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Veterinary Parasitology 134 (2005) 33–39

veterinary parasitology

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Effects of host maturity and prior exposure history on the production of *Neospora caninum* oocysts by dogs

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Received 21 March 2005; received in revised form 14 June 2005; accepted 14 June 2005

Abstract

To investigate whether dogs shed *Neospora caninum* oocysts more than once, five dogs with a previous history of shedding oocysts were fed infected bovine tissues. Two of three dogs shed oocysts when they were re-exposed 18–20 months after the first challenge; two other dogs re-exposed earlier, only 8 months after the primary exposure, did not produce oocysts. These results suggest that dogs may become refractory to shedding *N. caninum* oocysts for a period approximately between 8 and 18 months after a primary infection; however, this possibility requires statistical validation by testing of more dogs. The development of a high antibody titer did not ensure that a dog would completely resist shedding oocysts after consuming an infected meal. Oocyst production was also compared between puppies and adult dogs with primary infections. Twelve puppies (three from the present study and nine from a previous study) shed significantly more oocysts (mean: 166,400) compared with five adult dogs following primary exposure (mean: 2900), indicating that a dog's age can influence *N. caninum* oocyst production ($P = 0.02$).
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Keywords: *Neospora caninum*; Dogs; Oocysts; Transmission

1. Introduction

Neospora caninum is a protozoan parasite of domestic (Anderson et al., 1991; Barber and Trees, 1996) and wild animals (Woods et al., 1994; Dubey et al., 1996). Neosporosis is a frequent cause of bovine abortion worldwide (reviewed by Dubey, 1999). Oocysts of the parasite are shed in the feces of dogs (McAllister et al., 1998) and coyotes (*Canis latrans*) (Gondim et al., 2004c). Wolves are also suspected to be a definitive host (Gondim et al., 2004b). An association of the presence of dogs with high rates of *N. caninum* infection or abortion in cattle is supported in many epidemiological studies (Paré et al., 1998; Bartels et al., 1999; Mainar-Jaime et al., 1999; Wouda et al., 1999; Dijkstra et al., 2002; Moore et al., 2002; Otranto et al., 2003; Sanchez et al., 2003; Schares et al., 2003). Cattle, which are intermediate hosts of *N. caninum*, can be infected by ingesting oocysts (De Marez et al., 1999; Gondim et al., 2002; Trees et al.,

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0304-4017/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetpar.2005.06.011

2002). Transplacental transmission is common in naturally infected cows (Anderson et al., 1997; Schares et al., 1998; Davison et al., 1999; Bergeron et al., 2000), and transplacental infection and abortion were recently demonstrated experimentally in cows administered *N. caninum* oocysts (Gondim et al., 2004a).

Following consumption of tissues from a *N. caninum*-infected intermediate host, it is unknown if dogs may spontaneously re-shed oocysts after the initial patent period. It is also unknown if previously infected dogs may resist oocyst-production in the event that they ingest *N. caninum* infected tissues for a second time. McGarry et al. (2003) recently reported shedding of *N. caninum* oocysts by a naturally infected foxhound when examined twice in 4 months; the source of infection and the frequency that the dog was exposed to *N. caninum* was not shown, but the dog had a history of routinely consuming raw bovine tissues. Other factors that may influence *N. caninum* oocyst shedding have not yet been studied, such as dog age, sex, breed, pregnancy, lactation, and concurrent diseases.

The aims of the present experiment were to: (1) examine dogs for *N. caninum* oocyst shedding during a prolonged period, following ingestion of tissues of infected calves; (2) investigate if previously infected dogs are refractory to oocyst production when they ingest calf tissues infected with a heterologous strain of *N. caninum*; (3) compare the number of oocysts shed by puppies with the number shed by adult dogs.

2. Materials and methods

2.1. Infection of calves with N. caninum

Ten newborn dairy bull calves were obtained from the University of Illinois Dairy, after consuming colostrum. Each calf tested negative for antibodies against *N. caninum* by an immunofluorescent antibody test (IFAT) using a dilution of 1:25 as a cutoff (see serology section below). They were housed indoors on a slatted floor, without bedding, and fed commercial milk replacer without antibiotics. The calves were infected with 2×10^6 to 3.2×10^7 tachyzoites of *N. caninum* of the strains NC-beef (McAllister et al., 1998, 2000) or NC-Liverpool (Barber et al., 1995). Calves with *N. caninum* antibody titers $\geq$1:800 were killed by captive bolt between 6 and 8 weeks after infection.

