

Neospora caninum in free-range chickens (*Gallus gallus domesticus*) from southern Brazil

Neospora caninum em galinhas de vida livre (*Gallus gallus domesticus*) do sul do Brasil

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Abstract

Neospora caninum is an obligate intracellular parasite that can infect many domestic and wild animals, including birds. These animals are important sources for monitoring of environmental contamination, as they could become infected through sporulated oocysts; however, the real role of birds in the biological cycle of *N. caninum* remains uncertain. This study aimed to determine the prevalence of anti-*N. caninum* antibodies, evaluate associated factors, detect the parasite by molecular testing of free-range chickens from Brazil, and evaluate different techniques for its serological diagnosis. Blood samples of 366 chickens from 25 farms were collected for serological assays. The indirect fluorescent antibody test (IFAT) and the indirect enzyme-linked immunosorbent assay (ELISA) were used to detect anti-*N. caninum* antibodies. Chickens that tested seropositive by IFAT had their brain tissues and a pool of organs (heart, lung, and liver) submitted to PCR for molecular detection of the parasite. Out of 366 chickens, 65 (17.8%) and 163 (44.6%) were seropositive by IFAT and ELISA, respectively. Brain tissues (n=60) and the pools of organs (n=65) were negative in the PCR. Our results showed a high prevalence of antibodies in free-range chickens and that IFAT is the more sensitive technique for the detection of anti-*N. caninum* antibodies.

Keywords: Free-range chicken, indirect fluorescent antibody test, enzyme-linked immunosorbent assay, polymerase chain reaction.

Resumo

Neospora caninum é um parasita intracelular obrigatório que pode infectar vários animais domésticos e silvestres, incluindo as aves. Esses animais são importantes fontes para o monitoramento da contaminação ambiental, uma vez que eles podem se infectar por meio da ingestão de oocistos. Porém, o real papel das aves no ciclo biológico do *N. caninum* ainda é incerto. Este estudo teve como objetivos determinar a prevalência de anticorpos anti-*N. caninum*, avaliar fatores associados, detectar o parasita por meio de teste molecular em galinhas de vida livre do Brasil e avaliar diferentes técnicas para o diagnóstico sorológico. Amostras de sangue de 366 galinhas de 25 fazendas foram coletadas para testes sorológicos. A reação de imunofluorescência indireta (RIFI) e o ensaio de imunabsorção enzimática (ELISA) foram utilizados para detectar anticorpos anti-*N. caninum*. As amostras de tecidos (cérebro, coração, pulmão e fígado) de galinhas soropositivas na RIFI foram coletadas e submetidas à PCR para detecção molecular do parasita. Das 366 galinhas, 65 (17,8%) e 163 (44,6%) foram soropositivas pela RIFI e ELISA, respectivamente. Nenhuma amostra foi positiva na PCR. Esses resultados demonstram uma elevada prevalência de anticorpos em galinhas de vida livre e que a RIFI é uma técnica mais sensível para a detecção de anticorpos anti-*N. caninum*.

Palavras-chave: Galinhas de vida livre, imunofluorescência indireta, ensaio de imunabsorção enzimática, reação em cadeia da polimerase.

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Introduction

Neospora caninum is an Apicomplexa protozoan, of worldwide distribution, capable of infecting many domestic and wild animals, including birds (Dubey & Schares, 2011; Romero et al., 2016). The disease causes abortion in cattle and has considerable importance in cattle production, resulting in high economic losses worldwide (Reichel et al., 2013). In dogs, the disease is usually asymptomatic, although clinical manifestations that include muscular and neurological disorders have been described in young dogs (Silva & Machado, 2016).

In birds, serological evidence of *N. caninum* has been described in different species, including domestic and wild birds (Costa et al., 2008; Mineo et al., 2009; Gondim et al., 2010; Darwich et al., 2012; Salant et al., 2015; De Barros et al., 2017; Konell et al., 2019). A recent review showed that the seroprevalence in chickens (*Gallus gallus domesticus*) ranged from 7.2% to 83.6% according to the region (De Barros et al., 2018). The eating habits of birds increases their exposure to these pathogens, and since birds may be preyed by dogs, chickens could contribute to the epidemiology of the parasite. Moreover, previous studies have reported a positive correlation between the presence of birds on the farm and increased reproductive problems in cattle that could be related to *N. caninum* (Bartels et al., 1999; Otranto et al., 2003).

