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Veterinary Parasitology 206 (2014) 123–128

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Gallus gallus domesticus are resistant to infection with *Neospora caninum* tachyzoites of the NC-1 strain

Alexandre Dias Munhoz[a,*], Tatiane Fernanda do Amaral[b], Luiz Ricardo Gonçalves[b], Vera Maria Barbosa de Moraes[c], Rosangela Zacarias Machado[b,*]

[a] *Departamento de Ciências Agrárias e Ambientais, Universidade Estadual de Santa Cruz (UESC), Campus Soane Nazaré de Andrade Rodovia, Ilhéus Itabuna, Km 16, Salobrinho, Ilhéus, Bahia 45662-000, Brazil*
[b] *Departamento de Patologia Animal, Universidade Estadual Julio Mesquita Filho, UNESP/Jaboticabal, Rod. Paulo Castelanne s/n, Jaboticabal, São Paulo 14884-900, Brazil*
[c] *Departamento de Zootecnia, Universidade Estadual Julio Mesquita Filho, UNESP/Jaboticabal, Rod. Paulo Castelanne s/n, Jaboticabal, São Paulo 14884-900, Brazil*

ARTICLE INFO

Article history:
Received 2 November 2013
Received in revised form 7 October 2014
Accepted 16 October 2014

Keywords:
Neosporosis
Poultry
Resistance
DNA
PCR

ABSTRACT

The aim of this study was to experimentally evaluate infection in *Gallus gallus domesticus* with *Neospora caninum* tachyzoites of the NC-1 strain. Experimental infection was conducted in 90-day-old chickens, embryonated eggs and bioassays in dogs. In the first experiment, poults were randomly divided into four groups. Groups I and II were provided feed with coccidiostat, whereas groups III and IV received feed without coccidiostat. When the poults from groups I and III reached 90 days of age, they received a subcutaneous inoculation of *N. caninum*. Once the hens entered their egg-laying period, during the following 30 days, the eggs were collected, identified, weighed and placed in an incubator. On the 70th day after inoculation, all animals, including the chicks, were euthanized. Tissue samples from the adult poultry and chicks were collected for histopathology, immunohistochemistry (IHC) and PCR. Brain tissue and pectoral muscle samples from infected birds were fed to two dogs. Notably, the average weight of the group III eggs was lower than that of the group IV eggs ($p < 0.05$). No changes consistent with infection in adult poultry or chicks were detected by histopathology or IHC; moreover, no amplified parasite DNA was detected in the birds' tissues or dogs' feces. No dog eliminated oocysts. In the second experiment, the embryonated chicken eggs were inoculated with 1×10^2 *N. caninum* tachyzoites, on the 10th day of incubation, and chicks born from these eggs were housed in boxes suitable for the species and received commercial feed and distilled water *ad libitum*. On the 30th day after infection (DAI), the poultry were euthanized, and their organs were processed as described in experiment I. The amplification of parasite DNA was observed in the spleen and pectoral muscles of one of the birds. The ingestion of bird tissues by dogs did not result in oocyst elimination. These results indicate that the parasite may have been eliminated by the host and that the use of tachyzoites to induce chronic disease might be a poor source for hens.

* Corresponding authors. Tel.: +55 16 3209 2662; fax: +55 16 3209 2662.
E-mail addresses: munhoz@uesc.br, admruralvet@ig.com.br (A.D. Munhoz), zacarias@fcav.unesp.br (R.Z. Machado).

http://dx.doi.org/10.1016/j.vetpar.2014.10.009
0304-4017/

1. Introduction

Neospora caninum is an obligate intracellular parasite and is the primary cause of miscarriages in cattle (Dubey and Lindsay, 1996). The parasite infects a large number of intermediate hosts (Dubey, 2003), and currently, dogs, coyotes, Australian dingoes and gray wolves are considered to be definitive hosts (McAllister et al., 1998; Gondim et al., 2004; King et al., 2010; Dubey et al., 2014).

