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Risk factors associated with seroprevalence of *Neospora caninum* in dogs from urban and rural areas of milk and coffee production in Minas Gerais state, Brazil

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SUMMARY

This study was conducted to determine the seroprevalence of anti-*Neospora caninum* antibodies and to investigate the risk factors related to seroprevalence in dogs from urban and rural areas with distinct economic activities (milk and coffee production) in Minas Gerais state, Brazil. For this purpose, blood samples from 703 dogs were collected and questionnaires addressing epidemiological aspects were completed by dog-owners. The sera were analysed for anti-*N. caninum* antibodies by indirect fluorescent antibody tests (IFAT $\geqslant$ 1:50). Association between epidemiological aspects and seropositivity in dogs was evaluated with multivariate logistic regression models. A total of 80 (11·4%) dogs tested positive for *N. caninum*. In the multivariate logistic regression models, dogs aged >4 years, dogs used as guard dogs, dogs that spontaneously hunt, and history of bovine abortion were found to be greater risk factors for canine *N. caninum* infection. When we considered only dogs from rural areas, an association with seroprevalence was seen for milk farms, dogs not fed with commercial food, dogs that hunt, and dogs used as guard dogs.

Key words: Canine, dairy farms, indirect fluorescent antibody test, risk factors, seroepidemiology.

INTRODUCTION

Neospora caninum is an apicomplexan protozoan of the family Sarcocystidae [1]. Its definitive hosts are dogs (*Canis familiaris*) [1], coyotes (*Canis latrans*) [2], dingoes (*Canis lupus dingo*) [3] and grey wolves (*Canis lupus*) [4].

Neosporosis is known worldwide as one of the principal causes of abortion in cattle [1]. In dogs, the disease is transmitted by vertical and horizontal infection [5]. Studies conducted in Brazil, using different serological techniques, have demonstrated the presence of anti-*N. caninum* antibodies in dogs from different states, with considerable differences in the seroprevalence between the diverse population groups (pet dogs, stray dogs, dogs treated at veterinary clinics/hospitals, dogs from rural areas), varying between 3·1% and 67·6% [6]. In Minas Gerais state the presence of the parasite has been demonstrated immunohistochemically or by PCR in bovine fetuses [7] from milk farms and in caprines [8].

Some authors (e.g. [9]) suggest that prevalence can vary between regions and within the same region since the different populations can be exposed to several risk factors. Although studies conducted in

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Brazil have indicated that dogs in rural regions exhibit greater seroprevalence compared to those in urban areas [10], until now no research has compared the seroprevalence of different rural environments with distinct economic activities. In this context, the objective of this study was to determine the prevalence of anti-*N. caninum* antibodies in dogs in urban and rural areas from two microregions in Minas Gerais state: one mainly occupied by dairy farms (Lavras) and the other by coffee farms (Varginha), in order to analyse whether type of production, environment or other factors are important in terms of maintaining the disease.

In the microregion of Lavras, dairy cattle are reared mainly under intensive farming conditions (totally confined) or semi-extensive (grazing during part of the day and receiving some of their food and care in barns). The dogs are largely left to roam freely, as companions or guard dogs, with easy access to cattle, placentas and fetuses. Another important factor is that neosporosis may be maintained at a farm through transplacental transmission, since older cows are generally replaced with calves originating from the same herd.

By contrast, in the microregion of Varginha the study was conducted in farms which predominantly grow coffee and in a few farms that keep small numbers of beef cattle reared extensively. However, it seems that the significance of these bovines in the maintenance of a dog's seroprevalence to *N. caninum* is low due to bovine density and because these animals are mainly male which are reared in a growing and finishing system. There are probably other important factors, such as reports of various species of wild rodents and marsupials of nocturnal habits in coffee plantations [11]. Moreover, in some coffee farms, a small number of dairy cows may be present to sustain the family. Most of the dogs roam freely as working dogs or companions.

METHODS

Study area

This study was conducted in two microregions in the state of Minas Gerais, Brazil: Lavras and Varginha. Minas Gerais state, located in the southeastern region of Brazil, is currently the largest milk and coffee producer in the country [12]. The microregion of Lavras, which has a total area of 3430·72 km^2, produces ~123 million litres of milk per year, whereas the microregion of Varginha, which has a total area of 7599·36 km^2, is an important coffee area, producing ~157 000 tons of coffee per year. A few head of cattle can be found in Varginha microregion [12].

