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Antibodies to *Neospora caninum* in sheep from slaughterhouses in the state of São Paulo, Brazil

Anticorpos para *Neospora caninum* em ovinos de abatedouros do estado de São Paulo, Brasil

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Abstract

Neosporosis is an emergent disease responsible for considerable economic impact due to reproductive losses. Its zoonotic potential remains unknown. This study involved a survey of antibodies to *Neospora caninum* in slaughtered sheep and their association with epidemiological variables. Serum samples from 596 sheep from the states of São Paulo and Rio Grande do Sul, Brazil, were collected in two slaughterhouses located in São Paulo and evaluated by indirect fluorescence antibody test (IFAT), using cut-off titers of 25. Among these samples, 353/596 (59.23%; 95%CI 55.23-63.10) were positive and 263/353 (74.50%; 95%CI 69.71-78.77%) were from Rio Grande do Sul. Statistical associations were determined in the univariate analysis between the serological results and sex, breed and municipality of origin. Sheep that came from extensive breeding system showed higher chance (OR=2.09) of presenting antibodies to *N. caninum* in relation to those from semi-intensive system. Higher chance was also observed for the different studied breeds, except Bergamácia, in relation to Hampshire Down. The results revealed the presence of infection by *N. caninum* in sheep from slaughterhouses.

Keywords: *Neospora caninum*, serology, sheep, slaughterhouses.

Resumo

A neosporose é uma doença emergente responsável por considerável impacto econômico devido a perdas reprodutivas e seu potencial zoonótico permanece desconhecido. Este estudo envolveu a pesquisa de anticorpos para *Neospora caninum* em ovinos abatidos e sua associação com variáveis epidemiológicas. Amostras de soro sanguíneo de 596 ovinos, procedentes dos estados de São Paulo e Rio Grande do Sul, Brasil, foram colhidas em dois abatedouros localizados em São Paulo, e avaliadas pelo teste de reação de imunofluorescência indireta (RIFI), utilizando-se como ponto de corte o título de 25. Dentre essas amostras, 353/596 (59,23%; IC95% 55,23-63,10) foram positivas e dentre os ovinos positivos 263/353 (74,50%; IC95% 69,71-78,77%) eram procedentes do Rio Grande do Sul. Associações estatísticas foram determinadas na análise univariada entre os resultados sorológicos e o sexo, raça e município de origem. Ovinos oriundos de sistema de criação extensivo demonstraram maior chance (OR=2.09) de apresentarem anticorpos para *N. caninum* em relação aos de sistema semi-intensivo. Uma maior chance também foi observada para as diferentes raças estudadas, exceto Bergamácia, em relação à raça Hampshire Down. Os resultados revelaram a presença de infecção por *N. caninum* em ovinos de abatedouros.

Palavras-chave: *Neospora caninum*, sorologia, ovinos, abatedouros.

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Introduction

Neospora caninum is a cosmopolitan obligate intracellular apicomplexan protozoan, whose economic importance is tied to reproductive losses in animals of zootechnical interest. Initially described in Norway (BJERKAS et al., 1984) as a coccidium similar to *Toxoplasma gondii*, the species *N. caninum* was only described (DUBEY et al., 1988a) and isolated four years later (DUBEY et al., 1988b). The domestic dog is the main definitive host of *N. caninum*, playing an important role in the transmission of infection by eliminating oocysts in its feces (MCALLISTER et al., 1998). In infected dogs, clinical neosporosis can occur consistently, causing neuromuscular disorders. Similar disorders have been reported in cattle, which are commonly affected by reproductive problems, such as epidemic or endemic abortion around the fifth month of pregnancy (WOUDA et al., 1999; DUBEY, 2003).

Different animal species have been reported as susceptible to *N. caninum* infection, including more recent reports of intermediate hosts of the species *Gallus gallus domesticus* (COSTA et al., 2008) and *Sminthopsis crassicaudata* (KING et al., 2011), as well as definitive canid hosts of the species *Canis lupus dingo* (KING et al., 2010) and *Canis lupus* (DUBEY & SCHARES, 2011). Serological evidence of human exposure to *N. caninum* has also been demonstrated in several studies (NAM et al., 1998; TRANAS et al., 1999; LOBATO et al., 2006); however, the zoonotic potential of the parasite has not been completely elucidated.

