



Short communication

Survey for natural *Neospora caninum* infection in wild and captive birds

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ABSTRACT

Neospora caninum is a protozoan parasite that presents worldwide distribution and is mainly implicated as responsible for bovine abortion. Although the presence of birds in cattle-raising properties is positively correlated to higher infection rates, very little has been described about the role of these animals in the parasite's life cycle. In that sense, this work aimed to investigate the serological and histological positivity of different avian species sampled in its natural habitat or in captivity. No serological positivity was observed in the 294 tested serum samples. On the other hand, Apicomplexa-like cysts found in muscular tissues of two Psittaciformes were immunostained with *N. caninum* antisera. These findings indicate that *N. caninum* may infect a wider range of hosts than described to date, and that further studies should be performed in order to determine the presence of the infection in different avian species.

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1. Introduction

Apicomplexan parasites *Neospora caninum* and *Toxoplasma gondii* share many morphological features, however present distinct biological properties (Hemphill et al., 2006; Innes and Mattsson, 2007).

T. gondii has been widely researched in the last century and infection in birds was reported after three years of its first description (Nicolle and Manceaux, 1908; Splendore, 1908), in liver and spleen smears of a naturally infected pigeon (Carini, 1911). From that point on, in numerous reports have been published about *T. gondii* infection

in birds, with higher concentration of articles between 1940–60s, when researchers found out that avian *Toxoplasma* was the same parasite that caused illness in humans and other mammals (Dubey, 2002). On the other hand, natural infection by *N. caninum* in wildlife birds has been described only once, in captured sparrows (Gondim et al., 2010). An extensive number of wildlife species have been investigated for their role in *Neospora*'s life cycle, such as coyotes and Australian dingos, which were implicated as the parasite's definitive hosts (Gondim et al., 2004; King et al., 2010). Other wild animal species are also exposed to the protozoan, as demonstrated by serological, histological and/or PCR evidence (De Craeye et al., 2010; Dubey et al., 2007; Malmsten et al., 2010; Sedlak and Bartova, 2006).

Experimental infections have established that pigeons may be susceptible to infection, produce specific IgG antibodies, and are potential intermediate hosts of the parasite (McGuire et al., 1999; Mineo et al., 2009). Similarly,

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embryonated eggs have been shown as a promising experimental model due its differential susceptibility according to the incubation period (Furuta et al., 2007). On the other hand, carnivorous bird species experimentally infected with *N. caninum* did not present clinical signs of infection or shed oocysts (Baker et al., 1995), indicating that susceptibility to infection within birds may be species-specific. In that sense, this study aimed to observe the presence of *N. caninum* infection in wild birds maintained in captivity and free-ranging birds, using serological and histological assays.

2. Materials and methods

Serum samples from two hundred and ninety four animals, from 17 species representing 9 avian orders (Table 1) were analyzed for specific antibodies against *N. caninum*. These birds were patients in the Wildlife Animal Ambulatory of the Veterinary Hospital, FCAV/UNESP (Jaboticabal, São Paulo State), or from zoos and ecological reserves throughout Brazil, being collected from 1998 to 2005.

Patients that died or were euthanized during internment underwent necropsy, and its tissues were submitted to microscopic analysis, as routine diagnostic procedure. Tissues containing Apicomplexa-like tissue cysts were selected for immunoassays, as described below. All animal procedures were performed according to the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and to the 2000 Report of the AVMA Panel on Euthanasia (2001).

In order to check for the presence of *N. caninum* in the sampled birds, indirect fluorescent antibody technique (IFAT) and immunohistochemical (IHC) assays were undertaken as previously described (Mineo et al., 2009). Briefly, IFAT was performed with the incubation of test

sera in antigen slides containing formalin-fixed tachyzoites at 1:20 dilution. As secondary antibodies, an anti-chicken IgG antibody conjugated to FITC (Sigma, USA) was used at 1:50 dilution. Slides were mounted with carbonate-buffered glycerin (pH 9.5) and coverslips before being read in an epifluorescence microscope (Olympus, Japan). Only a bright fluorescence of the whole tachyzoite surfaces was considered as a positive result. Slides containing paraffin-embedded tissues from selected animals were submitted to IHC assays using polyclonal antibodies against *N. caninum* (1:1000), obtained from experimentally infected BALB/c mice, as primary antibodies. Tissue samples were also incubated with mAb 74.1.8, a monoclonal antibody against BAG1 antigen, commonly expressed in *N. caninum* and *T. gondii* tissue cysts (Weiss et al., 1999). For signal amplification, the avidin–biotin complex immunoperoxidase step was performed (DakoCytomation, Denmark) and the slides stained with diaminobenzidine tetrahydrochloride (DAB – DakoCytomation). Counter staining was performed with Harris hematoxylin (10%) and slides were later mounted on coverslips to be read under light microscope (Nikon, Japan). Direct detection was also attempted by the detection of Nc5 locus in the IHC positive samples, using DNA extraction, primer sets and amplification protocols as previously described (Furuta et al., 2007).

