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Original Article

Neospora caninum in properties in the west region of Paraná, Brazil: prevalence and risk factors

Neospora caninum em propriedades rurais da região Oeste do Paraná, Brasil: prevalência e fatores de risco

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Abstract

Neospora caninum is a heteroxenous protozoa, whose definitive hosts are canids and intermediate hosts are herbivores, and is of great importance in cattle. The objectives of this study were to determine the prevalence of *N. caninum* in dairy cattle and dogs, to detect the presence of the protozoa at the molecular level in aborted fetuses, and to identify the risk factors associated with infection in properties in the western region of the state of Paraná. For this study, 600 bovine serum samples from 60 properties, 163 canine serum samples from 52 properties and 17 bovine fetuses from nine properties were collected. Data were collected using an epidemiological questionnaire to verify the risk factors. Serum samples were analyzed using the indirect fluorescent antibody test. Fetal tissues were analyzed using polymerase chain reaction and subsequent DNA sequencing. Of the bovine samples, 23.67% were positive for *N. caninum*. Among the canine samples, 11.66% were positive for *N. caninum*. Risk factors in cattle were history of abortion, low milk production, extensive breeding, and Jersey breed ($p<0.05$). Protozoan DNA was detected in 52.94% of the 17 fetuses and the sequencing presented high similarity with *N. caninum*.

Keywords: Abortion, neosporosis, PCR, IFAT.

Resumo

Neospora caninum é um protozoário heteroxeno, cujos hospedeiros definitivos são os canídeos e os hospedeiros intermediários são os herbívoros, apresentando maior importância em bovinos. O objetivo desse trabalho foi determinar a prevalência de *N. caninum* em bovinos leiteiros e em cães, detectar molecularmente o protozoário em fetos abortados e identificar os fatores de risco associados à infecção em propriedades da região Oeste do estado do Paraná. Para isso, foram coletadas 600 amostras de soro de bovinos provenientes de 60 propriedades, 163 amostras de soro de cães de 52 propriedades e 17 fetos de nove propriedades. Por fim, foram coletados dados em um questionário epidemiológico para verificar os fatores de risco. As amostras de soro foram analisadas por meio da Reação de Imunofluorescência Indireta (RIFI) e os tecidos fetais foram analisados utilizando a PCR e posterior sequenciamento de DNA. Das amostras de bovinos, 23,67% foram positivas para *N. caninum*. Entre os cães, 11,66% foram positivos. Os fatores de risco nos bovinos foram histórico de aborto, baixa produção de leite, criação extensiva e raça Jersey ($p<0,05$). Dos 17 fetos analisados, em 52,94% foi possível detectar DNA do protozoário, e o sequenciamento mostrou alta similaridade com *N. caninum.*

Palavras-chave: Abortos, neosporose, PCR, RIFI.

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Introduction

Neospora caninum is an obligate intracellular protozoan that requires two hosts to complete its cycle. Canines are its definitive hosts and dairy cattle the main intermediate hosts, but also infect sheep, goats, horses, birds, and wild animals (DUBEY & SCHARES, 2011; GOODSWEN et al., 2013).

Canids become infected when they ingest tissues, such as placenta or fetal remains, contaminated with protozoan cysts that is at proliferative stages. Dogs exhibiting clinical signs, with diarrhea being the most common, usually associated with oocyst elimination, and neurological signs are rare (LINDSAY et al., 1999; MONNEY & HEMPHILL, 2014; DONAHOE et al., 2015). Intermediate hosts may acquire a disease by horizontal transmission when they ingest the sporulated oocysts present in water or contaminated food (GOODSWEN et al., 2013), and vertical transmission of the protozoan, which is more important than the maintenance of the parasite in the properties. This can lead to miscarriage or the birth of congenitally infected calves. The infection can also be transmitted to future offspring. Thus, infection by this protozoan is considered one of the main causes of abortion in cattle and is responsible for innumerable losses in properties (MCALLISTER, 2016).