The study area was selected because there is currently no available information about the risk factors and seroprevalence of dogs in rural areas. Moreover, the number of diagnosed bovine abortions caused by *N. caninum* at dairy farms has increased considerably [7].

Urban dogs were included in this study to compare their seroprevalence in relation to rural dogs, since some risk factors for *N. caninum* infection are mostly associated with rural environments [10].

Sampling

The dog sample number was based on the human population of the two regions under study, which is around 580000 inhabitants. For the dog:human ratio the proportion of one dog to ten inhabitants was used, based on national indicators for the canine population used in anti-rabies vaccination campaigns [13], giving an estimated total of 58 000 dogs. The sample size was calculated [14] using the Statcalc Epi Info program, version 7.0, with a 95% confidence interval (CI), a maximum error of 5%, a power of 80%, an expected odds ratio (OR) of 2·5 and an expected prevalence of 6% (not exposed dogs), in accordance with previous studies [15]. This resulted in a total of 551 dogs to be tested in the two microregions. However, a total of 703 blood samples were collected by puncture of the cephalic or jugular vein, representing 500 dogs from Lavras and 203 dogs from Varginha. The dogs were selected randomly from a subset of the population which was selected based on convenience. The samples were collected during the anti-rabies vaccination campaign for urban and rural dogs and despite the different sample size between the microregions the number of animals was representative of each region.

Questionnaire

During the blood collection, face-to-face interviews were conducted with all property owners and a questionnaire was completed which contained the variables relating to the animal and the property which could be associated with infection by *N. caninum*. The information collected comprised: gender; age; breed; place in which the animal was kept (urban, rural, or urban with temporary access to rural area during weekends); type of food (home-made food, commercial feed or raw bovine meat from the property itself or butcher/bovine placenta waste/aborted fetuses); constraint status (always constrained, always free-ranging or free-ranging for temporary periods); function of dog (companion, guard dog, herding, or dogs that according to the owner frequently and spontaneously hunt small mammals and birds); whether or not the dog had previously suffered neuromuscular disease; whether or not the animal was under immunosuppressive treatment (corticotherapy or chemotherapy) or suffering from a concomitant immunosuppressive disease; whether or not the dog had previously suffered or was currently suffering from any reproductive disturbance (abortions, stillbirths, weak litter or infertility); the predominant activity of the rural property (dairy cattle farming, beef cattle farming, coffee crop planting, livestock farming of other species); and whether or not there was a history of bovine abortion at the farm and how the aborted fetuses and placentas were disposed of.

For some variables it was not possible to obtain information or a history of the dogs (e.g. stray dogs cared for at veterinary clinics or adopted by an animal protection shelter) or else these questions were not answered by the property owners. In these cases, the animals were excluded from the study and analysis was performed only with the data on the dogs for which information could be obtained.

Indirect fluorescent antibody test (IFAT)

The sera were tested for *N. caninum* antibodies with an IFAT, using the NC-1 parasite strain [1]. As a secondary antibody a commercial FITC-labelled anti-dog IgG (Sigma-Aldrich, USA) was used. Positive and negative controls were applied. The sera were tested in twofold dilutions, starting at 1:50 [9, 10, 16]. Samples which showed complete peripheral fluorescence of the tachyzoites were considered to be positive. The cut-off dilution of 1:50 in the IFAT has a sensitivity of 80% and a specificity of 85% [16].

Statistical analysis

For statistical analysis, the database was developed in Epidata v. 3.1 (Epidata Association, Denmark) and the analysis was performed in SPSS v. 17.0 and PASW v. 18.0 (IBM, USA).

A descriptive analysis of all of the variables was performed. It analysed the diference of general seroprevalence between the two microregions and considered the animals in the urban and rural area by χ^2 test. Associations between seropositivity for *N. caninum* in dogs and the variables studied (included in the questionnaire) were evaluated by χ^2 test or Fisher's exact test (fewer than five observations in at least one cell in the contingency table). For the significant variables ($P<0{\cdot}05$), ORs were calculated with their 95% CIs. Variables that presented $P\leqslant 0{\cdot}2$ according to χ^2 or Fisher's exact test were selected for building the multivariate models by means of logistic regression with stepwise elimination (backward conditional method; PASW v. 18.0) with the aim of ascertaining the adjusted ORs.