3. Results and discussion

The need to observe *N. caninum* in wildlife animals has been pointed out as a possible way to understand some obscure aspects of the parasite's cycle (Gondim, 2006). There are indications that the presence of birds in cattle-raising farms could be associated with the increase of seroprevalence and abortions related to *N. caninum* (Bartels et al., 1999; Otranto et al., 2003). In *T. gondii* epidemiological chain, birds are considered parasite's reservoir, since those animals are frequently preyed upon by its definitive hosts, felids (Elmore et al., 2010). The same pattern of events may be observed in the relationship between dogs and birds, which may lead to speculations towards if birds may also perform the role of *N. caninum* reservoirs in nature. Epidemiological studies for these protozoa frequently employ antibody detection to estimate population infection rates. Serological positivity to *T. gondii* in birds is usually low, which is not compatible with direct detection in different tissues (Dubey, 2002).

Serological analysis by IFAT of samples gathered from wild birds maintained in captivity and free-ranging birds for the presence of antibodies to *N. caninum* were inconclusive, since specific IgG antibodies to *N. caninum* were not detected. The absence of detectable levels of specific IgG against *N. caninum* in birds is not a surprise, since it has been already shown that experimentally infected pigeons and different chicken models present an abrupt antibody seroconversion, despite a brief detection period (Furuta et al., 2007; Mineo et al., 2009). Additionally, the same phenomenon has been described in experimental infections of wild birds with *T. gondii* (Mineo et al., 2009; Vitaliano et al., 2010). The lack of detection of circulating antibodies specific to the parasite in the tested species may be partially attributed to the serological assay employed,

Table 1
Zoological classification and number of samples from captive and wild birds serologically assayed for *Neospora caninum* specific IgG antibodies.

Order	Species	Number assayed samples
Ciconiformes	<i>Coragyps atratus</i>	4
Columbiformes	<i>Columba livia</i>	50
	<i>Zenaidura macroura</i>	25
Falconiformes	<i>Caracara plancus</i>	8
Passeriformes	<i>Oryzoborus maximilliani</i>	6
	<i>Serinus canaria</i>	20
Piciformes	<i>Ramphastos toco</i>	2
	<i>Amazona aestiva</i>	29
	<i>Anodorhynchus hyacinthinus</i>	4
Psittaciformes	<i>Anodorhynchus leari</i>	2
	<i>Ara ararauna</i>	1
	<i>Ara chloropterus</i>	2
	<i>Melopsittacus undulatus</i>	64
Strigiformes	<i>Asio clamator</i>	2
	<i>Tyto alba</i>	1
Rheiformes	<i>Rhea americana</i>	37
Struthioniformes	<i>Struthio camelus</i>	37
	Total	294

which is based on a secondary antibody raised for chickens. Although the assay seems to work properly with some wildlife species, IgG domains of different bird species is variable and might not present the same homology with chicken antibodies, fact that may dampen the serological diagnosis in wild life animals. Unfortunately, it is uncommon to find commercial conjugates specific for wild life animals, which limits applied research focusing those species.

Post-mortem analysis through observation of gross and microscopic lesions is an important tool to assist the determination of death causes in wild animals, once availability of other support diagnostic techniques may not be as feasible. Interestingly, routine analysis of a red-and-green macaw (*Ara chloropterus*) and a blue-fronted Amazon parrots (*Amazona aestiva*), which died and were necropsied due to unrelated clinical conditions, presented Apicomplexan-like tissue cysts in HE stained tissue slides. The samples containing the unidentified parasite forms underwent IHC analysis, and showed to be positive for *N. caninum*. The cysts were found in the musculature around the cloacae of the red-and-green macaw (Fig. 1A) and in blue fronted Amazon's cervical musculature (Fig. 1B). Similar structures were found in the breast musculature of several pigeons although no positive staining was observed (Fig. 1C). None of the tissues from the three animals presented positive staining for *T. gondii*, however the tissues from the two psittacine birds presented a faint staining for BAG1, while positivity was not observed for the tissue cysts found in the breast musculature of the pigeon. Pigeons frequently bear *Sarcocystis* spp. infections (Olias et al., 2010, 2011), and morphological analysis of the slides is suggestive of that infection. Altogether, the reactivity in IHQ of the parasitic forms to *N. caninum* and BAG1 directed antibodies, associated to the lack of reactivity against *T. gondii* antisera and total absence of staining in *Sarcocystis* spp. bearing samples, suggests that the immunostaining protocol used in this work was specific for *N. caninum*.