N. caninum has a wide distribution, with cases in Asia, Africa, America, Europe, and Oceania (DUBEY et al., 2007; SPILOVSKÁ et al., 2009; YU et al., 2009; PANADERO et al., 2010; REICHEL et al., 2013). Its occurrence in several countries, as well as in Brazil, varies according to the region, risk factors, and diagnostic techniques (GOODSWEN et al., 2013).

In Brazil, there are four *N. caninum* isolates from cattle: BCN/PR3 (LOCATELLI-DITTRICH et al., 2003), BCN/PR1 (LOCATELLI-DITTRICH et al., 2004), Nc-Goiás 1b (GARCÍA-MELO et al., 2009) and Nc-SP1 (OLIVEIRA et al., 2017), being currently considered an important cause of abortion in cattle, bringing innumerable losses (CERQUEIRA-CEZAR et al., 2017). In recent years, the occurrence of *N. caninum* in cattle ranged from 9.1% to 97.2%, and these values were found in the states of Mato Grosso do Sul and Minas Gerais, respectively (GUEDES et al., 2008; MELLO et al., 2008; CERQUEIRA-CEZAR et al., 2017). In the state of Paraná, the occurrence varies from 12% to 34.8%, but most authors find results between 20 and 30% (LOCATELLI-DITTRICH et al., 2001; OGAWA et al., 2005; CERQUEIRA-CEZAR et al., 2017).

The state of Paraná in southeastern Brazil is the third largest producer of milk in Brazil, with the westernmost state being the most prominent region. Three cities (Marechal Cândido Rondon, Toledo, and Cascavel), in particular, stand out as being among the 15 largest producers in the country (IBGE, 2016).

Epidemiological studies involving pathogens that affect dairy cattle in this region are rare. Thus, the objective of this study was to determine the seroprevalence of *N. caninum* in dairy cattle and dogs, to detect molecularly the protozoan in aborted fetuses, and to identify the risk factors associated with infection in properties in the western region of Paraná.

Material and Methods

Committee of ethics on animal experimentation

This study is in accordance with the Ethical Principles of Animal Experimentation and was approved by the Commission of Ethics in the Use of Animals of the Universidade Federal do Paraná-Palotina Sector, with protocol number 50/2014.

Area of study and sampling calculation

The western region is the main dairy basin in the state of Paraná, with the cities of Marechal Cândido Rondon, Toledo, and Cascavel being the largest producers in the region (IBGE, 2016), which is the reason this region was chosen for the seroprevalence study of *N. caninum*.

Sampling was performed using the EpiInfo program (version 7.2.0.1), where an expected prevalence of 35% was used based on other studies in the state of Paraná, such as the study by Locatelli-Dittrich et al. (2008). An expected maximum error of 5% and 95% confidence interval and design effect (DEFF) of 1.5, which should be used when there is no random sample collection to reduce error, were used. This resulted in a minimum of 525 samples to be collected. However, 600 animals, which were distributed in four cities of the western region of Paraná (Cascavel, Marechal Cândido Rondon, Palotina, and Toledo), were included.

The number of rural cattle farms in each city was provided by the Agricultural Defense Agency of Paraná (ADAPAR), and the number of properties where samples will be collected was evaluated, with proportional distribution shown in Table 1.

The properties in each city were randomly selected, ranging from small- to large-scale production. It was stipulated that 10 animals would be sampled from each of the 60 properties.

Sample collection and application of the epidemiological questionnaire

Cow blood samples from caudal veins were collected using 40 X 1.2 mm needles and 10 mL syringes. After collection, blood samples were stored in properly labelled test tubes without anticoagulant and kept refrigerated until processing. Data on the age, race, and abortion history of each animal were also collected.

Table 1. Distribution of farms, cattle and dogs collected in different cities of the western region of the state of Paraná, Brazil.

City	Nº dairies	Cattle		Dogs	
		Nº of collected dairies	Nº of collected animals	Nº of collected dairies	Nº of collected animals
Cascavel	1902	18	180	17	68
Toledo	2000	19	190	19	53
M. C. Rondon	1862	17	170	11	27
Palotina	591	6	60	5	15
Total	**6355**	**60**	**600**	**52**	**163**

During the collection period, 17 bovine abortions occurred in nine properties: five in Palotina, two in Cascavel, one in Toledo and one in Marechal Cândido Rondon. Fragments of the heart, brain, and placenta of these fetuses were collected, packed in Microtubes, and stored at -20 ºC. After the collection of the fetuses, another visit was made to the farms to collect blood samples from the cows that had abortions, if they were not part of the 10 selected cows from the property. Thus, in addition to the 600 samples, eight blood samples were also collected.

