Seroprevalence and risk factors associated with *Neospora caninum* in crossbred dairy cows in Uberlândia

Soroprevalência e fatores de risco associados ao *Neospora caninum* em vacas leiteiras mestiças em Uberlândia, Minas Gerais

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Abstract

Neospora caninum causes reproductive disorders in dairy cattle such as repeat breeding, abortion, and anestrus, which are risk factors associated with the seroprevalence of *N. caninum* in Brazil and worldwide. This study aimed to verify the risk factors related to the seroprevalence of *Neospora caninum* in crossbred dairy cows in the city of Uberlândia, Minas Gerais. During, January to July 2013, 740 blood samples were collected from 57 herds with a history of reproductive disorders and determinations of antibody titers against *N. caninum* were obtained by ELISA assay. The seroprevalence of *N. caninum* was 18.4% (136/740). Risk factors for *N. caninum* were cows greater than 2 years of age (P = 0.034), mechanical milking (P = 0.012), and frequent purchase of animals (P = 0.004), in addition to individual history of abortion (P = 0.007) and repeat breeding (P = 0.030). The highest probability of occurrence of associated risk factors was associated with individual history of abortion and repeated breeding and the frequent purchase of animals, with odds of 12.8, 5.9, and 3.8 times, respectively. In conclusion, the risk factors associated with *N. caninum*: members of the female sex and cows with more than 2 years-of-age, mechanical milking, and the frequent purchase of animals, in addition to individual history of abortion and repeated breeding.
Key words: Abortion. Cattle. Neosporosis.

Resumo

Neospora caninum causa distúrbios reprodutivos na bovinocultura de leite como repetição de cio, abortamento e anestro, que são fatores de risco associados à soroprevalência do *Neospora caninum* no Brasil e no mundo. Objetivou-se verificar os fatores de risco relacionados à soroprevalência do *Neospora caninum* no município de Uberlândia, Minas Gerais em vacas leiteiras mestiças. No período de janeiro a julho de 2013 foram colhidas 740 amostras de sangue de vacas, em 57 propriedades com

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histórico de problemas reprodutivos e as determinações dos anticorpos contra o *N. caninum* foram obtidos pela técnica de ELISA. A soroprevalência foi de 18,4% (*136/740*) de positivos para o *N. caninum*. Os fatores de risco associados para o *N. caninum* foram fêmeas maiores de dois anos (*P=0,034*), ordenha mecânica (*P=0,012*), compra frequente de animais (*P=0,004*), além do histórico individual de abortamento (*P=0,007*) e repetição de cio (*P=0,030*). A maior probabilidade de ocorrência de fatores de risco associados foram: histórico de abortamento e repetição de cio individual e a compra frequente de animais, respectivamente, com chances de 12,8; 5,9 e 3,8 vezes. Concluiu-se que existem fatores de risco associados para o *N. caninum*: fêmeas maiores de dois anos, ordenha mecânica, compra frequente de animais, além do histórico individual de abortamento e repetição de cio.
Palavras-chave: Abortamento. Bovinos. Neosporose.

Introduction

There are several causes of embryonic loss and abortions in cattle, and the likely etiologic agents of these diseases should be identified (SARTOR et al., 2005). *Neospora caninum* is considered as one of the main causative agents of reproductive disorders (SARTOR et al., 2005) and is responsible for economic losses in dairy cattle resulting from abortions around the 5th and 6th months of pregnancy (MELO et al., 2006).

Neosporosis has been identified in several countries, with the following reported seroprevalences: Morocco - 8.52% (*15/176*) (LUCCHESE et al., 2016), China - 17.14% (*769/4487*) (SUN et al., 2015), Algeria - 19.64% (*157/799*) (GHALMI et al., 2012), Vietnam - 19% (*25/130*) (DUONG et al., 2008), Japan - 5.7% (*139/2420*) (KOIWAI et al., 2006), Thailand - 5.49% (*30/549*) (KYAW et al., 2004), United States - 10.34% (*9/87*) to 32.11% (*105/327*) (ORTEGA et al., 2007), Venezuela - 11.3% (*52/459*) (LISTA-ALVES et al., 2006), Paraguay - 29.8% (*262/879*) (OSAWA et al., 2002), and Spain - 35.9% (*402/1121*) (QUINTANILLA-GOZALO et al., 1999).