The epidemiology of neosporosis, especially in dogs and cattle, has been widely studied (Dubey and Schares, 2006; Dubey et al., 2007a). The dog–cattle–dog cycle has been demonstrated (De Marez et al., 1999; Dijkstra et al., 2001; Gondim et al., 2002), and the large elimination of oocysts by dogs which are fed organs of experimentally infected cattle reveals that bovine animals are natural parasitic hosts (Gondim et al., 2002). However, the contribution of other hosts in this epidemiology has not been fully elucidated and requires further study due to the possibility that canine predation in reservoirs is a factor associated with infection (Wouda et al., 1999; Jesus et al., 2006).

The characterization of hens as a natural host (Costa et al., 2008) and the elimination of oocysts in dogs fed experimentally infected embryonated chicken eggs (Furuta et al., 2007) refer to the possibility that hens are a good reservoir for *N. caninum* in the same manner that hens are for toxoplasmosis (Dubey et al., 2007b; Yan et al., 2010). This hypothesis is reinforced by the findings of Bartels et al. (1999) and Otranto et al. (2003), who identified that the presence of hens on the farm was a risk factor for cattle.

Most adult mammals exhibit no clinical signs of neosporosis (Dubey, 2003). However, there are reports of decreased milk production (Thurmond and Hietala, 1997), the early culling of cows (Thurmond and Hietala, 1996) and efficiency of vertical parasite transmission (Barber and Trees, 1998; Davison et al., 1999; Magalhães et al., 2014). Similar to observations in mammals, adult birds have no clinical signs of disease (McGuire et al., 1999; Furuta et al., 2007; Costa et al., 2008; Mineo et al., 2009; Gondim et al., 2010), even when they are challenged with high parasite doses in experimental infections (Furuta et al., 2007; Mineo et al., 2009).

Different patterns of pathogenicity among *in ovo* infections have been reported. These differences result in outcomes such as embryo death, normal birth with infections in chicks, and chicks showing neurological symptoms, such as ataxia and pedaling movements (Mansourian et al., 2009). These patterns are associated with the inoculum concentration and inoculation period in embryo (Furuta et al., 2007; Mansourian et al., 2009; Khodakaram-Tafti et al., 2012), the bird species involved (Mansourian et al., 2013), the number of parasite passages in cell culture (Namavari et al., 2012) and the type of cell culture used (Khordadmehr et al., 2013).

Previous studies have shown a greater isolation of the parasite in the brain (Dubey et al., 2007a; Gondim et al., 2001; Rodrigues et al., 2004; Cavalcante et al., 2011). In birds, the migration of the parasite from the organs to the muscles has been proposed by Furuta et al. (2007) and Mineo et al. (2011). Therefore, it is possible that *N. caninum* presents tropism for these sites in these animals.

In the poultry industry, the risk of clinical eimeriosis is prevented by the constant use of coccidiostats that are added to the diet (Carvalho et al., 2011). However, it is still unclear whether coccidiostat use can minimize the effect of *N. caninum* infection in commercial poultry. Therefore, little is known concerning the influence of infection over the zootechnical indices and the possibility of vertical transmission in these animals. Therefore, the aims of this study were threefold: first, to evaluate the experimental infection of 90-day-old chickens (*G. gallus domesticus*) with *N. caninum* tachyzoites fed diets with or without coccidiostat; second, to evaluate the experimental infection of embryonated eggs; and third, to characterize the possible elimination of oocysts in dogs fed poultry tissues that had been infected in the first and second experiments.

2. Materials and methods

2.1. Neospora caninum (NC-1) samples

Neospora caninum tachyzoites from the NC-1 isolate (Dubey et al., 1988), which were grown in CV-1 cells that were maintained according to the methodology described by Furuta et al. (2007), were used. The parasites were counted in a Neubauer chamber, and the final inoculum per bird was 3×10^6 tachyzoites.

2.2. Experimental infection in poultry

2.2.1. Experiment I

On the first day of embryogenesis, twenty embryonated pathogen-free chicken eggs (Hy-Line do Brasil Ltda, Nova Granada, SP, Brazil) were used in the experiments. The eggs were placed in a rotating incubator and maintained at a temperature of $37.7 \pm 2\,^{\circ}C$ and $55 \pm 5\%$ humidity. From the 7th day until hatching, eggs were taken to the ovoscope (i.e., an egg tester) daily to diagnose embryo viability. After hatching, twelve female chicks and eight male chicks were housed in boxes suitable for their species and were provided commercial feed and distilled water *ad libitum*.