Furthermore, samples from 163 dogs residing in 52 properties were collected (Table 1). Some properties did not allow collection of blood samples from dogs. Samples from the dogs were collected through the puncture of the cephalic vein or jugular vein using 20 X 0.55 mm needles and 5 mL syringes. After collection, blood was stored in labelled test tubes and kept under refrigeration until processing. During collection of blood samples, data from animals such as age, sex, and race were also collected.

In the laboratory, samples were centrifuged at 1500rpm for 10min for serum separation, after which they were stored in microtubes at -20°C until processing.

Data were collected using an epidemiological questionnaire for the 60 properties to evaluate the main risk factors associated with neosporosis. The data collected included presence of dogs on the properties, contact of the dogs with the cattle, size of the herd, reproductive and herd management, milk production, breed, age and history of cattle abortion, as well as data related to dogs, such as feeding, race, age, sex, whether they were released or trapped, use of vermifuge and vaccines.

Analysis of samples

For the serological analysis of the samples, the indirect fluorescent antibody test (IFAT) was performed for the bovine and canine samples (PARÉ et al., 1995).

The IFAT slides were adsorbed with *N. caninum* strain Nc-1, from the Laboratory of Parasitology of the University of São Paulo (USP), fixed with methanol, and stored at -20°C.

Each serum sample was diluted with the dilution solution (0.0084 M Na2HPO4, 0.0018 M NaH2PO4, 0.146 M NaCl, and 1% BSA). Then, 20μL was added to each well of the immunofluorescence slide, and they were incubated for 30min at 37°C. After that, three washes were performed with a wash solution (0.0268M Na2CO3, 0.0975M NaHCO3, and 0.036M NaCl). Then, the slides were oven dried at 37°C and then 20μL of the anti-bovine and anti-canine IgG conjugate *Sigma*, incubated for 30 min at 37 °C, then three washes were performed using the stock solution. Then, the slide was oven dried at 37°C. Afterwards, a coverslip was placed with two drops of buffered glycerin (50%) and viewed under an epifluorescence microscope at a magnification of 400X (PARÉ et al., 1995).

Samples with total fluorescence of tachyzoites from the 1:100 dilution for cattle and 1:50 for dogs were considered positive, while samples that only presented with apical reaction were considered negative (ACOSTA et al., 2016; CAMILLO et al., 2010). Positive sera were then diluted in ratio two series to the final titre.

For the analysis of the fetuses, polymerase chain reaction (PCR) was performed. About 5 g of the collected organs (heart, brain, and placenta) were individually macerated; then, DNA extraction was performed using the commercial kit DNeasy Blood and Tissue (Qiagen). For PCR, the Nc5 region was selected as the target sequence for DNA amplification. The primers Np21/Np6 (5'-CCCAGTGCGTCCAATCCTGTA-3')/ (5'-CTCGCCAGTCAACCTACGTCTTCT-3') (MÜLLER et al., 1996) were used. The reaction was carried out with a final volume of 50μL, containing 10X buffer, 200μM DNTP, 1.5mM $MgCl_2$, 20μM of each primer, 1.25U Taq DNA Polymerase, with initial denaturation at 95ºC for 5 min, 40 cycles at 94ºC for1 min/63ºC for 1 min/74ºC for 3,5 min and final extension at 74ºC for 10 min. Amplified samples were subject electrophoresis in a 1.5% agarose gel for visualization. The positive controls used were tachyzoites of the Nc-Bahia strain, and negative controls were autoclaved ultrapure water.