Neosporosis in sheep was first reported in 1990 (DUBEY et al., 1990), in a lamb born at term with clinical signs of neurological disease. In Brazil, there have been a few reports of natural infections of sheep by *N. caninum*. Given the importance of seroepidemiological surveys in evaluating the occurrence of infection in this species due to the expansion of sheep farming in the country, economic losses resulting from reproductive problems, and the need to elucidate doubts regarding infection in sheep, this study aimed to determine the presence of antibodies to *N. caninum* in sheep from slaughterhouses located in the state of São Paulo, Brazil, and their association with the epidemiological variables.

Materials and Methods

Origin of samples

The serum samples were collected from 596 sheep (*Ovis aries*) in two slaughterhouses located in the Midwestern region of the state of São Paulo, which process sheep from the states of São Paulo and Rio Grande do Sul, Brazil, regardless of sex, breed, age or origin. Data on the sheep's age, breed, sex, breeding system and origin were collected from the slaughterhouses. Blood samples were drawn by jugular venipuncture into sterile glass tubes sealed with rubber stoppers, which were sent to the Zoonosis Research Center (NUPEZO) of the Faculty of Veterinary Medicine and Animal Science (FMVZ) of São Paulo State University (UNESP). The samples were centrifuged at 1600 *g* for 10 min to obtain the serum, which was stored at -20 ºC until the serological tests were performed.

Antigen

The NC-1 strain of *Neospora caninum* was used as antigen, which was cultured in vitro in a VERO (African Green Monkey Kidney) cell line and RPMI 1640 (LGCbio®) medium supplemented with 10% antibiotic antimycotic solution (Vitrocell®) containing 100U.mL^{-1} of penicillin, 100 µg.mL^{-1} of streptomycin and 0.25 µg.mL^{-1} of amphotericin B. In the case of VERO cells that were not inoculated with NC-1 tachyzoites, the medium was supplemented with 10% sterile fetal bovine serum free of *Mycoplasma* (Cultilab®). The 25 cm^2 culture flasks were stored in an incubator at 37°C, in 5% CO_2 and under controlled humidity.

Indirect Fluorescence Antibody Test (IFAT)

Antibodies to *N. caninum* were surveyed using the indirect fluorescence antibody test (IFAT). This reference and gold standard test for detecting anti-*N. caninum* antibodies (BJÖRKMAN & UGGLA, 1999) was performed according to Dubey et al. (1988b). The serum samples were first screened using a dilution of 1:25 (DUBEY et al., 2005), the cut-off adopted, and positive samples were then titrated in duplicate dilutions of up to 1:400. Antibodies were detected using a commercial anti-IgG sheep conjugated to fluorescein isothiocyanate (Sigma-Aldrich®, USA). The slides were analyzed using a fluorescence optical microscope (Zeiss® SH250, Germany) equipped with a 40x lens, and the dilutions were considered positive when complete fluorescence was observed in at least 50% of tachyzoites (BJÖRKMAN & UGGLA, 1999). This was determined by the reactions of the positive and negative control sera used.

Data analysis

All the data were tabulated on an Excel spreadsheet. The serological results were associated with the epidemiological variables of origin (state and municipality) and sex, age, breed and breeding system, using univariate analysis by the Chi-square test (χ^2) and/or Fisher's exact test, adopting a significance level (α) of 5%.

Posteriorly, all the variables that were directly related to sheep that showed $P < 0.25$ in the univariate analysis were then included in the logistic regression model by multivariate analysis, excluding the variables that only described the location where the animals came from (state and municipality). The serological result was considered the dependent variable, positive or negative for *N. caninum*. Statistically significant difference was determined when the results showed $P<0.05$ in the univariate and multivariate analysis (TRIOLA, 2005; SOUZA et al., 2012). The statistical tests were performed with EpiInfo™ version 3.5.1 and IBM® SPSS® Statistics version 21 software.

Results

Table 1 lists the results of the survey of antibodies to *N. caninum* and Table 2 lists their association with the epidemiological variables, by univariate analysis. Among the tested samples, 353/596 (59.23%;

95%CI 55.23-63.10%) were positive, with titers ranging from 25 (129/353, 36.54%; 95%CI 31.69-41.69%) to 400 (8/353, 2.27%; 95%CI 1.17-4.40%).

Among the seropositive sheep, 74.50% (263/353; 95%CI 69.71-78.77%) came from Rio Grande do Sul. The highest percentage of seropositive sheep (25/30, 83.33%) was originated from the municipality of Pirajuí, in the state of São Paulo. Statistically significant differences were observed between the seropositivity and municipality (P=0.00) of origin.

