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Rapid Communication

Chickens (*Gallus domesticus*) are natural intermediate hosts of *Neospora caninum* ☆

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Abstract

Neospora caninum naturally infects many mammal species, but has not previously been demonstrated in birds. We examined sera for *N. caninum* antibodies from 200 outdoor chickens and from 200 chickens confined indoors in the state of Bahia, Brazil. Seroprevalence was greater in outdoor chickens (23.5% versus 1.5%, $P < 0.001$). PCR testing for *N. caninum* was positive in six of 10 seropositive chickens. Amplicons from two of these were sequenced and had 97–98% nucleotide identity with *N. caninum*. This finding extends the list of intermediate hosts of *N. caninum* to include birds and may have important epidemiological consequences.
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Keywords: *Neospora caninum*; *Toxoplasma gondii*; Chicken; Antibodies; Infection

Neospora caninum is a protozoan parasite that infects a large spectrum of domestic and wild mammals (Dubey, 2003; Gondim, 2006). Dogs and coyotes are, so far, the only confirmed definitive hosts of the parasite, but other wild canids are also suspected to be definitive hosts (McAllister et al., 1998; Gondim et al., 2004). *N. caninum* is a common cause of abortion in cattle and has a significant economic impact in the dairy and beef industries (Trees et al., 1999). There is no efficient vaccine or drug to prevent abortion or transplacental infection in cattle. Horizontal transmission can be minimised by the adoption of measures to reduce contact between carnivores and efficient intermediate hosts such as cattle (McAllister et al., 2000).

Among several risk factors associated with *N. caninum* infection in cattle, the combined presence of dogs and poultry was reported in two studies (Bartels et al., 1999; Otranto et al., 2003). However, *N. caninum* has not been detected in chickens (*Gallus domesticus*) or any other non-mammalian species. It is known that chickens are exposed to *Toxoplasma gondii*; this parasite has been detected and isolated in chickens from different countries, including Brazil (Dubey et al., 2002).

Chickens are cosmopolitan animals and serve as a food source for different animal species, including dogs. The present experiment aimed to investigate whether chickens are natural intermediate hosts of *N. caninum* and to determine if outdoor chickens are more exposed to *N. caninum* and *T. gondii* than indoor chickens.

Blood samples were collected by wing vein puncture from 400 chickens, with ages ranging from 6 months to 2 years. Two hundred animals were from two commercial companies, where chickens were confined indoors in

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suspended crates (indoor group). Another 200 animals were from five farms; these chickens were kept outdoors, either within group pens or without confinement (outdoor group). All chickens were bred in Bahia State, Brazil, between 100 and 400 km from the city of Salvador. Outdoor chickens were labeled with a metal ring on the left leg for further identification. All animal procedures were performed in compliance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation, and approved by the Institutional Bioethics Committee.

Sera were tested for *N. caninum* and also for the closely related parasite *T. gondii* using indirect fluorescent antibody tests (IFATs) according to methods previously described (Gondim et al., 1999), with minor modifications. IFATs for *N. caninum* and *T. gondii* were performed using tachyzoites of the strains NC-Bahia (Gondim et al., 2001) and RH as antigens, respectively, and a commercial fluorescein-labeled anti-chicken IgG (Sigma, USA) as the secondary antibody. The sera were screened at a 1:50 dilution for *N. caninum* and at 1:16 dilution for *T. gondii*. A reaction was considered positive if fluorescence occurred around the complete periphery of tachyzoites. Positive sera were diluted two-fold until the end point. Negative and positive control sera were included on each slide. *N. caninum*-positive control serum was obtained 15 days after s.c. inoculation of an experimental chicken with 1.5×10^4 tachyzoites of the strain NC-Bahia. *T. gondii* positive control serum was produced in a chicken inoculated s.c. with 1.5×10^4 tachyzoites of the RH strain. Negative control serum was obtained from a broiler chicken which tested negative for both parasites. The frequencies of seropositivities for *N. caninum* and *T. gondii* were compared between indoor and outdoor groups using the χ^2 test with a confidence interval of 95%.

A total of 10 *N. caninum*-seropositive chickens from two farms were purchased for PCR testing, depending upon the availability of the birds when the farms were revisited, and which chickens could be located and captured. The animals were killed, their brains were aseptically removed and DNA was extracted. Three-quarters of each brain was mixed in a tissue grinder and part of this sample was used for DNA extraction using a commercial DNA extraction kit (Easy DNA, Invitrogen, USA). PCRs were performed in 50 μl volumes containing the Np6/Np21 primer pair (Yamage et al., 1996) and the template in a commercial PCR Master Mix (25 U/ml TaqDNA polymerase in a proprietary reaction buffer, pH 8.5, 200 μM each deoxynucleoside triphosphate and 1.5 mM $MgCl_2$) (Promega, USA). DNA from *N. caninum* and sterile ddH_2O was used as positive and negative controls, respectively.