For the sequencing, a positive sample from each property was selected and sent to ACTGene Análise Moleculares Ltda. (Biotechnology Center, UFRGS, Porto Alegre, RS). For this, samples were sequenced by ACTGene Análises Moleculares Ltda. (Centro de Biotecnologia, UFRGS, Porto Alegre, RS, Brazil) using the automatic sequencer AB 3500 Genetic Analyzer armed with 50 cm capillaries and POP7 polymer (Applied Biosystems). DNA templates were labeled with 2.5 pmol of the primer Np21/Np6 and 0.5μL of BigDye Terminator v3.1 Cycle Sequencing Standart (Applied Biosystems) in a final volume of 10μL. Labeling reactions were performed in a LGC XP Cycler termocycler with a initial denaturing step of 96ºC for 3 min followed by 25 cycles of 96ºC for 10 sec, 55 ºC for 5 sec and 60ºC for 4 min. Labeled samples were purified by isopropanol precipitation 75% followed by and 60% ethanol rinsing. Precipitated products were suspended in 10μL formamide Hi-Fi (Applied Biosystems), denatured at 95ºC for 5 min, ice-cooled for 5 min and electroinjected in the automatic sequencer. Sequencing data were collected using the software Data Collection 2 (Applied Biosystems) programmed with the following parameters: Dye Set "Z"; Mobility File "KB_3500_POP7_BDTv3.mob"; BioLIMS Project "3500_Project1"; Run Module 1 "FastSeq50_POP7_50cm_cfv_100"; e Analysis Module 1 "BC- 3500SR_Seq_FASTA.saz".

Research of risk factors

The seroprevalence and variables related to epidemiological questionnaire data were analyzed in the Epi Info program (version 7.2.0.1) using the chi-square test and Odds Ratio (OR), with a 5% as a significant level.

Results and Discussion

Seroprevalence and risk factors

The IFAT-based seroprevalence of *N. caninum* in dairy cattle in the western region of the State of Paraná was 23.67% (142/600). Of the 60 properties included in this study, 80% (48) had at least one bovine seropositive to *N. caninum*. All the properties surveyed in the city of Palotina were positive, whereas the cities of Cascavel, Toledo, and Marechal Cândido Rondon presented positive results in 77% of properties (Table 2).

In the studies carried out in recent years in Brazil, the occurrence of *N. caninum* in cattle ranged from 10.9 to 50.74% depending on the state, method of diagnosis, cut-off point used, main risk factors, and number of samples analyzed can also influence the results of the research (TEIXEIRA et al., 2010; AGUIAR et al., 2011; AMARAL et al., 2012; BRUHN et al., 2013).

The seroprevalence of *N. caninum* is rare in dairy cattle in Paraná. One of the reasons is the number of samples collected, which most of the time only allows the detection of occurrence and not of prevalence. The last study was by Locatelli-Dittrich et al. in 2008, who reported a seroprevalence of 33% upon analysis of 1263 sera samples from the state of Paraná by the ELISA method. Rocha et al. (2015) evaluated 367 animals in the region of Francisco Beltrão (Southwest of Paraná) and found 35.1% of the samples to be positive, a higher percentage than the one found in the west region, which can be justified by the cutoff point of 1:50.

Camillo et al. (2010) analyzed samples from the southwest region of Paraná using the IFAT with 1:100 cutoff, and found a prevalence of 24.2%. The similar seroprevalence in the southwest and west regions can be explained by geographical proximity, as well as the constant purchase or sale of cattle between the two regions.

When the properties were analyzed, Locatelli-Dittrich et al. (2008) found that 77% of the properties had at least one seropositive animal in the state of Paraná, which is similar to the results of this study in the western region of Paraná. In the state of Rio Grande do Sul, Corbellini et al. (2005) found that 93.3% of 60 properties had at least one positive animal.

Cattle titers ranged from 100 to 12800, with most animals having titers of 800, followed by 1600 and 400. Only one animal had a titer of 6400 and another one had 12800. Koiwai et al. (2006) analyzed 2420 bovine sera using the IFAT for *N. caninum* and found more animals with a titer of 800, followed by 1600 and 400. However, other authors found a higher frequency of titers below 800 (KASHIWAZAKI et al., 2004). Titers of IgG antibodies equal to or greater than 800 are generally associated with clinical signs of the disease in cattle (DUBEY, 1999; LINDSAY et al, 1999). However, as verified by Teixeira et al. (2010), not all animals with high titers had a history of abortion, although they might have given birth to congenitally infected normal calves.