2.2. Infection of dogs

A total of 13 dogs were used in the present experiment. Five female mixed-breed hounds, that shed *N. caninum* oocysts between 5 and 28 days after ingestion of *N. caninum*-infected calf tissues, were selected from a previous study (Gondim et al., 2002). Another eight female mixed-breed hounds, consisting of five adults (2–3 years old), and three puppies (10–14 weeks old), were purchased from the same commercial class A breeder (Covance Inc., Madison, WI) and served as positive controls. These 8 positive control animals had never consumed raw meat and were seronegative for *N. caninum* by IFAT at a screening dilution of 1:25 (described in Section 2.5).

The five dogs that had previously shed *N. caninum* oocysts, identified as A–E, were re-challenged with infected calf tissues between 8 and 20 months after initial infection. Each dog ingested calf tissues infected with a different strain of *N. caninum* from the first challenge (Table 1). A total of 0.5–3 kg of calf tissues, which were cut in pieces of about 3 cm^3 and mixed, was consumed by each dog in a period of 1–3 days. Dogs A and B were fed only brain and spinal cord. Dogs C–E were fed a mixture of brain, spinal cord, heart, kidney, tongue, diaphragm, and other skeletal muscles. For each test dog, one naïve adult control dog simultaneously received identical amounts of mixed tissue from the same calves. The times between first and second exposures are included in Table 2. For three of the test dogs (C–E), three naïve puppies were fed an equal amount of the same tissues

Table 1
Strains of *Neospora caninum* in tissues of calves which were used to induce primary and secondary infections in dogs

Dog	First exposure with calf tissues (Gondim et al., 2002)	Second exposure with calf tissues
A	NC-2	NC-beef
B	NC-Illinois	NC-beef
C	NC-Illinois	NC-Liverpool
D	NC-beef	NC-Liverpool
E	NC-Illinois	NC-beef

Table 2
Production of *N. caninum* oocysts in dogs after repeated exposure to *N. caninum*-infected calf tissues

Re-exposed dogs				Control adults		Control puppies	
ID	Time between first and second exposure (month)	Oocysts produced after first exposure as puppies (Gondim et al., 2002)	Oocysts produced at second exposure	ID	Oocysts produced after first exposure	ID	Oocysts produced after first exposure
A	8	23500	0	Adult 1	0	NA	NA
B	8	25100	0	Adult 2	2000	NA	NA
C	19	345900	0	Adult 3	1200	Pup 1	504400
D	20	54100	1700	Adult 4	11400	Pup 2	45200
E	18	5700	11600	Adult 5	0	Pup 3	500

Dog IDs 7, 8, 12, 10, and 11 in Gondim et al. (2002) correspond to A, B, C, D, and E in the present study. Dogs A, B, adults 1, and 2 were fed only brain and spinal cord; all other animals consumed multiple tissue types.

from the same infected calves. In addition to comparing oocyst production between previously exposed and naïve dogs, oocyst production was also compared between naïve control puppies and naïve control adult dogs following primary infection.

After the initial patent period following the primary infection, fecal samples from dogs A to E were examined once a week. At the time of the second experimental infection, feces were examined for four consecutive days before ingestion of calf tissues, and daily from the 3rd to the 28th day after exposure; positive control dogs and pups were handled identically.

2.3. Fecal examination and isolation of oocysts

Each fecal sample, corresponding to a total fecal volume collected in 24 h, was homogenized and 5 g were examined for oocysts using a standard sucrose flotation technique, as previously described (Gondim et al., 2002). The total number of oocysts produced was extrapolated from the weight of the dog's feces. Fecal samples containing oocysts were mixed with five volumes of 2% H_2SO_4 in plastic bottles and aerated for 3 days at room temperature for oocyst sporulation. The samples were refrigerated at 4 °C for further use.

2.4. DNA extraction and PCR of isolated oocysts

Sporulated oocysts in acidic fecal samples were concentrated by sucrose flotation, and washed three times in water and 1 time in sterile PBS by centrifugation ($800 \times g$ for 10 min). The sediment was mixed with 700 μl of digestion buffer (100 mM NaCl, 10 mM Tris–HCl, pH 8.0, 25 mM ethylenediaminetetraacetic acid, 0.5% sodium dodecyl sulfate), 5 μl of proteinase K (20 mg/ml), and added to a 1.5 ml tube containing 500 μl of glass beads (0.5 mm in diameter). The mixture was vortexed for 10 min and incubated at 65 °C for 2 h. DNA was extracted by standard phenol/chloroform followed by 2-propanol precipitation.