Aiming to clarify the relationship of 'productive rural environments' (milk production, coffee planting and/or beef cattle) as determinant factors of the variables presented in the questionnaire and to control their confounding effects, the association between 'productive rural environments' and each of the variables studied was analysed by χ^2 or Fisher's exact test. Further, the existence of interaction in seroprevalence between dogs from the different rural areas was analysed. Then, multivariate logistic regression models were tested for seroprevalence of *N. caninum* in dogs stratified by 'productive rural environment', using the following variables: 'dogs originating from dairy farms', 'other farms than dairy cattle (coffee planting and/or beef cattle)' and 'all rural properties' to check the effect of the productive environment in seroprevalence. For these analyses only dogs from properties considered 'rural' were used ($n=421$).

Moreover, in the adjusted model for rural stratum and dairy farms, the dog's age was analysed as a confounding or as an effect modifier variable by stratification technique [17].

Ethical approval

The study was approved by the Bioethics Committee for Utilization of Animals of the Federal University of Lavras, Minas Gerais, Brazil.

RESULTS

Anti-*N. caninum* antibodies were found in 80/703 (11·4%) dogs, with positive animals showing titres which varied between 50 and 6400 (Table 1). There was no significant difference between general

Table 1. *Distribution of titres of anti-*Neospora caninum *antibodies detected by indirect fluorescent antibody test (IFAT ⩾ 1:50) in dogs from Minas Gerais state, Brazil*

Titres	*n* (positive samples)	%
50	23	28·75
100	14	17·5
200	18	22·5
400	12	15·0
800	3	3·75
1600	6	7·5
3200	2	2·5
6400	2	2·5
Total	80*	100·0

* Prevalence: 11·4% (80/703).

Table 2. *Prevalence of anti-*Neospora caninum *antibodies detected by indirect fluorescent antibody test (IFAT ⩾ 1:50) in dogs from urban, rural, and urban with access to rural areas from Minas Gerais, Brazil*

Area	Total sampling	Positive		Negative	
		n	%	*n*	%
Urban	182	15	8·24[a]	167	91·76
Rural	421	58	13·78[b]	363	86·22
Urban with access to rural	19	0	0[a,b]	19	100
Total	622	73	11·74	549	88·26

n, Number of animals.
[a,b]*Neospora caninum*-positive serology percentages with different superscripts are significantly different ($P<0.05$).

seroprevalence in animals from Lavras (59/500, 11·8%) and Varginha (21/203, 10·34%) ($P>0·05$).

Of the 703 dogs tested, 11·5% ($n=81$) were stray dogs, 25·9% ($n=182$) came from the urban area (112 from Lavras, 70 from Varginha), 59·9% ($n=421$) came from the rural (not urbanized or country town) region (291 from the milk production region and 130 from the coffee-growing region) and 2·7% ($n=19$) came from the urban area with access to rural areas (Table 2).

Dogs from the rural region demonstrated a greater risk of seropositivity than those from the purely urban region (OR 1·779, 95% CI 0·980–3·230, $P=0·05$). Of the animals which tested positive, 79·5% were from the rural region.

The results of the analyses for prevalence of anti-*N. caninum* antibodies and each variable related to the animals are given in Table 3. It was not possible to obtain complete datasets for all dogs and because of this the number of data points used varies widely in this table. Of the seropositive animals, dogs aged >4 years (OR 3·436, 95% CI 2·082–5·673, $P<0·001$), dogs with a history of reproductive dysfunctions (OR 2·607, 95% CI 1·177–5·775, $P=0·015$), and dogs which were not fed on commercial feed (OR 2·534, 95% CI 1·543–4·161, $P<0·001$) had a greater chance of infection by *N. caninum.*

Animals used as guard dogs (OR 2·905, 95% CI 1·768–4·773, $P<0·001$) or that spontaneously hunted small mammals and birds (OR 4·902, 95% CI 2·230–10·779, $P<0·001$) were at higher risk of infection compared to those which were not used for these purposes or did not have this habit. Animals which were not described by their owners as companions had a greater risk of infection (OR 2·010, 95% CI 1·155–3·500, $P=0·012$). Dogs which had contact (or the possibility of contact) with and/or ingested aborted fetuses or placentas had a 3·2-fold greater chance of being infected by *N. caninum* (OR 3·218, 95% CI 1·865–5·551, $P<0·001$) (Table 3). According to the property owners, all animals received treated water and contact with other water sources was not possible.