High antibody levels are associated with the reactivation and reconversion of bradyzoite into a tachyzoite, which will reach the placenta and the fetus through the circulatory system. This phase usually occurs from the fourth to the fifth month of gestation but may occur throughout the pregnancy period. This results in miscarriages or birth of congenitally infected calves. After abortion, antibody levels tend to decrease (CONRAD et al., 1993; MCALLISTER, 2016).

Of the 163 samples from dogs, 19 (11.66%) were positive for anti-*N. caninum* antibodies. When the properties were analyzed, 28.84% (15/52) had at least one seropositive dog. Palotina had a higher percentage than the other three cities (Table 2).

In some experiments, dogs that ingested animal tissue with *N. caninum* cysts did not seroconvert after eliminating oocysts in the feces (LINDSAY et al., 1999; DIJKSTRA et al., 2001; SCHARES et al., 2005). In another study, which was conducted by Barber & Trees (1998), dogs with low titers of antibodies against the protozoa became seronegative over time. Due to these factors, the actual protozoan seroprevalence in dogs may be higher than that found in this study.

Several authors have compared the seroprevalence of *N. caninum* in dogs from urban areas with those from rural areas, and this one is generally greater in the wandering dogs or domiciled in farms, fact that is related to the cycle of the parasite. As transmission to carnivores occurs through the consumption of raw meat or fetal remains with protozoan cysts, the chances of dogs becoming infected in rural areas are greater than that of dogs in urban areas (FERNANDES et al., 2004; LASRI et al., 2004; KING et al., 2012; NOGUEIRA et al., 2013).

Locatelli-Dittrich et al. (2008), analyzed 129 canine samples using IFAT from 35 properties from the entire state of Paraná and obtained 25% positive results. This percentage is considered high compared to that found in higher tham the present study.

Acosta et al. (2016) analyzed samples of 187 dogs from 30 properties in the state of Espirito Santo and found an occurrence of 11.76%, which is similar to the results of this study in the west of Paraná. Nogueira et al. (2013) evaluated the prevalence of protozoa in dogs from urban and rural areas in the State of Minas Gerais and found that 11.4% of the samples had positive results: 13.78% in dogs from rural areas and 8.24% in dogs from urban areas.

The titer of the dogs measured using IFAT ranged from 50 to 800, with a higher number of animals showing titers of 100 (47.36%), followed by 50 (21.05%) and 200 (15.79%). Only two animals showed titers of 400 (10.52%) and one animal had a titer of 800 (5.26%). However, Nogueira et al. (2013) found anti-*N. caninum* antibodies in dogs ranging from 50 to 6400, with a

Table 2. Occurrence of anti-*Neospora caninum* antibodies in cattle and dogs from dairy farms in the western region of the state of Paraná, Brazil.

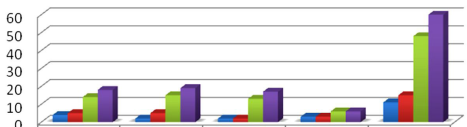
City	Cattle						Dogs					
	Animals			Dairies			Animals			Dairies		
	P	N	%	P	N	%	P	N	%	P	N	%
Cascavel	45	135	25.00	14	4	77.78	8	60	11.76	5	12	29.41
Toledo	40	150	21.05	15	4	78.95	5	48	9.43	5	14	26.31
M. C. Rondon	34	136	20.00	13	4	76.45	3	24	11.11	2	9	18.18
Palotina	23	37	38.33	6	0	100.00	3	12	20.00	3	2	60.00
Total	**142**	**458**	**23.67**	**48**	**12**	**80.00**	**19**	**144**	**11.66**	**15**	**37**	**28.84**

P: positive; N: negative.

higher number of animals showing titers of 50. Acosta et al. (2016) found a greater number of dogs with titers between 800 and 1600.