Antibodies against *N. caninum* were detected in serum from dairy cattle from herds located in several Brazilian states (RAGOZO et al., 2003), and a seroprevalence of 16.83% (*101/600*) was reported in the states of São Paulo and Minas Gerais (COSTA et al., 2001). Santos et al. (2009) reported a seroprevalence of 46.25% in calves and heifers from dairy herds in the state of Minas Gerais,

and there has been a report of 10% (*7/69*) to 20% (*34/174*) prevalence of *N. caninum* antibodies in dairy cows with a history of reproductive problems in properties in the region of the Triângulo Mineiro, Minas Gerais (MINEO et al., 2006).

More recently, several studies were done to identify the risk factors associated with the seroprevalence of *N. caninum*, with the aim to prevent and reduce the occurrence of neosporosis in herds. Poor quality of water, the presence of dogs, poor hygiene conditions, and a history of abortions have already been reported as risk factors (GHALMI et al., 2012; SUN et al., 2015). In Brazil, the type of herd, method of herd management, presence of definitive hosts (SARTOR et al., 2005), and reproductive problems such as repeated breeding, history of abortion, and anestrus (BRUHN et al., 2013) have been reported as risk factors. Abortion is considered the main clinical manifestation of cows infected with *N. caninum* (HUSSIEN et al., 2012).

Accordingly, the aim of this study was to identify the risk factors associated with seroprevalence of *N. caninum* in crossbred cows from the city of Uberlândia, Minas Gerais.

Materials and Methods

Study area

The study was conducted in the city of Uberlândia, located in the state of Minas Gerais, Southeastern Brazil, in the microregion of the Triângulo Mineiro, between January and July 2013.

A total of 57 properties distributed in five districts of the municipality (Martinésia, Cruzeiro dos Peixotos, rural perimeter of Uberlândia, Tapuirama and Miraporanga), were assessed.

Inclusion criteria

Only properties of the municipality with a history of reproductive problems, defined by abortion observed in any stage of pregnancy and repeated breeding, were included in the experiment. A maximum of 15 adult lactating crossbred dairy cows, born on the farm and raised under an extensive rearing system, were randomly selected for the study. These animals were immunized against brucellosis and foot-and-mouth disease, according to the requirements of the Brazilian Ministry of Agriculture, Livestock and Supply (MAPA).

Epidemiological survey

To identify potential associated risk factors, information concerning the following was collected from the properties: type of farming (dairy/mixed), total number of cows on the property ($\leq$100/>100 animals), number of cows over two years old ($\leq$100/>100 animals), total herd population ($\leq$100/>100 animals), type of milking (manual/mechanical), frequent purchase of animals (no/yes), completion of quarantine (no/yes), presence of birthing paddock (no/yes), presence of dogs (no/yes), history of abortion on the property (no/yes), repeat breeding, and history of individual abortion (no/yes).

Serology

The properties were visited once for the collection of blood sample. Blood was collected by caudal vein puncture, and the samples were collected in vacuum tubes without anticoagulant. The serum aliquots were maintained frozen and subsequently used in the enzyme-linked immunosorbent assay (ELISA).

Antigen

N. caninum tachyzoites (strain NC-1) were maintained under the same conditions by continuous passage of HeLa cells at 37 °C and 5% CO_2 in RPMI medium supplemented with 25 mM HEPES, 2 mM L-glutamine, 3 mM sodium bicarbonate, and an antibiotic/antimycotic solution (penicillin 100 IU/mL, streptomycin 100 μg/mL and amphotericin B 0.25 g/mL; Gibco) (MINEO et al., 1980).

