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## Short communication

## Quails are resistant to infection with *Neospora caninum* tachyzoites



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## ABSTRACT

The aim of this study was to characterize the role of quails (*Coturnix coturnix japonica*) as intermediate hosts for *Neospora caninum*. Fifty-eight 20-day old quails were individually identified with numbered rings and kept in cages adequate for the species. The birds were fed commercial feed and distilled water *ad libitum*. They were weighed daily during the first month and every seven days from then on. The birds were randomly distributed into 3 groups; 40 quails (group A) and 8 quails (group B) received  $3.5 \times 10^6$  and  $5 \times 10^6$  *N. caninum* tachyzoites, respectively, and 10 quails (group C) received placebo. Four quails from group A and one from group C had their blood collected and were euthanized on the 1st, 3rd, 5th, 7th, 14th, 21st, 30th, 60th, 120th day after infection (DAI), and 4 quails from group B were euthanized on days 60th and 120th DAI. Following euthanasia, the birds were submitted to necropsy, the organs were weighed and fragments were collected for histopathology, immunohistochemistry (IHC) and PCR. On the 60th DAI, two dogs were fed tissue obtained from group A quails, and one dog was fed quail tissues from group B. The dogs' blood was collected weekly for serology, and their feces were collected daily for 33 days for microscopy and molecular identification of oocysts. Student's *t*-test was used to compare the weights of the collected organs and the quail and cloacal temperature. No quail presented any clinical signs or died. Splenomegaly and hepatomegaly were the primary necropsy findings during the first week of infection ( $p < 0.05$ ). Positive serology, immunoreactivity through IHC and molecular identification of the parasite, were observed, especially during the first two weeks of infection. No dogs eliminated the oocysts or seroconverted. The infected quails gained more weight than the control quails. The results demonstrated that quails are resistant to infection with *N. caninum* tachyzoites under the conditions of this study.

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## 1. Introduction

*Neospora caninum* is an intracellular protozoa with a heteroxenous life cycle. It can cause infections in mammals (Dubey et al., 2007) and birds (McGuire et al., 1999; Furuta et al., 2007; Costa et al., 2008; Mineo et al., 2009, 2011; Martins et al., 2011; Molina-López et al., 2012).

The relationship between *N. caninum* and birds has already been observed in experimental (Furuta et al., 2007; Mineo et al., 2009) and observational studies (Mineo et al., 2011; Molina-López et al., 2012) and is a risk factor for bovine infection (Otranto et al., 2003). However, gaps remain regarding the role of birds in the cycle of *N. caninum* because to date, only experimentally infected embryonated chicken (*Gallus domesticus*) eggs were capable of causing infection and the elimination of oocysts in dogs (Furuta et al., 2007).

Quails (*Coturnix coturnix japonica*) have been used scientifically as laboratory birds due to the many advantages they offer in relation to other gallinaceous birds. Their anatomy and physiology are similar to those of domestic chickens (Ichilcik and Austin, 1978). Because chickens were identified as hosts of the *N. caninum* parasite (Costa et al., 2008; Gonçalves et al., 2012) and belong to the same order as quails, the objective of this study was to characterize *N. caninum* infections in quails and determine the potential of quails as intermediate hosts to the parasite and as a biological model for infections caused by the parasite.

## 2. Materials and methods

### 2.1. Origin and management of quails and dogs

Fifty-eight female quails aged 20 days were acquired from a commercial breeder, individually identified with numbered rings and kept in cages adequate for the species. The birds were fed commercial feed and distilled water *ad libitum*. They were weighed daily during the first month and every seven days from then on. The birds were randomly separated into the following three groups: 40 quails (Group A), eight quails (Group B) and 10 quails (Group C).

Three healthy female mongrels aged two months and negative for *N. caninum* were utilized in the study. The dogs were vaccinated against canine distemper, parvovirus, coronavirus, adenovirus type 2, parainfluenza, canine infectious hepatitis and leptospirosis (Duramune® Max 5CvK/4L, Fort Dodge, USA); received anthelmintic treatment with pyrantel pamoate, praziquantel and febantel (Canex plus 3®, Vetbrands Brasil Ltda, Brazil); and were fed commercial feed and distilled water *ad libitum*.

### 2.2. Experimental infection of quails

The quails in group A received  $3.5 \times 10^5$  *N. caninum* tachyzoites, group B received  $5.0 \times 10^6$  and group C received placebo, through the subcutaneous route. The *N. caninum* tachyzoites originated from an NC-Bahia isolate (Gondim et al., 2001).