The suggestive findings of *N. caninum* tissue cysts in two psittacine birds are very relevant, since those structures are considered rare histological findings, been found mostly in nervous tissues of dogs and ruminants (Dubey et al., 2002). Few reports demonstrate intramuscular tissue cysts in dogs and cattle, where parasites are usually located inside myofibers (Peters et al., 2001). Experimental attempts were made in different species to visualize the parasite in its cystic forms inside the musculature, but none succeeded (Dubey, 2002). This fact was intriguing to researchers, since it is common sense that the definitive hosts of *N. caninum* begin to play their role in the parasite's cycle after predation, which is usually performed by primary ingestion of offal and muscles. The detection of latent parasitic forms in the musculature of birds might shed some light into the discussion related to *Neospora's* wildlife life cycle.

Immunohistochemical protocols based on HRPO and FITC conjugates, using polyclonal antibodies to *N. caninum* and *T. gondii*, showed to be parasite specific once no cross-reactivity was demonstrated, as observed previously (van Maanen et al., 2004). Moreover, morphologically similar tissue cysts found in other musculature samples

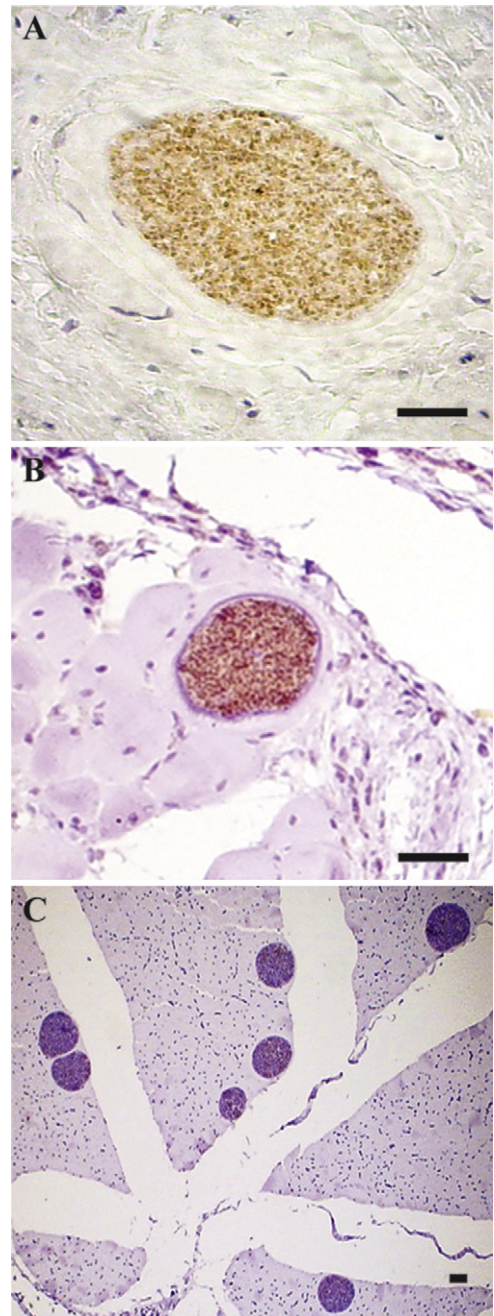


Fig. 1. Positive immunostaining of Apicomplexa-like tissue cysts with anti-*Neospora caninum* polyclonal antibodies: (A) in the cloacae musculature of a red-and-green macaw (*Ara chloropterus*); (B) in the cervical musculature of a blue-fronted Amazon parrots (*Amazona aestiva*). Apicomplexa-like tissue cysts contained (C) in the pectoral muscles of a pigeon (*Columba livia*) are not reactive in immunohistochemical assay to detect *Neospora caninum* antigens. Scale bars = 20 μ m.

did not react to any of the primary antibodies used. Posterior morphological analyses suggested *Sarcocystis* spp., commonly found in these species (Kutkiene and Sruoga, 2004). To further confirm direct parasite detection in bird species, parasite-specific DNA amplification and/or parasite isolation is desirable. However, many obstacles may

turn that task ungrateful. Parasite forms evidenced by immunoenzymatic assays were findings of histopathological examination of animals that were taken in with other clinical conditions. In that sense, with no macroscopic evidences of infection, tissue collection for PCR assays becomes nearly random. DNA extraction of positive paraffin-embedded tissues was tried in our laboratory, however yielded poor quality DNA, independently of the extraction procedure. This phenomenon was previously observed in an interlaboratory comparison of diagnostic methods for *N. caninum* infection in bovine fetuses (van Maanen et al., 2004).