A suspension of the parasite was washed twice (720 × g, 10 minutes, 4 °C) in phosphate buffered saline (PBS 0.01 M, pH 7.2), treated with protease inhibitors (Complete, Roche) and then subjected to 10 cycles of freezing-thawing and sonication (60 Hz, amplitude of 90%, ice bath). After centrifugation (10 000 × g, 30 min, 4 °C), the supernatant was collected and filtered through a 0.22 m membrane (Millex, Millipore, USA). The supernatant, containing soluble *N. caninum* antigens (NLA), was collected and the concentration of protein was estimated using the Bradford assay. The aliquots were stored at -20 °C until completion of the assay.

Indirect ELISA

Indirect ELISA was used to detect IgG antibodies as described by Silva et al. (2007), with modifications. High affinity microtiter plates were coated with NLA (10 μg/ml) in 0.06 M carbonate (pH 9.6) overnight at 4 °C. The plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T) and blocked with PBS-T plus 5% MOLICO (PBS-TM) for 1 hour at room temperature. Subsequently, after three more washes, the plates were incubated with serum samples diluted in PBS-TM (1: 200) for 1 hour at 37 °C, with known seropositive and seronegative samples as the reaction control group. Plates were washed six times and incubated for 1 hour at 37 °C after addition of peroxidase-labeled IgG anti-bovine antibody, diluted in PBS-TM. The reaction was developed after a new wash cycle by adding the enzymatic substrate (0.03% H_2O_2) and chromogen (0.01 M 2,2-azino-bis-3-

ethyl-benzothiazolinesulfonic acid [ABTS: Sigma Chemical Co.]) in 0.07 M citrate-phosphate buffer (pH 4.2). Optical density (OD) was read at 405 nm after a 40 min incubation period on a plate reader (M2e, Molecular Devices, USA).

ELISA index values

The cutoff of the reaction was determined by the mean optical density (OD) with negative control serum with three deviations. Antibody titers were expressed by the ELISA index value, according to the formula IE = OD sample/OD cutoff, as described by Silva et al. (2007). Samples with ELISA index ≥ 1.2 were considered positive.

Statistical analysis

Using the binary logistic regression model in the SPSS program, the result (positive/negative) the output variable was selected as the dependent variable (binary) and as covariates, predictive variables: type of farming (dairy or mixed), total number of cows in the property (≤100/>100 animals), number of cows over 2 years old (≤100/>100 animals), total number of herd (≤100/>100 animals), type of milking (manual or mechanical), frequent purchase of animals and completion of quarantine, presence of birthing paddock (yes/no), and presence of reproductive problems such as abortion and repeat breeding (yes/no). The Hosmer-Lemeshow goodness of fit test was used to evaluate the model and compare the observed and expected frequencies.

Ethics Committee

The study was approved by the Ethics Committee on Animal Use (CEUA) of the Universidade Federal de Uberlândia under protocol no. 011/13.

Results

Seven hundred and forty blood samples were collected from the 57 properties used in the experiment. The properties were divided according to district: Martinésia (*n=98*), Cruzeiro dos Peixotos (*n=88*), rural perimeter of Uberlândia (*n=277*), Tapuirama (*n=64*), and Miraporanga (*n=213*). The seroprevalence for *N. caninum* was 18.4% (*136/740*) (Table 1), with prevalence in the districts ranging between 12.2% and 24.2%: 14.3% (*14/98*) in Martinésia, 20.5% (*18/88*) in Cruzeiro dos Peixotos, 24.2% (*67/277*) in the rural perimeter of Uberlândia, 17.2% (*11/64*) in Tapuirama and 12.2% (*26/213*) in Miraporanga.

Of the 12 factors analyzed (Table 1), five risk factors associated with seroprevalence of *N. caninum* were identified. Animals from properties with more than 100 cows over two years old had a threefold higher risk and the frequent purchase of animals was associated with a 3.8-fold higher risk. Individual histories of abortion increased the risk 12.8-fold and repeated breeding in animals by 5.9-fold. Manual milking was associated with a 49.4% lower risk than was mechanical milking (Table 2). All the properties had dogs in their premises.

Table 1. Percentage distribution of potential risk factors associated with seroprevalence for *Neospora caninum* in unvaccinated crossbred cows in the city of Uberlândia, Minas Gerais, Brazil, by the ELISA technique.