Animals from farms using semi-intensive livestock breeding systems showed a lower percentage of seropositivity, with 27/60 (45.00%) positive sheep, compared to those from intensive (27/45, 60.00%) and extensive (299/491, 60.90%) breeding systems. Additionally, no statistically significant differences were observed between breeding system and seropositivity (P=0.06), but it was observed when extensive and semi-extensive systems were compared in the univariate analysis (P=0.02).

With regard to sex, ewes presented higher seropositivity, with 220/349 (63.04%), while 133/247 (53.84%) rams were positive. Significant associations (P=0.03; OR=1.46) were observed between seropositivity and sex in the univariate analysis. No significant differences were observed in relation to age (P=0.17) and all three sheep between 2 and 3 years of age were seropositive in the present study.

Table 1. Occurrence of positive sheep among the 596 animals of this study, based on neosporosis serological titers by IFAT.

IFAT (Titers)	Frequency	Percentage (%)	95%Confidence Interval
25	129	36.54	31.69 - 41.69
50	131	37.11	32.24 - 42.27
100	65	18.41	14.72 - 22.79
200	20	5.67	3.71 - 8.59
400	8	2.27	1.17 - 4.40
TOTAL	353	59.23	55.23 - 63.10

Table 2. Association (univariate analysis) between epidemiological data and serological test results for *Neospora caninum* infection by the indirect fluorescence antibody test (IFAT).

Variables		N1	IFAT2	Variable(%)*; 95CI%3	*P*4	OR (95CI%)5
State	Rio Grande do Sul	426	263	61.88[a]; 57.17 - 66.37	0.056	1.43 (0.98- 2.08)
	São Paulo	170	90	52.63[a]; 45.16 - 59.98		
Municipality	Holambra (SP)	1	0	0.00[d]; 0.00 - 0.00		
	Avaré (SP)	17	4	23.53[d]; 9.70 - 47.64		
	Manduri (SP)	27	8	29.63[d]; 15.88 - 48.67		
	Duartina (SP)	25	8	32.00[d]; 17.21 - 51.79		
	Itapira (SP)	3	1	33.33[bcd]; 6.76 - 80.59	0.007	-
	Ourinhos (SP)	15	6	40.00[cd]; 19.75 - 64.57		
	São Manuel (SP)	53	38	71.70[ab]; 58.36 - 82.02		
	Pirajuí (SP)	30	25	83.30[a]; 66.27 - 92.55		
	Bagé/Uruguaiana (RS)	75	40	53.33[c]; 42.13 - 64.21		
	Sant'Ana do Livramento (RS)	350	223	63.71[b]; 58.5 - 68.58		
Sex	Female	349	220	63.04[a]; 57.85 - 67.93	0.036	1.46 (1.05–2.04)
	Male	247	133	53.84[b]; 47.61 - 59.96		
Age	Up to 1 year old	407	243	59.71[a]; 54.87 - 64.36		
	1 < n ≤ 2 years old	74	48	64.87[a]; 53.46 - 74.78	0.177	-
	2 < n ≤ 3 years old	3	3	100.0[a]; -		
	No information	112	59	52.68[a]; 43.48 - 61.70		
Breed	Hampshire Down	17	4	23.53[b]; 9.70 - 47.64		
	Bergamácia	3	1	33.33[ab]; 6.76 - 80.59		
	Corriedale	104	56	53.85[a]; 44.28 - 63.13		
	Ille de France	104	57	54.81[a]; 45.22 - 64.05	0.027	-
	Ideal	175	111	63.43[a]; 56.06 - 70.21		
	Crossbreed	156	99	63.46[a]; 55.65 - 70.61		
	Santa Inês	37	25	67.57[a]; 51.35 - 80.37		
Breeding system	Semi-intensive	60	27	45.00[b]; 33.06 - 57.55		
	Intensive	45	27	60.00[ab]; 45.66 - 73.27	0.067	-
	Extensive	491	299	60.90[a]; 56.51 - 65.11		

[1]N: number of sampled animals; [2]Number of positive animals for antibodies to *Neospora caninum* by IFAT when titers were ≥ 25; [3]Frequency of reactive sheep based on the variable studied, at a 95% level of confidence; [4]*P* value for α = 5%; [5]OR: *Odds ratio*; [6]Fisher's exact test; [7]Chi-square test (χ^2); *Different superscript letters in the same column: P<0.05 in the analyzes of variables 2 by 2.