Dogs from rural properties where dairy cattle farming was carried out (OR 3·460, 95% CI 1·189–10·069, $P=0·016$) or farms which had a history of bovine abortion (OR 3·087, 95% CI 1·468–6·491, $P=0·002$) were found to have a higher risk of infection by *N. caninum*, whereas dogs from properties where beef cattle farming or coffee cultivation were performed showed no significant association with seropositivity ($P>0·05$) (Table 3).

In the multivariate analysis, the dog's age (>4 years old), dogs that spontaneously hunt, dogs used as guard dogs, and dogs from rural properties with a history of bovine abortion had a significant association ($P \leqslant 0·05$) with seropositivity (Table 4).

The comparison between seroprevalence in dogs from three different rural environments (dairy cattle, beef cattle, coffee crops) ($n=421$) revealed a statistical difference only between dogs from properties which were predominantly dairy farms compared to other environments (beef cattle, crops) (OR 3·396, 95% CI 1·163–9·911, $P=0·018$).

Considering the 'productive rural environments' as a dependent variable and the others as independent

Table 3. *Factors associated with seroprevalence of* Neospora caninum *by univariate* χ^2 *test in dogs from Minas Gerais, Brazil* ($P \leqslant 0{\cdot}05$)

	IFAT: *Neospora caninum*						
	Positive		Negative				
Factors	*n*	%	*n*	%	OR	95% CI	*P*
Age (*N* = 620)							<0·001
⩽4 years	30	7·77	386	92·23	1		
>4 years	43	21·08	161	78·92	3·436	2·082–5·673	
History of reproductive disturbance (*N* = 611)							0·015
Yes	9	24·32	28	75·68	2·607	1·177–5·775	
No	63	10·98	511	89·02	1		
Type of food (*N* = 610)							
Commercial feed							<0·001
Yes	37	8·71	388	91·29	1		
No	36	19·46	149	80·54	2·534	1·543–4·161	
Function of dog (*N* = 617)							
Companion							0·012
Yes	52	10·30	453	89·70	1		
No	21	18·75	91	81·25	2·010	1·155–3·500	
Guard							<0·001
Yes	38	20·43	148	79·57	2·905	1·768–4·773	
No	35	8·12	396	91·88	1		
Dog that spontaneously hunts							<0·001
Yes	11	36·67	19	63·33	4·902	2·230–10·779	
No	62	10·56	525	89·44	1		
Possibility of contact with/ingestion of aborted fetuses/placenta (*N* = 583)							<0·001
Yes	46	18·04	209	81·96	3·218	1·865–5·551	
No	21	6·40	307	93·60	1		
Activity of the rural property (*N* = 276)							
Dairy cattle farming							0·016
Yes	42	19·53	173	80·47	3·460	1·189–10·069	
No	4	6·56	57	93·44	1		
History of bovine abortion (*N* = 202)							0·002
Yes	22	28·21	56	71·79	3·087	1·468–6·491	
No	14	11·29	110	88·71	1		

IFAT, Indirect fluorescent antibody test; OR, odds ratio; CI, confidence interval.

verified the association between dairy farms and certain variables ($P \leqslant 0{\cdot}05$), such as 'fed on commercial feed' (OR 0·333, 95% CI 0·165–0·670), 'fed on homemade food' (OR 0·309, 95% CI 0·091–1·050), 'contact with placenta and aborted bovine fetuses' (OR 3·419, 95% CI 1·822–6·414) and 'history of bovine abortion' (OR 0·126, 95% CI 0·027–0·603).

Regarding the analysis stratified by rural productive environments without considering the dog's age, it was observed that the stratum dairy farms in the adjusted model demonstrated that dogs that hunt and those that were not fed with commercial feed have a greater chance of being seropositive to *N. caninum* (Table 5).

When we considered all the rural farms in the adjusted model, it was noted that the factors which increased the chance of seropositivity were only: 'dogs that spontaneously hunt small mammals and birds', 'dogs used as guard dogs' and 'dogs from dairy cattle farms' (Table 5).

