2011). The observation that some infected dams can exhibit negative antibody values during any gestational period, particularly *Neospora* at parturition or abortion was previously demonstrated (BENAVIDES et al., 2012; NOGAREDA et al., 2007). It has been reported that 5% (8/170) (PARÉ et al., 1996) and 3% (1/33) (MORE et al., 2009) of seronegative cows gave birth to seropositive calves. This shows that caution must be taken in

the interpretation of serologic results if they are to be used in control strategies for bovine neosporosis (CONRAD et al., 1993).

It has been demonstrated that abortions caused by *N. caninum* may occur from 3 months of gestation to term (DUBEY & LINDSAY, 1996). The average (±SD) gestational age of abortions that was suggestive for neosporosis in this study was 7.22±0.66 months, ranging from 6 to 8 months; similar results have been described (MEERSCHMAN et al., 2002; SAGER et al., 2001).

Although the histopathological alterations seen within the brain are non-specific, positive IHC staining for *N. caninum* was observed either in the cerebrum or in the myocardium of six (60%) fetuses that also had non-specific histopathological changes. In contrast, typical *N. caninum*-induced lesions were reported within the brain of fetuses that were experimentally infected (BENAVIDES et al., 2012). Additionally, *N. caninum*-induced necrosis was observed within the skeletal muscles of aborted fetuses whose dams were experimentally infected, but without similar lesions present within the brain, lung, kidneys, and myocardium (McCANN et al., 2007). Nevertheless, these results confirm the

superiority of IHC relative to histopathology for the identification of *N. caninum* within tissue fragments.

A combination of maternal ELISA and fetal ELISA, PCR and IHC gave a better indication of transmission of the parasite to the fetus than maternal ELISA results alone. The PCR assay is considered as a valuable technique for the diagnosis of *N. caninum* (SERRANO-MARTINEZ et al., 2007), and the results from this study have demonstrated that 38.8% (14/36) of the aborted fetuses were positive. Similar findings were described by nested PCR where 80% and 57.7% (MEDINA et al., 2006; YAO et al., 2009) of aborted fetuses were positive for *N. caninum*, in Mexico and China, respectively. However, other studies (PEREIRA-BUENO et al., 2003; RAZMI et al., 2006, 2010; SAGER et al., 2001) have described values that ranged from 11.9 to 33%. Thus, the results of this investigation are in agreement with the diagnosis of *N. caninum*-associated abortions in dairy cattle worldwide, where it was demonstrated that 12 to 45% of aborted fetuses are infected by this protozoan parasite (DUBEY, 2005).

In summary, the present study observed a high frequency of *N. caninum* in abortions from dairy cows from southern Brazil, with a higher *N. caninum* prevalence found in cows that were older than 36 months. In addition, serology, PCR, and IHC should be used in combination for better diagnosis of neosporosis in cattle.

Acknowledgements

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