Reaction conditions were 1 cycle at 94 °C for 5 min; 40 cycles at 94 °C for 1 min, 53 °C for 1 min, 72 °C for 1 min; and a final extension step at 72 °C for 7 min. The expected size of the amplicon is 328 bp. PCR for *T. gondii* was performed similarly, except by using the primer pair TgB1-1/TgB1-4 (Burg et al., 1989), an annealing temperature of 55 °C, and *T. gondii* DNA as positive control. The expected size of the amplicon is 195 bp. PCR products were analysed by electrophoresis on a 2% agarose gel stained with ethidium bromide and visualised under UV light.

PCR fragments were cloned into a pGEM-T Easy plasmid (Promega, USA), according to the manufacture's instructions using *Escherichia coli* XL-1 blue cells as hosts. Cloning was confirmed by colony PCR, using a Np6/Np21 primer pair. The DNA inserts were sequenced, using a T7 primer, by the dideoxide method and analysed in an ABI 3100 automatic sequencer (Applied Biosystems, USA). Only sequences with a PHRED index greater than 20 were considered. The sequences of *N. caninum nc5* gene amplified from chicken brains were submitted to homology search by BLASTN at the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/). These sequences were also aligned with *nc5* partial sequences from other hosts by Clustal-W.

The number of chickens that tested positive for *N. caninum* in the outdoor group, (47/200 [23.5%]) was significantly higher ($P < 0.001$) compared with the indoor chickens (3/200 [1.5%]). The antibody titers for *N. caninum* ranged from 1:50 to 1:400. The seropositivity for *T. gondii* was significantly higher in the outdoor group (50/200 [25%]), compared with the indoor chickens (6/200 [3%]) ($P < 0.001$), with titers ranging from 1:16 to 1:1024. In a total of 50 *N. caninum*-positive serum samples, 33 sera reacted solely with *N. caninum* and 17 sera reacted with both *N. caninum* and *T. gondii*.

Six of 10 chickens that tested positive for *N. caninum* by IFAT were also positive by PCR using the *N. caninum*-specific Np6–Np21 primer pair. None of the 10 *N. caninum*-seropositive chickens were positive for *T. gondii* by PCR. After plasmid cloning, amplicons from two chickens were sequenced using the T7 primer. The two sequences (chicken 197: GenBank Accession No. EU073599, and chicken 152: GenBank Accession No. EU073600) shared a 97% match with each other and matched closely with previously deposited *N. caninum nc5* gene sequences from mammals; chicken 197: (GenBank Accession No. EU073599) matched 97–98% with the sequences EF202082.1, EF202080.1, EF202081.1, EF202079.1, and chicken 152 matched 97–98% with the sequences EF463098.1, EF463099.1 and EF581827.1.

In this study, the seropositivities for *N. caninum* and *T. gondii* in outdoor chickens were significantly higher than in indoor chickens, indicating that the former group is more exposed to both parasites. *N. caninum* infection was confirmed by PCR in six of 10 seropositive chickens. To the authors' knowledge, this is the first confirmation of natural *N. caninum* infection in chickens and also the first demonstration of naturally occurring *N. caninum* infection in birds. These results are epidemiologically important because chickens are cosmopolitan animals that are consumed by many other animal species, including dogs which are definitive hosts of the parasite (McAllister et al., 1998). It is possible that other avian species can also be infected with *N. caninum*.

Other authors (Bartels et al., 1999; Otranto et al., 2003) previously reported an epidemiological association of poultry with abortion storms or with *N. caninum* seropositivity in cattle. Bartels et al. (1999) found an association between the presence of dogs, the presence of poultry and the feeding of moldy maize-silage during summer with neosporosis abortion outbreaks in cattle in the Netherlands. Otranto et al. (2003) reported that a higher seropositivity in cattle in southern and northern Italy was associated with a higher number of dogs on farms, with farm size and with the presence of poultry.

In two studies *N. caninum* infection was experimentally induced in birds. McGuire et al. (1999) inoculated *N. caninum* tachyzoites in six birds and caused infection in three domestic pigeons (*Columbia livia*), whereas three zebra finches (*Poephila guttata*) resisted infection. In a recent report (Furuta et al., 2007), chickens and embryonated eggs were inoculated with *N. caninum* tachyzoites, and dogs shed oocysts after ingesting the infected chicken embryos.

Many studies have been done on *T. gondii* infection in chickens worldwide, including Brazil (Dubey et al., 2002), and the main route of infection for chickens is supposed to be through ingestion of oocysts in the soil. Chickens probably become infected with *N. caninum* by the same route. Besides many similarities between *N. caninum* and *T. gondii*, significant differences in host susceptibility to disease have been observed for these parasites. For example, cattle are highly susceptible to neosporosis but have low susceptibility to toxoplasmosis, whereas the opposite occurs with sheep, which are highly susceptible to toxoplasmosis, but have low susceptibility to neosporosis (Innes et al., 2007).

Despite the confirmation of *N. caninum* infection in chickens, further studies are necessary to determine the efficiency with which chickens may transmit infections to carnivorous hosts, and also to isolate the parasite from chickens for comparisons with mammalian isolates.

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