On the 75th day of age, the poultry were randomly divided into four groups of five birds (three females and two males). Groups I and II were provided feed containing coccidiostat, whereas groups III and IV received feed without coccidiostat. When the poultry from groups I and III reached 90 days of age, they received a subcutaneous inoculation of *N. caninum*, whereas those from groups II and IV were inoculated with a placebo.

One week before inoculation, and on days 0, 7, 14, 21, 28, 35, 42, 56 and 70 after inoculation, the birds' weight was recorded (using a pediatric weight balance). Blood samples were concurrently collected for serology. Every three days, rations were provided, and their intake was recorded. After the egg-laying period was initiated and during a period of 30 days, the eggs were collected, identified, weighed and placed in an incubator. On the 70th day after inoculation, all birds were euthanized as well as their chicks, after birth, following the procedures standardized by the Brazilian College of Ethics and Animal Welfare (*Colégio Brasileiro de Ética e Bem-Estar Animal*—COBEA).

2.2.2. Experiment II

On the first day of embryogenesis, six embryonated pathogen-free chicken eggs (Hy-Line do Brasil Ltda, Nova Granada, SP, Brazil) were used in the experiments. These eggs were placed and packaged as in experiment I. On the 10th day, five eggs were inoculated with 1×10^2 *N. caninum* tachyzoites and one with a placebo. Four birds were born (three from infected eggs and one from the control egg) and housed in boxes suitable for their species. The chicks received commercial feed and distilled water *ad libitum*. On the 30th day after infection (DAI), blood collections were performed for serology, and the animals were euthanized following the procedures determined by COBEA.

2.3. Histopathology and immunohistochemistry

Sections of brain, liver, heart and pectoral musculature of adult birds and chicks from experiment I and brain, cerebellum, liver, heart, thymus, lung, kidney and pectoral musculature tissue of birds from experiment II were placed in 10% buffered formalin for 24 h and then transferred to 70% alcohol to perform histopathology (HP) and immunohistochemistry (IHC) according to Furuta et al. (2007) with some modifications. For IHC, antigen retrieval was performed in a citrate buffer solution (pH 6.0) in a water bath at 95 °C for 30 min, and endogenous peroxidase blocking was performed in a solution of methyl alcohol with hydrogen peroxide at 8% for 20 min. Polyclonal serum from a naturally infected bovine animal was used as the primary antibody (Munhoz et al., 2011) at a dilution of 1:1000.

2.4. Bioassays in dogs

Four dogs aged two months were accommodated in individual stalls and vaccinated against distemper, parvovirus, leptospirosis, influenza, infectious hepatitis, coronavirus. All pups were from the same litter and the bitch was negative for anti-*N. caninum* antibodies. The animals were dewormed (Praziquantel 5 mg/kg and Pyrantel Pamoate 14.4 mg/kg single dose and repeated after 15 days), treated with giardia protozoacide (metronidazole, 10 mg/kg, twice daily for 5 days) and were seronegative for *Babesia canis, Toxoplasma gondii, N. caninum, Leishmania chagasi*, and *Ehrlichia canis*. Further to this treatment, a diet based on commercial feed was assured, without the possibility of raw meat ingestion, or of any other feed, in order to avoid possible coccidian infection. Every day the stalls were cleaned and disinfected with a solution of formaldehyde at 1% and sodium hypochlorite at 2%, and the animals were submitted to a physical and clinical assessment.

The dogs were fed with infected bird tissue as follows: dogs 1 and 2 received a pool of brains and pectoral muscles of birds from groups I and III (experiment 1), respectively. Each dog was fed with approximately 12 g of pooled brain and 20 g of muscular tissues. In the 2nd experiment, dog number 3 received pooled organs (approximately 60 g) collected from the infected birds, whereas dog number 4 received the pooled organs from the control bird. Every week during a 28 day period, blood was collected from the jugular vein of dogs to obtain serum, which was stored at −20 °C until its use in the indirect fluorescent antibody test (IFAT) for anti-*N. caninum* antibodies.