The presence of *N. caninum* seropositive dogs was not considered a risk factor for seropositivity in cattle, as it was not statistically significant (Figure 1). Other studies have also evaluated the seropositivity of dogs as a risk factor for bovine neosporosis and did not find a significant difference (GUIMARÃES et al., 2004; AGUIAR et al., 2006; LOCATELLI-DITTRICH et al., 2008; BENETTI et al., 2009). However, some authors reported a higher seroprevalence of *N. caninum* in cattle when they had contact with dogs, especially if the dogs were seropositive. Thus, the dogs could be involved in the transmission of neosporosis (PARÉ et al., 1998; WOUDA et al., 1999; SANCHEZ et al., 2003).

The insignificant difference may be an indication that the protozoan is kept in the herd by vertical transmission, not necessarily needing a definitive host (PARÉ et al., 1997; MCALLISTER, 2016). Another factor that must be considered is the possibility of the disease being transmitted by stray dogs or wild canids that can feed on fetal remains with the *N. caninum* bradyzoites and then disseminate the oocysts excreted in the feces through grass or water (BENETTI et al., 2009, MCALLISTER, 2016). It can be related to seroconversion, since some dogs may not seroconvert or may not be seronegative, which would influence the results of occurrence of the protozoan in dogs and its correlation with seroprevalence in bovines (BARBER & TREES, 1998; LINDSAY et al., 1999; DIJKSTRA et al., 2001; SCHARES et al., 2005).



	Cascavel	Toledo	M. C. Rondon	Palotina	Total
Dairies with positive dogs and cattle	4	2	2	3	11
Dairies with positive dogs	5	5	2	3	15
Dairies with positive cattle	14	15	13	6	48
Total of dairies	18	19	17	6	60

Figure 1. Correlation of the results of the properties with seropositive dogs and cattle, properties with seropositive dogs, properties with seropositive cattle, and the total data collected from properties in the west of the state of Paraná, Brazil.

The risk factors related to *N. caninum* infection in dairy cattle in the western region of the state of Paraná included the following. Milk production: properties that produce 100 to 200 liters are 1.96 times more likely to have *N. caninum* than other properties. Reproductive management: properties that use artificial insemination and bull are 2.02 times more likely to have the protozoan than properties that use other reproductive management, such as bull only, or the association of bull and artificial insemination at fixed times. The type of production: cattle raised in extensive production are 1.76 times more likely to have neosporosis. A history of abortion: animals with a history of abortion have about 3.81 times more chances of having *N. caninum*. The breed Jersey are 2.18 times more likely to have *N. caninum* than other breeds (Table 3).

The main risk factors are associated with the production e reproduction profile, usually family farms with low milk production, and extensive farms, where animals raised mainly in pasture have a higher chance of contact with the protozoan.

Although the Jersey breed in this study was identified as a risk factor for *N. caninum* infection, this result reflects the sampling, since blood samples were collected from a small number of animals

Table 3. Risk factors related to the presence of anti-*Neospora caninum* antibodies in dairy cattle from rural properties in the western region of the state of Paraná, Brazil.

Variables		Positive	Negative	%	OR	P
Milk production	1-100L	7	23	23.33	Ns	1.000
	100-200L	25	45	35.71	1.96 (1.15-3.33)	0.017
	200-500L	55	185	22.92	Ns	0.799
	500-1000L	32	118	21.33	Ns	0.507
	>1000L	23	87	20.91	Ns	0.529
Reproductive management	Bull	44	126	25.88	Ns	0.486
	IATF	12	51	19.05	Ns	0.450
	IA	37	111	25.00	Ns	0.742
	IA and IATF	24	105	18.6	Ns	0.158
	IA and bull	22	38	36.67	2,02 (1,15-3,55)	0.019
	IA, Bull and IATF	3	27	10.00	Ns	0.112
Type of production	Extensive	47	102	31.54	1.76 (1.14-2.60)	0.012
	Intensive	29	90	24.37	Ns	0.935
	Semi-intensive	66	266	19.88	0.62 (0.42-0.91)	0.019
Contact with dogs		115	377	23.37	Ns	0.814
Dogs pos. in daires		41	109	27.33	Ns	0.267
Abortion sistory	yes	59	72	45.04	3.81 (2.50-5.78)	<0.01
Breed	Holstein	121	422	22.28	0.49 (0.27-0.87)	0.021
	Jersey	20	32	38.46	2.18 (1.20-3.95)	0.014
	Braunvieh	0	5	0	Ns	0.598

Ns: not significant; OR: Odds Ratio; P: probability of significance; IA: artificial insemination; IATF: fixed-time artificial insemination.

of this breed. Only two properties had Jersey and most of those were seropositive.