When considering the dog's age in the multivariate model and the same variables as in Table 5, it was observed that only 'age' and 'not fed with commercial feed' demonstrated association with *N. caninum* ($P < 0{\cdot}05$) seropositivity. The analysis showed that

Table 4. *Final multivariate logistic regression for risk factor to* Neospora caninum *infection in dogs from Minas Gerais, Brazil* ($P \leqslant 0{\cdot}05$)

Factors	OR	95% CI	*P*
Dog used as guard dog (*n* = 186)	2·840	1·181–6·831	0·020
Dog that spontaneously hunts (*n* = 30)	4·436	1·239–15·873	0·022
History of bovine abortion (*n* = 78)	3·301	1·462–7·456	0·004
Dog aged >4 years (*n* = 204)	2·820	1·256–6·332	0·012

OR, Odds ratio; CI, confidence interval.
Goodness-of-fit: $\chi^2 = 3{\cdot}498$, $P = 0{\cdot}624$.
Variables tested in the model ($P \geqslant 0{\cdot}05$): 'fed on commercial feed', 'use of dog as a companion', 'possibility of contact with/ingestion of aborted fetuses/placenta', 'dairy farm', 'beef cattle farm'.

Table 5. *Adjusted analysis of the possible factors associated with seropositivity for* Neospora caninum *in dogs from rural areas in Minas Gerais state, Brazil stratified by rural environments and by dairy farms*

Stratum	Associated factors	aOR	95% CI*	*P* value*
Dairy cattle farms	Dog not fed with commercial feed (*n* = 95)	2·110	1·004–4·484	0·051
	Dog that spontaneously hunts (*n* = 16)	4·449	1·420–13·943	0·010
All rural properties	Dairy cattle farming (*n* = 207)	3·471	0·999–12·051	0·050
(dairy cattle, beef cattle,	Dog used as guard dog (*n* = 159)	3·385	1·168–4·871	0·017
coffee planting farms)	Dog that spontaneously hunts (*n* = 29)	5·807	1·987–16·975	0·001

aOR, Adjusted odds ratio; CI, confidence interval.
Goodness-of-fit (dairy cattle farms): $\chi^2 = 0{\cdot}563$, $P = 0{\cdot}905$.
Goodness-of-fit (all rural properties): $\chi^2 = 2{\cdot}972$, $P = 0{\cdot}396$.
* Multivariate logistic regression.
Variables evaluated in the model ($P > 0{\cdot}05$): 'possibility of contact with/ingestion of aborted fetuses/placenta', 'beef cattle farm', 'use of dog as a companion', 'dog used as guard dog'.

age should be considered an effect modifier variable, since it is not associated with other variables included in the model, and the variables from Table 5 showed differentiated seropositivity by age stratum. Next, besides maintaining Table 5 which demonstrates significant associations with the function of the dog and dairy farms, a new multivariate analysis stratified by the dog's age was performed. The results found were (1) for dogs up to age 2 years there were no associated factors; (2) for dogs aged 2–4 years only animals not fed with commercial food showed an association (OR 6·667, 95% CI 1·541–28·571, $P = 0{\cdot}011$); and (3) for dogs aged >4 years only dogs that spontaneously hunt demonstrated an association (OR 5·430, 95% CI 1·198–24·606, $P = 0{\cdot}028$).

DISCUSSION

Most of the animals which tested positive came from the rural region. Several studies have also demonstrated that seroprevalence is greater in dogs from rural areas than from urban areas [10, 18]. This can be explained by the greater exposure of these animals to protozoans, since they hunt small mammals, wild birds and chickens which are potential intermediary hosts for these parasites, and they also have more chance of ingesting environmental oocytes or cysts present in bovine tissue.

Since most of the dogs roam freely in both microregions, they may have contact with those intermediary hosts, which could explain the similar general seroprevalence in both regions. Moreover, in Lavras there is a greater density of dairy cattle, increasing the chance of ingesting aborted fetuses and placentas. By contrast, in coffee farms the presence of various species of wild rodents and marsupials of nocturnal habits have been reported [11]. The importance of wild animals in the life cycle of *N. caninum* has long been described in the literature [19–21]. However, it was not possible to investigate the influence of wild animals in seroprevalence in this study, although the presence of those animals may have contributed to the occurrence of the risk factor 'dogs that spontaneously hunt' in all models and strata.