It has been shown in the present work that *N. caninum* may be present in wildlife bird species, and more studies should be performed to measure the actual susceptibility and infection rates of wildlife birds to the infection.

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References

- Baker, D.G., Morishita, T.Y., Brooks, D.L., Shen, S.K., Lindsay, D.S., Dubey, J.P., 1995. Experimental oral inoculations in birds to evaluate potential definitive hosts of *Neospora caninum*. *J. Parasitol.* 81, 783–785.
- Bartels, C.J., Wouda, W., Schukken, Y.H., 1999. Risk factors for *Neospora caninum*-associated abortion storms in dairy herds in The Netherlands (1995 to 1997). *Theriogenology* 52, 247–257.
- Carini, A., 1911. Infection spontanéie du pigeon et du chien due au *Toxoplasma cuniculi*. *Bull. Soc. Pathol. Exotique* 4, 518–519.
- De Craeye, S., Speybroeck, N., Ajzenberg, D., Darde, M.L., Collinet, F., Tavernier, P., Van Gucht, S., Dorny, P., Dierick, K., 2010. *Toxoplasma gondii* and *Neospora caninum* in wildlife: common parasites in Belgian foxes and Cervidae? *Vet. Parasitol.*
- Dubey, J.P., 2002. A review of toxoplasmosis in wild birds. *Vet. Parasitol.* 106, 121–153.
- Dubey, J.P., Barr, B.C., Barta, J.R., Bjerkas, I., Bjorkman, C., Blagburn, B.L., Bowman, D.D., Buxton, D., Ellis, J.T., Gottstein, B., Hemphill, A., Hill, D.E., Howe, D.K., Jenkins, M.C., Kobayashi, Y., Koudela, B., Marsh, A.E., Mattsson, J.G., McAllister, M.M., Modry, D., Omata, Y., Sibley, L.D., Speer, C.A., Trees, A.J., Uggla, A., Upton, S.J., Williams, D.J., Lindsay, D.S., 2002. Redescription of *Neospora caninum* and its differentiation from related coccidia. *Int. J. Parasitol.* 32, 929–946.
- Dubey, J.P., Schares, G., Ortega-Mora, L.M., 2007. Epidemiology and control of neosporosis and *Neospora caninum*. *Clin. Microbiol. Rev.* 20, 323–367.
- Elmore, S.A., Jones, J.L., Conrad, P.A., Patton, S., Lindsay, D.S., Dubey, J.P., 2010. *Toxoplasma gondii*: epidemiology, feline clinical aspects, and prevention. *Trends Parasitol.* 26, 190–196.
- Furuta, P.I., Mineo, T.W., Carrasco, A.O., Godoy, G.S., Pinto, A.A., Machado, R.Z., 2007. *Neospora caninum* infection in birds: experimental infections in chicken and embryonated eggs. *Parasitology* 134, 1931–1939.
- Gondim, L.F., 2006. *Neospora caninum* in wildlife. *Trends Parasitol.* 22, 247–252.
- Gondim, L.F., McAllister, M.M., Pitt, W.C., Zemlicka, D.E., 2004. Coyotes (*Canis latrans*) are definitive hosts of *Neospora caninum*. *Int. J. Parasitol.* 34, 159–161.
- Gondim, L.S., Abe-Sandes, K., Uzeda, R.S., Silva, M.S., Santos, S.L., Mota, R.A., Vilela, S.M., Gondim, L.F., 2010. *Toxoplasma gondii* and *Neospora caninum* in sparrows (*Passer domesticus*) in the Northeast of Brazil. *Vet. Parasitol.* 168, 121–124.
- Hemphill, A., Vonlaufen, N., Naguleswaran, A., 2006. Cellular and immunological basis of the host–parasite relationship during infection with *Neospora caninum*. *Parasitology* 133, 261–278.
- Innes, E.A., Mattsson, J.G., 2007. *Neospora caninum* emerges from the shadow of *Toxoplasma gondii*. *Trends Parasitol.* 23, 43–44, discussion 44–45.