Since the role of birds in the epidemiology of neosporosis is not fully understood, the present study aimed to detect *N. caninum* in free-range chickens from Brazil, and evaluate different techniques for its serological diagnosis.

Material and Methods

Sampling and animals

Tissue (heart, brain, lung, and liver) and blood samples of 366 free-range chickens were collected between 2012 and 2014 from 25 farms of Paraná state, south Brazil (Figure 1). For sample size calculation, we assumed a prevalence of 50%, a confidence level of 95%, and efficacy 5% by the Epi Info version 3.5.4. An epidemiological questionnaire was applied for each farmer to determine possible factors associated with exposure to *N. caninum* in chickens. All procedures involving animals were approved by the Ethics Committee of the State University of Londrina (protocol number 64/10).

Indirect fluorescent antibody test (IFAT)

Serum samples from chickens were tested by IFAT according to the methodology described previously (Conrad et al., 1993). Tachyzoites of NC-1 strain of *N. caninum* were used as antigen, while anti-chicken IgY conjugate labeled with fluorescein isothiocyanate (FITC) (F8888 - Sigma-Aldrich, USA) was used as a secondary antibody. Serum samples from chickens experimentally inoculated with *N. caninum* (NC-1 strain) and non-infected chickens were

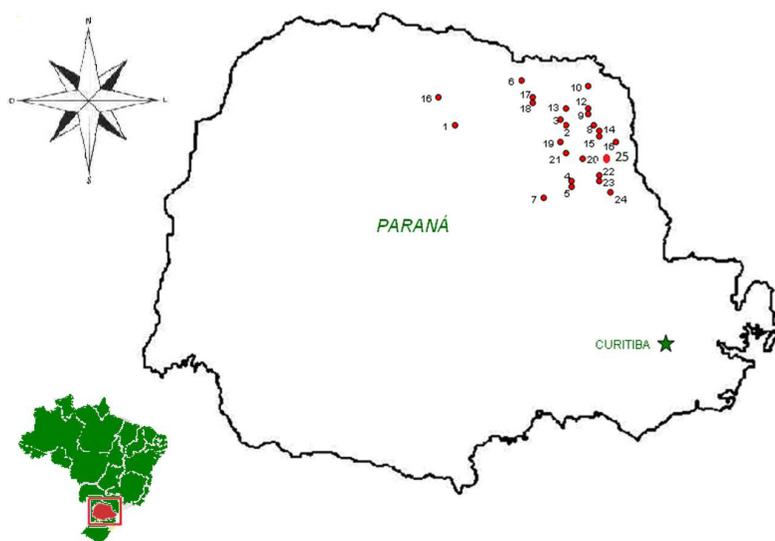


Figure 1. Location of free-range farms in Paraná state, Brazil, selected for collection of the animals used in the present study.

used as the positive and negative control, respectively. Samples that showed fluorescence over the total contour of the tachyzoite were considered positive. Since there is not an established cut-off for chickens, we used the cut-off ≥ 16 to improve the sensitivity of the test.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA was performed according to the methodology described previously (Paré et al., 1995) with few modifications. Tachyzoites of NC-1 strain grown in Vero cells were used to produce the total antigen. The tachyzoites obtained were passed through 27G needles, centrifuged at 10,000 g for 10 min, and the pellet was mixed with ceramic beads via vortexing three times for 10 min each. The resultant solution was centrifuged under the same conditions as described before, and the pellet was discarded. Finally, the protein concentration was quantified by a commercial kit (Pierce™ BCA, Thermo Fisher Scientific, USA), and the antigen was stored at -20°C until further analysis.