2.5. Coproparasitological examinations

One week before and thirty days after the dogs were fed (experiments I and II) with chicken organs, feces were examined daily using the centrifugal flotation method with a sucrose solution (density of 1.26 g/cm^3) and Sheather's method. Only 20 μL of the meniscus was removed for observation of *N. caninum* oocysts in an optical microscope. The remnant stools were homogenized in distilled water at 2 g per 10 mL, filtered through a double layer of gauze and centrifuged for 10 min at 735 × *g*. The supernatant was discarded, the sucrose solution (1.26 g/cm^3 density) was added to the pellet, and the solution was again centrifuged for 10 min at 735 × *g*. A 3 mL aliquot of the supernatant was removed, and 10 mL of distilled water was added. The material was centrifuged as described above. The precipitate was resuspended in a 2.5% potassium dichromate solution, and, regardless of the microscopy result, was kept under aeration for three days and stored at −20 °C until DNA extraction was performed.

2.6. Serology

Sera were tested for anti-*N. caninum* antibodies by IFAT according to the methods of Yamane et al. (1997) using NC-1 strain tachyzoites (Dubey et al., 1988) and a serum dilution of 1:20 (birds) and 1:25 (dogs). Chicken anti-IgG (Sigma®-Aldrich F4137 Inc., St Louis, MO, USA) and dog anti-IgG (Sigma®-Aldrich F4012 Inc., St Louis, MO, USA) were used as secondary antibodies and were diluted 1:50 and 1:32 in PBS, respectively. Dog and bird positive controls were by Furuta et al. (2007).

2.7. DNA extraction and PCR for N. caninum

Collected tissues fragments (Section 2.3) from both experiments I and II and 200 μL the dog stool samples (Section 2.5) were stored at −70 °C, in DNAase- and RNAase-free tubes, and the DNA was extracted using the DNeasy kit (Qiagen™) and DNA Stool kit (Qiagen™) according to manufacturers' recommendations. After extraction, the DNA concentration of each sample was determined using a Nanodrop™. In the dog stool samples, before the addition of proteinase K, eight 1-min cycles were performed (−70 °C, 96 °C), followed by stirring with 0.5 mm glass beads for 5 min.

PCR was conducted using the following primer pair: 5′-CAG TCA ACC TAC GTC TTC T-3′(Np6) and 5′-GTG CGT CCA ATC CTG TAA C-3′ (Np21), which amplified a 328 bp segment (Yamage et al., 1996). The amplification reactions were performed with a final 25 μL volume as follows: 5 μL of DNA sample, 0.5 μM of each oligonucleotide primer, 1 mM of $MgCl_2$, 200 μM of a mixture of dNTPs and Taq polymerase (Invitrogen 1 U/reaction) and finally sufficient ultrapure distilled water to reach the total reaction volume. The amplification process was performed on a PTC-200 thermocycler (MJ Research) with a 40-cycle program that included the following steps: denaturation (94 °C/1 min),

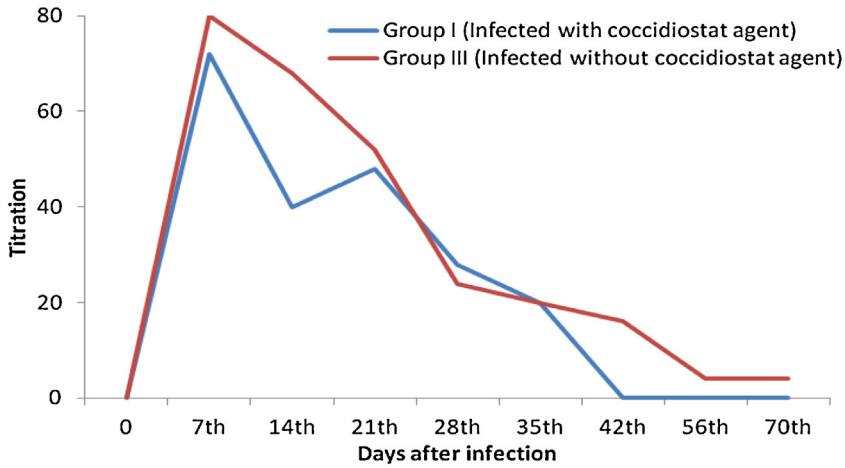

Fig. 1. The mean antibody titration of *Gallus gallus domesticus* that were experimentally infected with *Neospora caninum* tachyzoites and fed diets with or without coccidiostat agents.

annealing (50 °C/1 min) and extension (74 °C/3.5 min). We used NC-1 strain tachyzoite DNA as a positive control in the PCR and sterile double distilled water as a negative control.