- King, J.S., Slapeta, J., Jenkins, D.J., Al-Qassab, S.E., Ellis, J.T., Windsor, P.A., 2010. Australian dingoes are definitive hosts of *Neospora caninum*. *Int. J. Parasitol.* 40, 945–950.
- Kutkieni, L., Sruoga, A., 2004. *Sarcocystis* spp. in birds of the order Anseriformes. *Parasitol. Res.* 92, 171–172.
- Malmsten, J., Jakubek, E.B., Bjorkman, C., 2010. Prevalence of antibodies against *Toxoplasma gondii* and *Neospora caninum* in moose (*Alces alces*) and roe deer (*Capreolus capreolus*) in Sweden. *Vet. Parasitol.*
- McGuire, A.M., McAllister, M., Wills, R.A., Tranas, J.D., 1999. Experimental inoculation of domestic pigeons (*Columba livia*) and zebra finches (*Poephila guttata*) with *Neospora caninum* tachyzoites. *Int. J. Parasitol.* 29, 1525–1529.
- Mineo, T.W., Carrasco, A.O., Marciano, J.A., Werther, K., Pinto, A.A., Machado, R.Z., 2009. Pigeons (*Columba livia*) are a suitable experimental model for *Neospora caninum* infection in birds. *Vet. Parasitol.* 159, 149–153.
- Nicolle, M., Manceaux, L., 1908. Sur une infection a corps de Leishman (ou organismes voisins) du gondi. *C. R. Acad. Sci. Hebd. Seances. Acad. Sci.* D 147, 736.
- Olias, P., Gruber, A.D., Kohls, A., Hafez, H.M., Heydorn, A.O., Mehlhorn, H., Lierz, M., 2010. *Sarcocystis* species lethal for domestic pigeons. *Emerg. Infect. Dis.* 16, 497–499.
- Olias, P., Olias, L., Krucken, J., Lierz, M., Gruber, A.D., 2011. High prevalence of *Sarcocystis calchasi* sporocysts in European Accipiter hawks. *Vet. Parasitol.* 175, 230–236.
- Otranto, D., Llazari, A., Testini, G., Traversa, D., Frangipane di Regalbano, A., Badan, M., Capelli, G., 2003. Seroprevalence and associated risk factors of neosporosis in beef and dairy cattle in Italy. *Vet. Parasitol.* 118, 7–18.
- Peters, M., Lutkefels, E., Heckerroth, A.R., Schares, G., 2001. Immunohistochemical and ultrastructural evidence for *Neospora caninum* tissue cysts in skeletal muscles of naturally infected dogs and cattle. *Int. J. Parasitol.* 31, 1144–1148.
2001. *J. Am. Vet. Med. Assoc.* 218, 669–696.
- Sedlak, K., Bartova, E., 2006. Seroprevalences of antibodies to *Neospora caninum* and *Toxoplasma gondii* in zoo animals. *Vet. Parasitol.* 136, 223–231.
- Splendore, A., 1908. Un nuovo protozoa parassita deconigli incontrato nelle lesioni anatomiche d'una malattia che ricorda in molti punti il Kala-azar dell'uomo. *Revista da Sociedade Scientifica de São Paulo* 3, 109–112.
- van Maanen, C., Wouda, W., Schares, G., von Blumroder, D., Conraths, F.J., Norton, R., Williams, D.J., Esteban-Redondo, I., Innes, E.A., Mattsson, J.G., Bjorkman, C., Fernandez-Garcia, A., Ortega-Mora, L.M., Muller, N., Sager, H., Hemphill, A., 2004. An interlaboratory comparison of immunohistochemistry and PCR methods for detection of *Neospora caninum* in bovine foetal tissues. *Vet. Parasitol.* 126, 351–364.
- Vitaliano, S.N., Mineo, T.W., Andre, M.R., Machado, R.Z., Mineo, J.R., Werther, K., 2010. Experimental infection of crested caracara (*Caracara plancus*) with *Toxoplasma gondii* simulating natural conditions. *Vet. Parasitol.* 172, 71–75.
- Weiss, L.M., Ma, Y.F., Halonen, S., McAllister, M.M., Zhang, Y.W., 1999. The in vitro development of *Neospora caninum* bradyzoites. *Int. J. Parasitol.* 29, 1713–1723.