### 2.3. Collecting biological samples

Four quails from group A and one quail from group C had their blood collected from a puncture to the brachial vein prior to euthanasia on the 1st, 3rd, 5th, 7th, 14th, 21st, 30th, 60th (here were eight quails from group A and two quails from group C) and 120th day after infection (DAI). The quails from group B were euthanized on the 60th and 120th DAI. The blood samples were centrifuged, and the serum was removed for the indirect fluorescent antibody test (IFAT). After euthanasia, the birds were submitted to a necropsy, and the following organs were removed: spleen, bursa of Fabricius, brain, cerebellum, heart, liver, tongue, pectoral muscles, ovary, lungs and kidneys. These organs were weighed, and fragments were collected for histopathology (HP), immunohistochemistry (IHC) and polymerase chain reaction (PCR). Blood was collected from each dog on day 0 and on the 7th, 14th, 21st and 28th DAI. The serum was stored at  $-20^\circ\text{C}$  until it was used for *N. caninum* antibody testing by IFAT.

### 2.4. Ingestion of experimentally infected quail tissue by dogs

The dogs were fed a pool of euthanized quail tissue on the 60th DAI. Two dogs were fed quail tissue from group A, and one dog was fed quail tissue from group B. The tissue pool offered to the dogs was composed of the remainder of the organs following the retrieval of fragments for HP and PCR and contained at least 50% of the total amount of each organ, except for the bursa of Fabricius, which amounted to 20% of the organ.

### 2.5. Indirect fluorescent antibody test (IFAT) for the detection of anti-*N. caninum* antibodies

The sera were tested for anti-*N. caninum* antibodies by IFAT according to the methods of Yamane et al. (1997) using the strain NC-BA (Gondim et al., 2001). Chicken anti-IgG (Sigma®-Aldrich F4137 Inc., USA) and dog anti-IgG (Sigma®-Aldrich F4012 Inc., USA) were used as secondary antibodies. The cut-off points were 1:10 for quails and 1:50 for dogs.

### 2.6. Copro-parasitological examinations

The stool test was performed three days before the dogs were fed the quail tissues and then daily for 30 days thereafter using the centrifugal flotation method with a sugar solution (density of  $1.26\text{ g/cm}^3$ ). The quantity of feces used was 3 g, and the final precipitate was resuspended, regardless of the microscopy result, in a 2% potassium dichromate solution, kept under aeration for three days and then stored at  $-20^\circ\text{C}$  until the DNA was extracted.

### 2.7. Histopathology (HP) and immunohistochemistry (IHC)

The tissue fragments collected were kept in a 10% buffered formalin solution for 24 h and then transferred to a 70% ethyl alcohol solution. HP and IHC were performed

according to Furuta et al. (2007). The primary antibody used in the IHC was polyclonal serum from a naturally infected bovine (Munhoz et al., 2011) at a 1:6000 dilution.

### 2.8. Extracting DNA from experimentally infected quail tissues and the feces of dogs

Fragments of the collected tissues were transferred to sterilized and labeled cryotubes and stored at  $-80^{\circ}\text{C}$  for PCR. Before the DNA extraction, liquid nitrogen was added to each of the Eppendorf tubes containing the tissues so that they could be macerated with a sterilized glass mortar. After maceration, the DNA was extracted with a commercial kit (Easy-DNA<sup>TM</sup>, Invitrogen, USA) following the manufacturer's recommendations.

The stool samples were submitted to eight heating ( $96^{\circ}\text{C}$ ) and freezing ( $-80^{\circ}\text{C}$ ) cycles lasting 1 min each. Glass pearls (0.5 mm) were then added, and the material was vortexed for 5 min. All of the other phases were performed according to the manufacturer's instructions (Stool Mini Kit<sup>TM</sup>, Qiagen, USA). After the extraction, the DNA samples were stored at  $-20^{\circ}\text{C}$  until they were used in PCR.

### 2.9. Detection of *N. caninum* DNA by PCR on quail tissue fragments and dog feces

To detect *N. caninum* DNA, we performed PCR using the primers Np6 (5'CAGTCAACCTACGTCTTCT3') and Np21 (5'GTGCGTCCAATCCTGTAAC3'), according to Yamage et al. (1996). We used NC-Bahia strand tachyzoite DNA as a positive control (Gondim et al., 2001). Amplification reactions and the electrophoretic run were performed as described by Munhoz et al. (2011).