2.8. Statistical analysis

The data related to feed intake and the bird and egg weights were statistically analyzed by Tukey's test with a 95% confidence interval.

3. Results

3.1. Experimental infection in poultry

3.1.1. Experiment I

All infected animals on infected groups were serologically positive on the 7th DAI, with their titers ranging from 20 to 160. On the 14th DAI, the titers began to decline quickly. Within one month of infection, most poultry were seronegative, and only one hen from group III remained seropositive (1:20) until the end of the experiment (Fig. 1). All control animals were negative throughout the experiment. There were no significant differences in feed intake or bird weight among the groups. Nonetheless, the average weight of the group III eggs during the experimental period (infected and fed without coccidiostat agents) was lower compared with that of the group IV eggs ($p < 0.05$) (Table 1).

Table 1
The mean weight of hens' eggs from the control group and experimentally infected group with *Neospora caninum* tachyzoites.

Groups	Mean egg weight
Group I (infected with coccidiostat agent)	$40.80 \pm 4.62^{a,b}$
Group II (control with coccidiostat agent)	$40.76 \pm 2.52^{a,b}$
Group III (infected without coccidiostat agent)	38.81 ± 6.01^{b}
Group IV (control without coccidiostat agent)	43.83 ± 7.56^{a}

Different letters $p < 0.05$.

No changes consistent with the infection were detected by histopathology or IHC. No PCR amplified DNA parasite was observed on collected and analyzed tissues.

3.1.1.1. Chick evaluation. Four chicks per group were born clinically normal and exhibited no noticeable clinical signs consistent with infection. Neither PCR amplified parasite DNA nor a change in histopathology or immunoreactivity by IHC was detected in the analyzed animal tissues (Section 2.4).

3.1.2. Experiment II

Three chicks were born clinically normal (two embryos died 72 h after inoculation, with compatible alterations with infection) and exhibited no positive serology at the time of euthanasia. There were no microscopic changes consistent with infection and no immunoreactivity by IHC. Amplified *N. caninum* DNA was detected in the spleen and pectoral muscles of one of birds.

3.2. Infection in dogs

In both experiments, no dogs exhibited clinical signs of infection, seroconverted, or eliminated oocysts that could be visualized by microscopy or were molecularly diagnosed by PCR in their stool samples.

4. Discussion

The choice of a 1×10^2 tachyzoites dose (experiment II) was based on a previous study (data not available) to determine the best inoculum to be used for the experiment. Embryos were inoculated with 1×10^2 tachyzoites on day 8 of development, and other groups were inoculated with 1×10^1, 1×10^2 and 1×10^3 tachyzoites on the 10th day of development. No higher inoculum was performed at 1×10^3 because the undesirable effect related to high mortality of the embryo, and the birth of chicks with neurological signs was described by Furuta et al. (2007).

As a result, all embryos, which received 1×10^2 tachyzoites on the 8th day of development, died by the 9th DAI. Embryos that received 1×10^3 engaged in similar behavior to those observed by Furuta et al. (2007), except for the immediate death of the chicks that hatched. The groups inoculated with 1×10^1 and 1×10^2 have similar behavior together with 60% hatching of chicks without clinical signs, resembling the study Mansourian et al. (2009) which recommended an inoculum dose of $1 \times 10^{2,3}$ tachyzoites for broiler chickens. Thus, we chose 1×10^2 dose for allowing infection with a low mortality rate with high hatching rate of asymptomatic animals.