The Santa Ines breed showed the highest occurrence of antibodies, with 25/37 (67.57%) seropositive sheep, and statistically significant difference was determined in the univariate analysis in relation to breed (P=0.02). Most breeds presented statistically significant differences in the seropositivity in relation to Hampshire Down breed in the univariate analysis and in the multivariate logistic regression (P<0.05; OR>1.00) (Table 3), except Bergamácia (P=0.36). Additionally, the multivariate analysis confirmed the significant differences observed in the univariate analysis between the semi-intensive and extensive systems (P=0.02; OR=2.09).

Discussion

Neospora caninum infection has been reported in different regions of the world and it is currently observed on all five continents (DUBEY, 1999). Seroepidemiological studies in sheep are scarce, with seropositivity (titers ≥ 50) of 0.45% (3/660) reported in England (HELMICK et al., 2002), while the prevalence in Brazil ranges from 1.8% in the state of Rio Grande do Norte (SOARES et al., 2009) to 64.2% in Pernambuco (TEMBUE et al., 2011).

The seropositivity determined in this study was 59.23%, with titers ≥ 25, differing significantly from those of 8.0% and 12.8% previously reported by Machado et al. (2011) and Langoni et al. (2011), respectively, in the state of São Paulo and using the same cut-off. Some studies in Brazil have reported low percentages of seropositive sheep with titers ≥ 50, as 8.1% (SALABERRY et al., 2010) in Minas Gerais, 9.2% (FIGLIUOLO et al., 2004) and 13.9% (MUNHÓZ et al., 2010) in São Paulo and 9.5% (ROMANELLI et al., 2007) in Paraná. In this study, considering as cut-off the titer of 50, the seropositivity decrease to 37.58% (224/596; 95%CI 33.79-41.45%), but stills remains higher than the other studies cited above. However, a high prevalence has been reported in the southeastern region, e.g., by Rossi et al. (2011), who described 78.0% (titers ≥ 50) in the state of Minas Gerais.

Comparisons of percentages of seropositivity should be cautious, because numerous differences may occur between studies, as a result of different sample sizes, laboratory equipment, cut-off points, and test sensitivity and specificity (BJÖRKMAN & UGGLA, 1999; MODOLO et al., 2008). In addition, factors intrinsic to the technique, such as the specific characteristics of the secondary antibody, alterations in the conditions of antigen-antibody reaction, the diversity of antigen preparations and the degree of subjectivity of the IFAT results, given that the interpretation of serological reactions is performed visually (BJÖRKMAN & UGGLA, 1999). Furthermore, larger numbers of seropositive sheep may be detected in studies using samples from problem herds (VOGEL et al., 2006) and although *N. caninum* is closely related to *Toxoplasma gondii*, *Sarcocystis* spp.,and other apicomplexans, cross-reactivity has not been a major issue in serologic assays (DUBEY & LINDSAY, 2006).

Higher occurrence of seropositive sheep were observed in this study in the state of Rio Grande do Sul, in strong contrast to the study by Vogel et al. (2006), who found a prevalence of only 3.2% in sheep by ELISA. The municipalities with higher occurrences of seropositive sheep (Bagé/Uruguaiana, Sant'Ana do Livramento, São Manuel and Pirajuí) were statistically significant different from the other studided municipalities and from each other when analyzed in pairs (2×2) in the univariate analysis, except São Manuel and Sant'Ana do Livramento. These results may be ascribed to the different farming systems adopted in different states and municipalities, as well as the presence of domestic and wild canids and other risk factors to *N. caninum* infections.

The occurrence of infections by *N. caninum* is not influenced by breed, age or the number of animals in the herd (BARBER, 1998; BECK et al., 2010) and no evidence of any association was observed between these variables and the seropositivity in the studies conducted by Figliuolo et al. (2004), Romanelli et al. (2007), Soares et al. (2009), Ueno et al. (2009), Salaberry et al. (2010) and Rossi et al. (2011). However, in this study, a statistically significant difference was found between the serological results and breed, but not age. Although the percentage of positive sheep sampled increased from the lowest to the highest age, no statistically significant difference was determined. Furthermore, because it is a cross-sectional study, the serological results refer to the age at which sheep were slaughtered, not infected.

Ewes presented more frequent positive serological reactions than rams, which is similar to the findings of Ueno et al. (2009), but in disagreement with those of Soares et al. (2009). In the present study, ewes showed a 1.46-fold greater chance of presenting antibodies to *N. caninum*, without taking into account the risk factors to which they may have been exposed.