Flat-bottom 96-well polystyrene microtitration plates (Nunc-Immuno Plate Maxisorp, Denmark) were coated with 100 μ l of crude antigen (2.5 μ g/ml) diluted in 0.1 M carbonate buffer (pH 9.6) and incubated at 4°C overnight. After incubation, the plates were washed three times with PBS-Tween 20 (50 mM Tris, 100 mM NaCl, and 0.05% Tween 20), blocked with carbonate buffer plus 8% nonfat dry milk and incubated at 37°C for one hour. Serum samples were diluted 1:100 in PBS-Tween 20% plus 5% nonfat dry milk, and 100 μ l of this mixture were added in duplicate into the microplates and incubated for one hour at 37°C. After another washing cycle, 100 μ l peroxidase-labeled anti-chicken IgY antibody (A9046; Sigma-Aldrich, USA) diluted in PBS-Tween 20% plus 5% nonfat dry milk was added in each well, followed by incubation for one hour at 37°C.

The plates were washed three times, and the peroxidase activity was revealed by adding 100 μ l of tetramethylbenzidine solution (TMB Single Solution; Thermo Fisher Scientific, USA). Then, 50 μ l of hydrochloric acid (1N) was used to stop the reaction, and the optical density (OD) was read at 450 nm through a microplate reader (iMark Microplate Absorbance Reader, Bio-Rad, USA). Serum samples from chickens experimentally inoculated with *N. caninum* (NC-1 strain) (positive control) and non-infected chickens (negative control) were added to all plates. The OD was corrected to control plate-to-plate variation, and the cut-off was calculated by the OD mean of the negative controls plus two times the standard deviation (Garcia et al., 2006b). The corrected OD were grouped into nine levels, where each subsequent level was determined by an increment of 35% (Machado et al., 1997).

Enzymatic digestion

Pooled organs (10 g of each tissue, including heart, lung, and liver) were digested as previously described (Dubey, 1998). Briefly, the organs were crushed in saline solution and added to artificial digestive fluid (0.78 g pepsin, 0.5 g NaCl, 0.7 ml HCl and 150 ml of distilled water) for one hour at 37°C. The product of digestion was filtered and centrifuged at 1,200 g for 10 min. The supernatant was discarded, and the pellet neutralized with a sodium bicarbonate solution 1.2%. The solution was resuspended in physiological solution and centrifuged again. Then the supernatant was discarded, the solution was resuspended in 1 ml, with 1000 UI of penicillin and 100 μ g of streptomycin per ml added and stored at -20°C until molecular analysis.

DNA extraction

Animals considered positive by IFAT had their tissue samples submitted to polymerase chain reaction (PCR). Samples of 60 brains (25 mg) and 65 pools of organs (heart, lung, and liver) (300 μ l of enzymatic digestion) were used for DNA extraction. The samples were homogenized and transferred to microtubes with an equal volume of extraction buffer (200 mM NaCl, 20 mM Tris, 50 mM EDTA, proteinase K 1 mg/ml and 2% SDS), followed by incubation at 56°C for one hour. Then, with buffered phenol (300 μ l) added, it was centrifuged at 13,000 g for 5 min. The supernatant was transferred for another microtube, and with phenol:chloroform:isoamyl alcohol added, it was centrifuged at 13,000 g for 5 min (Garcia et al., 2006a). Then, DNA was precipitated by sodium acetate and ethanol (Sambrook et al., 1989).

PCR

For the molecular detection of *N. caninum*, a conventional PCR, targeting the Nc5 gene, was performed using the primers Np6 plus (5'-CTCGCCAGTCAACCTACGTCTTCT-3') and Np21 plus (5'-GGGTGTGCGTCCAATCCTGTAAC-3'),

which amplify a fragment of 337 bp (Hughes et al., 2006). The reaction was performed in 25 µl using 100 ng/µl of DNA, 25 mM MgCl₂, 200 µM dNTP (Thermo Fisher Scientific, USA), 1 µM of each primer, 1x PCR buffer (20 mM Tris-HCl, 50 mM KCl, pH 8.4) 1.25 U of Taq-DNA polymerase (Thermo Fisher Scientific, USA) and ultrapure sterile H₂O. The thermocycler program was run in appliance TC-512 (Techne, USA) according to the program: 95°C for 5 min for initial denaturation, 35 cycles of 95°C for 30 sec for denaturation, 63°C for 30 sec for annealing, and 72°C for 1 min for the extension. A final extension at 72°C for 5 min was included. Positive and negative controls were added in all reactions. PCR products were submitted to electrophoresis in agarose gel 1.5% stained with Sybr® Safe DNA Gel Stain (Thermo Fisher Scientific, USA), visualized and photo-documented under UV (L – PIX EX, Locus Biotechnology, Brazil).