### 2.10. Statistical analysis

The comparisons of the quails' mean live weight and organ weight were carried with Student's *t*-test with a 95% level of significance. To analyze the organs' live weight, we considered their relative weight (organ weight  $\times$  100/quail's live weight) as the most appropriate for analyzing possible changes.

## 3. Results

In this study, we did not observe cases of morbidity or mortality or signs that could be associated with the presence of *N. caninum* in these species during the experimental period. There was a significant ( $p < 0.05$ ) difference in weight gain between the control group and the two infected groups from the end of the 3rd week of infection. The final average difference between the groups was over 30 g (Fig. 1).

The relative mean value of the weight of the infected quails' spleens was higher than that of the control group ( $p < 0.05$ ) on the 3rd, 7th and 14th DAI, whereas a significant difference ( $p < 0.05$ ) in liver weight was observed only on the 3rd DAI. The presence of *N. caninum* antibodies in the quails was observed beginning on the 7th DAI, and the titration peak occurred on the 14th DAI. After the 30th DAI,

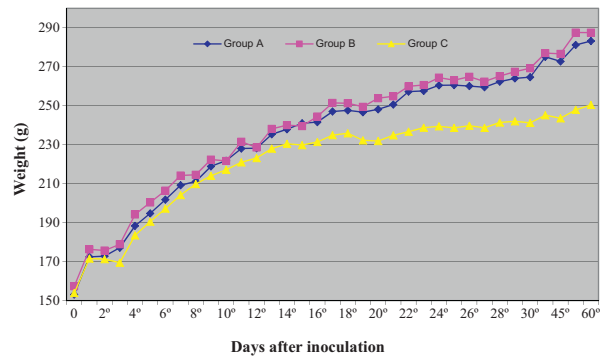


Fig. 1. Mean weight of quails infected experimentally with *N. caninum* tachyzoites (groups A and B) compared to the control group (C) quails.

no quails were found to be positive for *N. caninum*. The dogs remain seronegative by *N. caninum* throughout the study.

The bursa de Fabricius, brain, spleen, liver and lungs were the principal organs that tested positive for IHC and PCR, and they were the most positive during the first two weeks of infection. The histopathology was inconsistent, with the exception of the livers of three infected quails

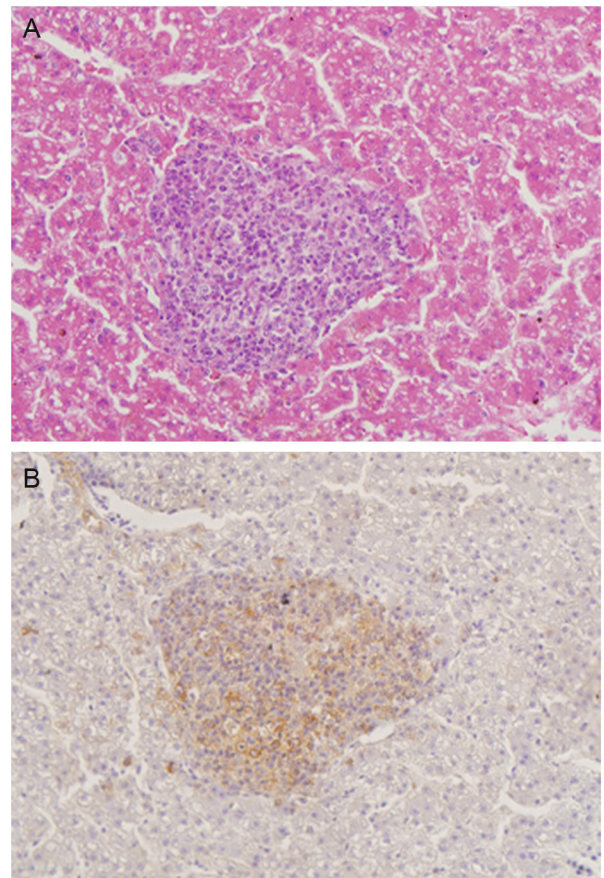


Fig. 2. Histopathological and immunohistochemistry (IHC) findings in quails infected experimentally with *N. caninum*. (A) Mononuclear inflammatory infiltrate in the liver of a quail on the 3rd day after infection, 400 $\times$ , with positive immunoreactivity by IHC, 400 $\times$  (B).

on the 3rd DAI (2 quails) and one on the 7th DAI (1 quail) in which we observed a mononuclear inflammatory infiltrate associated with immunoreactivity by IHC (Fig. 2). No oocysts were found in the dog stools examined during the experimental period. We also did not observe any amplification of parasite DNA using the dog stool samples.