At the end of the first experiment, the absence of anti-*N. caninum* antibody titers, the detection of DNA and immunoreactivity by IHC are evidence that the parasite may have been eliminated by the host. The absence of clinical signs in the adult birds corroborates the findings of other studies involving birds (McGuire et al., 1999; Furuta et al., 2007; Mineo et al., 2009; Oliveira et al., 2013). During all experiment stages, the infected animals from different groups exhibited no changes in weight gain and feed intake.

In contrast, the presence of parasite DNA in one animal from experiment II implies that either there was a shorter infectious period of the parasite into the vertebrate host that lacked timely parasite elimination or that parasite dissemination and proliferation in the embryo, whose immune system was still in formation, had easily occurred.

Although the quantity of bird tissues provided was lower than that provided in other studies (Gondim et al., 2002; Cavalcante et al., 2011) and might have been insufficient to promote cyst ingestion, the absence of oocyst shedding in dogs associated the other findings, reinforces the resistance of poultry to infection because the infected tissues of an effective intermediate host result in the elimination of thousands of oocysts (Gondim et al., 2002). Therefore, these results indicate that hens may be a poor intermediate *N. caninum* host, similar to the study of Gonçalves et al. (2012) and Oliveira et al. (2013).

In this context, the success of Furuta et al. (2007) in the oocysts shedding in dogs fed embryonated eggs was due to the high concentration of parasites in these tissues (embryo and membranes), thus demonstrating that infection susceptibility in birds "in ovo" appears to be distinct in adult animals.

The maintenance of strain utilized in the study (CV-1 cell culture in less than 30 passages) suggests that it was not attenuated (Bartley et al., 2006; Namavari et al., 2012; Khordadmehr et al., 2013). This condition corroborates the hypothesis that the resistance of certain bird groups to infection, as this same strain was able to induce death in pigeons (Mineo et al., 2009).

A subcutaneous route has been successfully used in the infection of *T. gondii* tachyzoites in birds with a subsequent parasite isolation in various tissues (Albuquerque et al., 2002; Munhoz et al., 2004). Therefore, the hypothesis that a subcutaneous route would provide better maintenance and infection conditions for hens has emerged. However, regardless of the inoculation route, the results of this study have demonstrated resistance to infection by tachyzoites, as observed by Oliveira et al. (2013) in quails (subcutaneous route) or Furuta et al. (2007) in chickens (intraperitoneally route). Unlike observations for other animal species, the tachyzoite infection does not appear to be appropriate to induce chronic infections in hens (Gondim et al., 2002).

One hypothesis that could explain the resistance of poultry is that high body temperatures would encourage tachyzoites to form cysts or even contribute to their destruction. Hence, once the parasite is detected in naturally infected hen brains (Costa et al., 2008) and infection by tachyzoites seems to be lasting, it is likely that the oocysts infection leads to different parasite behavior with a migration of infectious forms, which allows better development of the parasite either through the circulatory or the lymphatic system.

Importantly, although there were significant differences in egg weight, changes occurred only in groups receiving diet without coccidiostat; thus, it was not possible to make a definitive conclusion. Further studies are required to evaluate this condition because in commercial poultry production, either extensive or subsistence systems, it is possible that female breeders are fed diets without the presence of coccidiostat agents.

The egg-laying period occurred approximately 30 days after infection in conjunction with a decline in anti-*N caninum* antibody titers. Moreover, the birds might have eliminated the infection, which might have contributed to the absence of vertical transmission. This result obviated the need for further studies because it was not possible to confirm whether the transmission would have occurred if the infection of the birds had been held during the egg-laying period, as occurs in experimentally infected cows on 30th week of gestation (Williams et al., 2000).

In this context, the infection of hens with oocysts may serve as a model to evaluate the relevance of hens as a good reservoir of infection by observing oocysts in the feces of dogs that were fed tissues of these birds, the feasibility of vertical transmission, and the organ predilection of parasites. Understanding these issues necessitates further studies on this subject.

Acknowledgements

We are thankful to Hy-Line do Brasil Ltda, Rações Fri-Ribe S/A, Prof. Dr. Antonio Carlos Paulilo (Universidade Estadual Julio Mesquita Filho, campus de Jaboticabal) and to Programa Nacional de Cooperação Acadêmica—Novas Fronteiras no. 1512/2007-NF/CAPES).

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