Variables	Animals tested	Positive	Seroprevalence (%)
Type of farm			
Dairy	583	111	19.0
Mixed	157	25	15.9
Total number of cows			
≤100	317	53	16.7
> 100	423	83	19.6
Total number of cows > 2 years old			
≤100	506	100	19.8
> 100	234	36	15.4
Total number of animals			
≤100	170	28	16.5
> 100	570	108	18.9
Type of milking			
Manual	380	61	16.1
Mechanical	360	75	20.8
Purchase of animals property			
No	69	15	21.7
Yes	671	121	18.0
Quarantine - property			
No	550	107	19.5
Yes	190	29	15.3
History of abortion- property			
No	266	42	15.8
Yes	474	94	19.8
Birthing paddock			
No	353	70	19.8
Yes	387	66	17.1
History of abortion- animal			
No	724	134	18.5
Yes	16	2	12.5
Repeated breeding - animal			
No	716	132	18.4
Yes	24	4	16.7

*Presence of dogs in 100% of the properties.

Table 2. Risk factors associated with seroprevalence for *Neospora caninum* in unvaccinated crossbred cows in the city of Uberlândia, Minas Gerais, Brazil.

Risk Factors	Coefficient	P-value	Exp (B) Odds ratio	95% C.I.
Constant	-6.857			
Females > 2 years old	1.113	0.034	3.042	(1.088-8.507)
Type of milking	-0.682	0.012	0.506	(0.296-0.863)
Purchase of animals	1.335	0.004	3.800	(1.545-9.345)
History of abortion - animal	2.552	0.007	12.830	(2.041-80.632)
Repeat breeding - animal	1.782	0.030	5.941	(1.192-29.608)

*Exp (B) - adjusted Odds ratio, with Exp (B) > 1 indicating that as the predictor increases, the probability of an outcome increases; a value less than 1 indicates that as the predictor increases, the likelihood of an outcome decreases.

Discussion

The region of the Triângulo Mineiro, where the city of Uberlandia is located, has an important role in beef and milk production. However, as with various regions in Brazil, there is a lack of epidemiological data relative to diseases of cattle. This is exacerbated in the case of diseases that affect the reproductive performance of herds. Consequently, this study constitutes an important contribution to the identification of the seroprevalence of neosporosis, using representative and well distributed sampling of the Uberlândia region, which has a cattle herd of 234 490 animals, 78 904 of which are cows (34 336 heifers between 12 and 24 months old and 28 244 heifers over 24 months old) according to data from the Minas Gerais Institute of Agriculture (IMA, 2012).

The seroprevalence in properties with a history of reproductive problems and mostly extensive breeding systems was 18.4% (*136/740*). This prevalence is similar to that revealed by surveys conducted in the state of Minas Gerais (COSTA et al., 2001; MINEO et al., 2006) and from other states of Brazil (BENETTI et al., 2009; CORBELLINI et al., 2006; MELO et al., 2006; RAGOZO et al., 2003; SOUSA et al., 2012). There are reports of higher prevalence in cows aged less than 24 months: 43.66% (*93/213*) and 47.97% (*154/321*) in two dairy herds in Minas Gerais (SANTOS et al., 2009), and 50.4% (*412/812*) in dairy cattle in the state of Maranhão (TEIXEIRA et al., 2010).

In other countries, higher seroprevalences of *N. caninum* were reported in dairy cattle than that determined in the present study, varying between 19.64% (GHALMI et al., 2012) and 35.2% (TALAFHA; AL-MAJALI, 2013); however, lower seroprevalences of 5.49% (KYAW et al., 2004) to 8.52% (LUCCHESE et al., 2016) were also reported.

The risk factors identified in this study, such as the number of cows over two years old, abortion history, and frequent purchase of animals were previously reported (GHALMI et al., 2012; SUN et al., 2015). The main clinical manifestation of cattle infected with *N. caninum* is abortion (HUSSIEN et al., 2012), which was also observed in this study, because the animals that presented a history of abortion had a 12.8 fold greater risk of infection. Although abortion is a clinical sign present in neosporosis, it may be due to other infectious causes, that could be either bacterial (e.g. *Brucella, Leptospira*) and/or viral (e.g. BoHV-1, BVDV) (LUCCHESE et al., 2016).