Statistical analysis

The kappa coefficient was used to evaluate the agreement between the serological techniques, and the interpretation was performed as previously described (Landis & Koch, 1977). Sensitivity and specificity were calculated using IFAT as the gold standard (Casartelli-Alves et al., 2014). All the variables were analyzed by chi-square test or Fisher's exact test using a computer software (GraphPad Prism, v.6.0), and p-values ≤ 0.05 were considered significant.

Results

Out of 366 serum samples, 65 (17.8%) were positive by IFAT (Table 1), with the following titers: 16 (86.2%), 64 (12.3%) and 256 (1.5%). The occurrence of anti-*N. caninum* antibodies on each farm ranged from 7% to 62%, while 72% (18/25) of the visited farms had at least one positive animal. According to the ELISA test, 163 (44.6%) samples were positive, with the most frequent OD level among the positive samples at level four (Table 2). A

Table 1. Results of serological tests (IFAT and ELISA) for the detection of *Neospora caninum* in free-range chickens from northern Paraná state, Brazil.

		IFAT			SE (%)	SP (%)	PPV (%)	NPV (%)	Kappa
		Positive	Negative	Total					
ELISA	Positive	32	131	163					
	Negative	33	170	203	49.23	56.48	19.63	83.74	0.036
	Total	65	301	366					

SE: sensitivity; SP: specificity; PPV: positive predictive value; NPV: negative predictive value.

Table 2. Classification of positive animals for *Neospora caninum* in ELISA at different levels of optical density increasing by increments of 35%.

	ELISA Level		Total	%
0	0.00	0.274	203	55.5
1	0.275	0.371	16	4.4
2	0.372	0.502	20	5.5
3	0.503	0.679	28	7.7
4	0.68	0.918	31	8.5
5	0.919	1.241	26	7.1
6	1.242	1.677	14	3.8
7	1.678	2.265	13	3.5
8	2.266	3.059	6	1.6
9	≥ 3.060		9	2.5

comparison between the serological techniques showed that ELISA had lower sensitivity and specificity than IFAT, presenting 49% and 56%, respectively. The kappa coefficient of 0.036 demonstrates a slight agreement between the techniques. Factors associated with exposure to *N. caninum* in chickens were slaughter above 90 days ($p=0.02$), contact with animal wastes ($p=0.05$), and chickens raised free all-day ($p=0.005$) (Table 3). Through PCR, no samples were considered positive.

Table 3. Variables associated with *Neospora caninum* serum positive at IFAT in free-range chickens.

Variables		IFAT		Total	p-value
		Positive	Negative		
Presence of dog	Yes	58	273	331	0.89
	No	7	28	35	
Slaughter above 90 days	Yes	65	274	339	0.02
	No	0	27	27	
Contact with animal wastes	Yes	25	77	102	0.05
	No	40	224	264	
Chickens raised near the forest	Yes	24	94	118	0.45
	No	41	207	248	
Chickens raised free all-day	Yes	20	46	66	0.005
	No	45	255	300	
Water protection	Yes	60	281	341	0.97
	No	5	20	25	