Semi-intensive production and Dutch breed were found to be protective factors. These results may have been influenced by the number of samples collected that did not fit these conditions, such as Jersey breed and extensive production that are risk factors.

Among the seropositive cattle (59/142), 41% had a history of abortion, which is an important risk factor (p <0.001) and is the main clinical sign of neosporosis in cattle. No significant difference was found when this factor was correlated with the titers, although the majority of the animals with a history of abortion presented titers from 400 to 1600 (Table 4).

Other factors analyzed through the epidemiological questionnaire, such as presence of dogs on the property, contact with dogs, number of dogs on the farm, age of cattle, and replacement of cattle, among others, did not have significant differences.

The major risk factors for *N. caninum* infection in dogs found by other authors are as follows: age, older dogs are generally more likely to have the protozoan; feeding, dogs that consume food and/or raw meat are more likely to acquire the parasite and have contact with cattle, as they are the main intermediate hosts of *N. caninum* (NOGUEIRA et al., 2013).

However, in the present study, dogs did not present risk factors associated with *N. caninum*. The main factors investigated were age of the animal, contact with cattle, feeding, sex, if the dog is arrested or released, vaccination, race, use of vermifuge, and history of diseases.

Aborted bovine fetus

Seventeen dairy cattle fetuses were collected from nine properties in the region studied: four from Cascavel, two from Toledo, one from Marechal Cândido Rondon, and ten from Palotina. Of these, 52.94% (9/17) were positive for *N. caninum* using the PCR technique and all of the samples showed a high similarity (99%) to the protozoan by DNA sequencing.

The percentage found is considered high compared to that reported by other studies. Antoniassi et al. (2013) performed histopathological analyses of 490 fetuses and found 32.6% to be positive for *N. caninum*. Corbellini et al. (2000) found 10% of the fetuses to be positive when they analyzed tissues of 30 fetuses using the immunohistochemical technique (IHC). Orlando et al. (2013), after analyzing tissues of 60 fetuses through PCR and IHC, found 23.33% of the samples to be positive for the protozoan. Meanwhile, analyzing the brain, lung, liver, heart and intrathoracic fluid of 30 fetuses of dairy cattle from the State of Santa Catarina, Macedo et al. (2017) found 38.8% positives through the PCR technique, 25% through immunohistochemistry and 26.7% through of the ELISA.

Table 4. Correlation of titers of seropositive cattle for *Neospora caninum* and history of abortion in rural properties in the western region of the state of Paraná, Brazil.

Titration	nº animals	%	Abortion history	%
100	14	9.86	3	21.42
200	25	17.60	9	36.00
400	29	20.42	14	48.27
800	41	28.90	18	43.90
1600	29	20.42	13	44.83
3200	2	1.40	1	50.00
6400	1	0.70	1	100.00
12800	1	0.70	0	0
Total	142	100	59	41.54

The high positivity found in the present study may be due to the technique used, since PCR is a more sensitive and more specific technique than histopathology and IHC, especially in autolysis fetuses. Another factor that may have influenced the result is the number of fetuses collected. Additionally, two properties located in Palotina had many symptomatic animals that were seropositive for *N. caninum*. The animals had abortion frames, and these fetuses were collected.

We analyzed the brains and hearts of the 17 fetuses and the placenta of only three fetuses because of the difficulty in collecting placenta. The 17 fetuses were tested using PCR: nine were positive in the brain, five in the heart, and three in the placenta. All fetuses that showed positive results in the heart and placenta also presented positive results in the brain (Table 5).