The fact that *N. caninum* seropositive animals may not present a history of abortion may be due to undetected abortions, possibly at the beginning of pregnancy, or due to embryo resorption. This reinforces the importance of other risk factors such as individual repeated breeding, which increased the risk 5.9 fold in this study. This clinical sign was associated with the seroprevalence of *N. caninum*, as was previously described in dairy cattle from southeastern Brazil (BRUHN et al., 2013). Dogs were constantly present on the properties, and could be infected due to the ingestion of tissue containing bradyzoites (DUBEY et al., 2007).

As with abortion, repeated breeding also has other infectious causes such as campylobacteriosis, trichomoniasis, and genital mycoplasmosis, as well as non-infectious causes such as trauma, genetic and hormonal disorders, and ovarian cysts (JUNQUEIRA; ALFIERI, 2006).

The presence of dogs is considered a risk factor, as they are the definitive hosts of *N. caninum* (DUBEY et al., 2007; GHALMI et al., 2012). The resistant stage of the parasite, the oocyst, is excreted in the feces of dogs unsporulated (LINDSAY et al., 1999; MCALLISTER et al., 1998). *N. caninum* is transmitted in cattle by horizontal transmission by means of ingestion of sporulated *N. caninum* oocysts (DUBEY et al., 2006).

In the present study, the presence of at least one dog as companion or working dogs was observed on all properties, which may have contributed to the prevalence of *N. caninum* on the farms. The

presence of dogs is common on properties in Brazil and there is little concern on the part of the owners, or even veterinarians; there is no specific legislation regarding the control or prohibition of access of these animals to cattle breeding grounds.

The frequent purchase of animals had a 3.8 fold higher risk when animals were imported from other farms, representing a more elevated likelihood of introducing infected animals on the property, as described by Schares et al. (2004). The proportion of recently purchased animals stood at 90.7% (671/740) of the properties, as this is a common practice among owners in this region; this was done without performing diagnostic tests and quarantine. In the present study, only 34.5% (190/550) of the cows came from quarantined properties.

Animals from properties with more than 100 cows that were more than two years of age had a threefold higher risk of being infected, which could be related to the constant presence of animals in milk production. Factors predisposing herds to infection by N. caninum were found in 19-30 month old cows (MELO et al., 2001), and these authors stated that there is greater productive pressure (more stress) in herds of A/B milk producers.

The fact that manual milking was associated with a 49.4% lower risk than was mechanical milking may be correlated to the hygiene conditions at small properties. Ghalmi et al. (2012) indicated that cattle populations that inhabited a poor environment had a higher risk associated with N. caninum infection than did populations inhabiting sites with good hygiene practices.

Important predisposing factors, such as the water quality on the property, were also described. Running water was reported as a possible source of infection for cattle (SUN et al., 2015) because, according to these authors, cattle could consume water contaminated with N. caninum oocysts from feces of infected dogs. Dogs can have access to drinking water and at the same time defecate within or near the water source.

In the present study, the origin of the water was not considered, because most properties used more than one water source. However, it should be emphasized that according to the Instituto Trata Brasil (OLIVEIRA, 2017), the municipality of Uberlândia ranks second with respect to sanitation among the 100 largest cities of Brazil, in terms of the evolution of the indicators water, sewage, investments, and water loss. According to the Municipal Department of water and sewage in Uberlândia (DMAE, 2010), the municipality has the capacity to treat 100% of the sewage collected, with 98% of the total produced sewage collected in Uberlândia, including the districts of Miraporanga, Tapuirama, Martinésia, Cruzeiro dos Peixotos, and the rural area.

Conclusion

It was concluded that the risk factors associated with N. caninum are the presence of cows older than two years, mechanical milking, and the frequent purchase of cattle, as well as an individual history of abortion and repeated breeding.

Acknowledgments

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