Discussion

We observed that 17.8% of free-range chickens from Paraná State from Brazil were seropositive for *N. caninum*, demonstrating that the parasite is circulating in this region, and chickens are exposed to parasitic stages. Previous studies on free-range chickens in Brazil have also described a similar prevalence by IFAT, in Bahia with 17 - 23.5% of seropositivity (Costa et al., 2008; Gonçalves et al., 2012), 36.5% in Rio Grande do Sul (Camillo et al., 2015), and 20% in São Paulo (Martins et al., 2011). In other countries, the seroprevalence ranged from 23.1% to 41.5% (Martins et al., 2011; Feng et al., 2016; Rocchigiani et al., 2017). A similar prevalence was detected in waterfowl birds (17.5%, 32/182) from Italy, indicating that, as these bird species feed directly on the ground, they have a higher seroprevalence (Nardoni et al., 2019). However, in an urban park from Brazil, only one goose (*Anser* sp.) (out of 149) was found to be seropositive to *N. caninum*, suggesting that this seroprevalence may be due to a low seroconversion rate or a different type of feed in this species (Konell et al., 2019).

Based on the variable analysis, slaughter above 90 days was a variable associated with exposure to *N. caninum* in chickens. This result indicates that chickens more than 90 days of age have a higher chance of infection due to longer lifespan; however, a previous study showed no significant association between the *N. caninum* infection and age of chickens (Feng et al., 2016). Chickens that have contact with animal wastes and raised free all-day were also associated with infection. We suggest that these variables contribute to a higher exposition to the parasitic stages present in the environment since outdoor chickens have higher seropositivity (Costa et al., 2008). The presence of dogs on the farm showed no significant association with seropositivity in chickens; however, the presence of wild canids could contribute to the epidemiology of neosporosis (Melendez et al., 2020).

In the present study, we evaluated the ELISA technique using the total antigen; however, it showed a low sensitivity and specificity. Additionally, the Kappa coefficient showed a slight agreement with IFAT and ELISA, which makes this technique not appropriated for the screening test in epidemiological studies in chickens since cross-reaction with apicomplexan parasites may be present (Gondim et al., 2017). The protein disulfide isomerase (PDI), heat-shock protein (HSP70), and ribosomal proteins (RP1) have been identified as cross-reactive antigens and could be related to the low specificity of the serological test (Liao et al., 2005; Zhang et al., 2011). An ELISA using the surface antigen 1 of *N. caninum* (NcSAG1t) was tested in serum from chickens, and was a good option for serological diagnosis

of neosporosis in animals since the use of species-specific recombinant proteins avoids cross-reaction (Ibrahim, 2013; Gondim et al., 2017). An agglutination test (N-MAT) showed high sensitivity and specificity for naturally and experimentally infected chickens and could be an alternative tool (Sayari et al., 2016).

According to molecular analyses, no samples were positive. In chickens from China, no positive samples have been observed by molecular analyses in myocardium digestion liquids (n=25) (Feng et al., 2016). However, the presence of *N. caninum* DNA in chickens was first reported in the brain of six animals, confirming that chickens are natural intermediate hosts of the parasite (Costa et al., 2008). In wild birds that tested seropositive with a low titer of the antibody, a high rate of positive PCR samples was detected, indicating that these birds could also be involved in the epidemiology of *N. caninum* (Nardoni et al., 2019). DNA of *N. caninum* was also detected in brain and heart tissues from wild waterfowl from Italy, showing that these animals can become infected and could contribute to the spread of the parasite. (Rocchigiani et al., 2017).

Animals experimentally infected with tachyzoites and oocysts of two different strains of *N. caninum* yielded negative results at PCR (Oliveira et al., 2018); however, this resistance to chronic infection could be related to the strain of parasite as the parasite had a low capacity for multiplication at temperatures above 39°C to 41.5°C (Rezende-Gondim et al., 2017). Since we could not detect positive samples by PCR, we suggested that chickens were infected by a low dose of parasites, or they are resistant to chronic infection, being the tissue cysts eliminated due to high corporal temperature of free-range chickens. Moreover, previous studies have demonstrated that chickens present a transient *N. caninum* infection, being seropositive without the establishment of infection (Furuta et al., 2007; Munhoz et al., 2014).

Conclusions

Our results showed that *N. caninum* is circulating among free-range chickens on the studied farms; however, the importance of these animals on the epidemiology of neosporosis should be elucidated. The ELISA demonstrated a slight agreement with IFAT, being the IFAT a more appropriate technique for epidemiological studies.

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