#### 4. Discussion

The quails were resistant to infection by *N. caninum*, and no morbidity or mortality was observed, similar to the results of Furuta et al. (2007) in chickens. This differs from the study with pigeons by Mineo et al. (2009) that reported that one animal died on the 25th DAI. This suggests that pigeons may be more susceptible to *N. caninum*, and this observation was in agreement with a study by McGuire et al. (1999) that identified *N. caninum* in all pigeons analyzed.

The rapid decline of the *N. caninum* antibody titer indicates infection control by the host. Other experiments have confirmed these findings with titration peaks occurring between the second and third week (Furuta et al., 2007; Mineo et al., 2009). In these studies, the authors observed titrations higher than those found in the quails in the present study, and these differences could be due to the infection route used (intraperitoneal), which tends to induce a more intense response from the host. McGuire et al. (1999) found high antibody titers in pigeons on the 6th week after infection with a higher cutoff point than in the present study, which is another indication of the possibility that pigeons are more susceptible to infection. Molina-López et al. (2012) demonstrated that *N. caninum* also has a high prevalence in crows (*Corvus corax*), which belong to the same clade as pigeons.

The weight gain of both infected groups of quails remained similar throughout the experiment and showed no reduction in relation to the control group even during the acute phase of infection, demonstrating that the infection was not capable of decreasing the birds' growth rate. In fact, we observed a gradual increase in the average weight of the infected animals compared to the control animals, beginning with the third week of the infection. The mechanisms responsible for this phenomenon should be the subject of future studies. Our hypothesis is that the weight gain is related to a possible cross-immunity with other species of intestinal coccidian present in birds.

Organs, such as the spleen, bursa of Fabricius, brain, lungs and livers, were positive for *N. caninum* by both PCR and IHC, demonstrating that the parasite may be able to disseminate following infection by the subcutaneous route. However, the number of organs that tested positive was proportionally lower than in other experiments using the intraperitoneal route (Furuta et al., 2007; Mineo et al., 2009). What became evident in our study is that although the parasite was disseminated, its maintenance in the host was limited, and we were not able to identify its preferred organs or to visualize cysts in tissues, as observed by Mineo et al. (2011).

With the concentration of the inoculum used in this study, we expected a large number of birds and their respective tissues to be parasite-positive with the

techniques used. However, this was not the case, and, for example, the tissues of the quail that had the highest IFAT titrations (1:320) did not result positive results by PCR or IHC. Similarly, the organs that were positive for the parasite by technique were negative when another technique was used. A low concentration of parasites or an irregular distribution of parasites may have been responsible for these discrepancies, and using real time PCR may minimize the chances of false negative results during both natural and experimental infections.

Low levels of parasitism or the probable absence of the parasite from tissues demonstrates the resistance of these birds to infection, and this characteristic is supported by the fact that none of the dogs in the study eliminated oocysts or seroconverted. This strengthens the hypothesis that cysts were absent in the tissues, and it is in agreement with the fact that only Furuta et al. (2007) have observed the elimination of oocysts by dogs fed embryonated eggs. The elimination of oocysts in the Furuta study may be due to the high parasite load present in the chorioallantoic membrane.

Quails belong to the clade Gallonserae, the same clade as chickens. Pigeons, which are considered good experiment models (Mineo et al., 2009), belong to the clade Neoaves. Therefore, evolutionary differences may be responsible for the differences in their susceptibility to *N. caninum*. This hypothesis is strengthened by the fact that some species and orders that have been investigated tested negative for exposure to the parasite (McGuire et al., 1999; Mineo et al., 2011; Darwich et al., 2012).

Our study was unable to demonstrate that quails are models of infection and good intermediate hosts for *N. caninum*. However, our study did indicate that the birds may have differing degrees of susceptibility to the parasite, as occurs with mammals. Therefore, new studies will be necessary to verify the course of infection in different groups of birds and to determine their resistance to the parasites. These studies should take into account that factors such as the number of animals used from each species, diagnostic techniques, cutoff, age, degree of exposure and environment may influence the host's response to the parasite.

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