Farms where intensive or semi-intensive breeding systems are adopted usually have lower occurrences of neosporosis, in view of their better sanitation, separation of the sheep from the herd according to age, sex and production purpose, as well as their more skilled workforce (BARLING et al., 2001). On the other hand, extensive breeding can expose sheep to greater contact with canids, the definitive hosts (DUBEY et al., 2007).

Table 3. Multivariate logistic regression analysis of epidemiological variables.

Variable		OR (95%CI)1	*P*2
Sex	Male	1.00; -	-
	Female	1.23 (0.84-1.81)	0.28
Age	Up to 1 year old	1.00; -	-
	1 < n ≤ 3 years old*	1.11 (0.65 – 1.89)	0.69
	No information	0.88 (0.55-1.42)	0.61
Breed	Hampshire Down	1.00; -	-
	Bergamácia	3.57 (0.23-54.49)	0.36
	Corriedale	3.81 (1.13-12.86)	0.03**
	Ille de France	4.71 (1.41-15.69)	0.01**
	Ideal	4.99 (1.52-16.41)	0.01**
	Crossbreed	6.01 (1.83-19.72)	0.00**
	Santa Inês	8.91 (2.25-35.24)	0.00**
Breeding System	Semi-Intensive	1.00; -	-
	Intensive	1.66 (0.69-3.95)	0.25
	Extensive	2.09 (1.11-3.93)	0.02**

[1]*Odds Ratio* (95% confidence interval); [2]*P* value for α = 5%; *Due to the small number of sheep between 2 < n ≤ 3 years old (N = 3), this age group was included in a single group with sheep between 1 < n ≤ 2 years old; **Statistically significant difference (P<0.05).

Nevertheless, a higher seropositivity was observed in this study in sheep from breeding systems with distinct characteristics, extensive and intensive, and no statistically significant difference was determined between seropositivity and breeding system. However, analyzing the breeding systems in pairs (2×2) in the univariate analysis, the seropositivity in sheep from extensive breeding system was statistically significant different from those of semi-intensive system, with a 2.09-fold greater chance of presenting serological evidence of infection by *N. caninum*, demonstred by the multivariate logistic regression.

This results indicates that exposure of sheep to different epidemiological conditions and risk factors for *N. caninum* infections can occur in the different breeding systems, although it is known that the extensive system can expose sheep to risk factors more frequently and the adoption of sanitary measures may be necessary to control and prevent *N. caninum* infections.

Sheep of the Santa Ines breed presented higher seropositivity, though similar values were observed in crossbred sheep and in the Ideal breed. It is known that sheep from these breeds were mostly raised in the extensive breeding systems of Sant'Ana do Livramento, a municipality in the state of Rio Grande do Sul, where a higher percentage of seropositive sheep was determined.

On the other hand, sheep of the Hampshire Down breed presented lowest seropositivity, and the other studied breeds, except Bergamácia, showed significant differences in the serological results in relation to this breed, with higher chance (OR>1.00) of presenting antibodies to *N. caninum*. Sheep of Santa Inês breed presented 8.91-fold greater chance of presenting positive serological results in relation to Hampshire Down.

Cross-sectional studies may reflect the distribution of a disease and other relevant conditions, including biological factors in the population (GREINER & GARDNER, 2000). Thus, a statistical association was determined in this study between the serological results and epidemiological variables sex, breed and municipality of origin. However, it is noteworthy that risk factors for *N. caninum* infection can only be determined using a study design that includes the systematic collection of samples and epidemiological information about the herd.

Conclusions

An analysis of the findings revealed serological evidence of *N. caninum* infection in sheep herds from the states of São Paulo and Rio Grande do Sul, Brazil, which were destined for slaughter. Statistical associations were determined between the serological results and certain epidemiological variables. Sheep that came from extensive breeding system showed higher chance of presenting antibodies to *N. caninum* in relation to those from semi-intensive system. Higher chance was also observed for the different studied breeds, except Bergamácia, in relation to Hampshire Down. However, it is noteworthy that risk factors for *N. caninum* infection can only be determined using another study design. Evidence of the occurrence of neosporosis in humans has not yet been proven, but seroconversion in this species has been reported and additional studies involving animals destined for slaughter are particularly important for public health, given the relatively little information available about *N. caninum* infection in sheep and the fact that their meat is intended for human consumption. Furthermore, studies to investigate the possible zoonotic potential of neosporosis are equally important.

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