The most affected animal tissue by *N. caninum* is the central nervous system (MCALLISTER, 2016). In Brazil, there are four *N. caninum* isolates from bovine brain: BCN/PR3 (LOCATELLI-DITTRICH et al., 2003), BCN/PR1 (LOCATELLI-DITTRICH et al., 2004), Nc-Goias 1b (GARCÍA-MELO et al., 2009) and Nc-SP1 (OLIVEIRA et al., 2017). Thus, it is where the parasite is most commonly found through molecular techniques (SANTOS et al., 2011; ORLANDO et al. 2013, BROM et al., 2014). The protozoan can also be found in the heart, as it is one of the muscular tissues of predilection of *N. caninum*. Amaral et al. (2012) found, using PCR, a higher positivity in the heart than in the brain in fetuses coming from slaughterhouses. Therefore, it is important to evaluate and to associate the results found in both organs to decrease the number of false negatives in the diagnosis.

The placenta is an important organ that should be evaluated when possible to study the causes of abortion. It is essential to emphasize the collection of adequate material, since it is in cotyledons that most of the pathogens responsible for abortion can be found (MCALLISTER, 2016).

The gestational age with the highest occurrence (35.29%) of the abortions was eight months; however, when the age with the highest number of fetuses positive for *N. caninum* was analyzed, it was five months (75%), followed by four months (66.66%), seven months (50%), and eight months (33.33%). These results confirm those found by other authors. in addition to having a correlation with the gestation time in which there is a greater chance of reactivation and reconversion of the bradyzoite into a tachyzoite in the maternal organism, reaching the placenta and then the fetus (ORLANDO et al., 2013; MCALLISTER, 2016).

When the fetuses were analyzed based on sex, most were females (9/14), but only 33.33% of them were positive for the protozoan studied. Of the males, 60% (3/5) were positive for *N. caninum*. It was not possible to identify the sex of three fetuses since many structures were degraded.

One animal had negative serology results for *N. caninum*, but the aborted fetus was found to be positive; this can be explained

Table 5. Result of polymerase chain reaction of *Neospora caninum* from 17 bovine fetuses collected from rural properties in the western region of Paraná, Brazil.

City	Dairies	Cow titration	Fetal age	Sex	PCR brain	PCR heart	PCR placenta
Cascavel	P01	Negative	8	Male	Positive	Negative	NR
	P01	Negative	8	Male	Negative	Negative	NR
	P01	Negative	8	Female	Negative	Negative	NR
	P02	Negative	5	Female	Negative	Negative	NR
Toledo	P03	Negative	8	Female	Negative	Negative	NR
	P03	Negative	8	Female	Negative	Negative	NR
M. C. Rondon	P04	Negative	7	Female	Negative	Negative	NR
Palotina	P05	3200	5	Female	Positive	Positive	Positive
	P06	1600	4	NI	Positive	Negative	Positive
	P06	Negative	7	Female	Negative	Negative	NR
	P06	1600	8	Female	Positive	Positive	NR
	P07	800	7	Female	Positive	Negative	NR
	P07	1600	5	NI	Positive	Positive	NR
	P07	400	4	NI	Positive	Positive	Positive
	P07	400	7	Male	Positive	Negative	NR
	P08	800	5	Male	Positive	Positive	NR
	P09	200	4	Male	Negative	Negative	NR

NI: unidentified; NR: unrealized.

by the false negatives that the serology technique can generate. Another animal had positive serology results, but its aborted fetus was negative for the protozoan. This can be justified by the mummified condition of the fetus, which may have degraded the *N. caninum* DNA and generated a false negative result; it is also possible that there was a different cause for the abortion.

Conclusion

N. caninum is widely distributed in the western region of the state of Paraná both in cattle and in dogs.

There is no correlation between the presence of seropositive dogs and the serology of the bovines in the same properties.

The main risk factors for neosporosis in cattle in the western region of the state of Paraná are related to family production (small, low milk production, and extensive properties). History of abortion is also an important risk factor for the disease.

No risk factors associated with *N. caninum* infection were identified in dogs from rural farms that were included in this study.

The protozoan can be commonly found in several fetal tissues, especially the brain. The occurrence of *N. caninum* DNA in aborted fetuses was greater in those aged between four and five months.

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