In this study, dogs which are seropositive tend to be aged >4 years. The role of age in seropositivity suggests that most dogs acquire the infection during the postnatal period, i.e. horizontal transmission is more important than vertical transmission in the canine population [22]. Further, dogs with a history of reproductive diseases have a 2·6-fold greater chance of being seropositive. Some authors have noted that *N. caninum* can cause reproductive dysfunctions in dogs, including fetal death, mummification, reabsorption, abortion, stillbirth and the birth of weak litters [23].

When the function of the dog was analysed, those animals which were used as guard dogs or dogs that hunt had the greatest seroprevalence ($P \leqslant 0{\cdot}05$). This study indicates that the association of dog's age, especially with hunting habit is a very important risk factor for *N. caninum*. It was observed that younger dogs depend on the quality of the food provided by their owners. By contrast, older dogs have more opportunities for contact with intermediate hosts of *N. caninum*. Further, most of the hunting dogs and guard dogs are free-ranging and have contact in the field with carcasses which are frequently eviscerated [24].

Regarding the type of feed provided, dogs which did not receive commercial feed showed a 2·5-fold greater risk than those which did receive this type of feed, according to univariate analysis. Some studies have demonstrated that the ingestion of raw or undercooked meat can increase the chance of intake of tachyzoites or cysts present in the tissue of intermediary hosts [25]. In addition, the dogs whose owners reported the possibility of contact with or ingestion of fetuses or placental waste showed no significant association with the seropositivity of *N. caninum* in the multivariate analysis. This may be related to the fact that the real number of dogs which ingest this material could be underestimated owing to the low level of attention paid by most owners to dogs left to roam freely in the rural areas analysed.

The findings of this study indicate that dairy cattle farms, mainly those with a history of bovine abortion are indicators of a risk of neosporosis in dogs (Tables 3–5). From the analysis of the adjusted data, it was observed that the risk of being seropositive in dogs was highest for those from dairy farms and lowest for those from coffee farms (Table 5). Although the sample size of the dogs originating from beef cattle farms was small, the statistical difference observed may be attributable to the fact that the rearing methods and bovine management approaches differ greatly in the regions in question. On the beef cattle farms the system of rearing is predominantly the complete cycle type (rearing, growing, finishing) or growing and finishing, in an extensive manner (animals, mainly male, free to roam on large areas of land). By contrast, cattle from dairy farms are kept in an intensive form (where neosporosis is maintained in the bovine animals through transplacental transmission), which allows the dogs to have closer contact with the cattle and, consequently, with the placentas or fetuses originating from infected cows.

Analysing dichotomically the type of exclusive use of property, no significant difference was found ($P > 0{\cdot}05$). However, seroprevalence of dogs from dairy farms was higher than in dogs from coffee farms. This result needs to be investigated in greater depth since it may have occurred because of the number of mixed farms in the region, which reduced the number of properties in each stratum investigated.

The dogs on dairy farms show a greater tendency to be infected by *N. caninum*. The wild Canidae which have been shown to be definitive hosts for *N. caninum* are not found in Brazil [26], but wild mammals or synanthropes, like rodents [19] or rabbits [20], or wild [21] and domestic [27] birds, can be considered as intermediate hosts and a potential source of infection for dogs. In this regard, both environments are exposed to these intermediate hosts, i.e. it is likely that the variation in seroprevalence between the rural dogs of the dairy farms and properties carrying out other rural activities is caused by the close association between cattle and dogs and by the access of the former to sources of infection (aborted fetuses, placentas). However, the role of domestic and wild birds as well as small mammals as sources of infection in dogs cannot be completely discarded especially on coffee farms.

The seroprevalence found in this study was greater in dogs from rural areas than from urban areas. This finding suggests that bovines from milk farms with a history of bovine abortion are important for the horizontal transmission of *N. caninum* and its maintenance within the canine population, mainly when dogs are left to roam freely and are not fed with commercial feed. Moreover, dogs that spontaneously hunt were shown to be an important risk factor in all models and strata. Further investigation should be performed to evaluate the participation of Brazilian wildlife in the life cycle of *N. caninum*.

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DECLARATION OF INTEREST

None.

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