PCR for *N. caninum* was performed using the specific primer pair Np21/Np6 (Yamage et al., 1996);

Table 3
Antibody titers to *N. caninum* in dogs exposed twice to *N. caninum*-infected bovine tissues, and in matched control dogs and control puppies after primary exposure

Re-exposed dogs			Control adults		Control puppies	
ID	IFAT at time of second exposure	IFAT 4 weeks after second exposure	ID	IFAT 4 weeks after first exposure	ID	IFAT 4 weeks after first exposure
A	1:400	1:400	Adult 1	1:400	NA	NA
B	1:200	1:400	Adult 2	1:200	NA	NA
C	<1:50	1:100	Adult 3	1:200	Pup 1	<1:50
D	1:1600	1:1600	Adult 4	<1:50	Pup 2	1:800
E	<1:50	1:800	Adult 5	1:200	Pup 3	1:800

All dogs were seronegative to *N. caninum* at 1:25 by IFAT before the first exposure to *N. caninum*-infected bovine tissues.

the PCR conditions comprised an initial denaturing step at 94 °C for 1 min, followed by 40 cycles at 94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min, with a final extension step at 72 °C for 2 min. A positive control (*N. caninum* DNA) and a negative control (Vero cell DNA) were included in the test. PCR to detect *Hammondia heydorni*, which has similar oocysts to those of *N. caninum* (Ellis et al., 1999; Šlapeta et al., 2002), was conducted using the species-specific primers JS4/JS5 and PCR conditions described by Šlapeta et al. (2002); a positive control (*H. heydorni* DNA, kindly provided by Dr. John Ellis) and a negative control (Vero cell DNA) were included in each test. PCR products were electrophoresed on 2.0% agarose gel stained with ethidium bromide in a 0.5× TBE buffer, at 100 V for 1 h, and visualized under ultra-violet illumination.

2.5. Serology

Calf and dog sera were tested for *N. caninum* antibodies by IFAT. Tachyzoites of the NC-beef strain (McAllister et al., 1998, 2000) were used as antigen. A fluorescein isothiocyanate (FITC)-conjugated anti-bovine IgG (Bethyl, Montgomery, TX) was employed as a second antibody for calf sera, and a FITC-conjugated anti-dog IgG (Bethyl, Montgomery, TX) was used for dog sera. Sera of calves and dogs were screened at a conservative cut-off (1:25) prior to infection. After infection, sera were tested at 1:50, and maximum antibody titers were determined.

2.6. Statistics

Oocyst production was compared between the 5 control adult dogs and 12 puppies (3 puppies from the present experiment, combined with data from 9 identically handled puppies from a previous experiment (Gondim et al., 2002)) using a two-sided Mann–Whitney test with a confidence interval of 95%.

3. Results

3.1. Oocyst production after the second challenge

No oocysts were detected in dogs A, B, and C, which were re-infected after 8, 8, and 19 months, respectively (Table 2). Dogs D and E shed *N. caninum* oocysts after re-exposure to infected-bovine tissues 20 and 18 months after the primary infection, respectively. Oocysts were confirmed to be *N. caninum* by microscopic examination and using specific PCR primers (Yamage et al., 1996). PCR for *H. heydorni* using the same oocyst DNA yielded negative results.

3.2. Spontaneous re-shedding of oocysts

Two dogs (A and B) spontaneously re-shed a small number of oocysts 2 months after the first exposure. Once oocysts were detected in the weekly fecal exam, fecal samples were examined on the following 2 days to obtain higher numbers of oocysts for molecular tests. Dog A shed an estimated total of 640, 665, and 170 oocysts on the 3 days, respectively. Dog B shed 130, 0, and 0 oocysts, respectively. Thereafter, neither dog was observed to shed oocysts in weekly checks.

3.3. Oocyst shedding in control puppies and control adults after primary exposure

Six of eight positive control animals shed oocysts after consuming infected calf tissues (Table 2). PCR tests for *N. caninum* and *H. heydorni* were performed on oocysts shed by the three control animals that shed the most oocysts; the tested oocysts were positive for *N. caninum* and negative for *H. heydorni*. Sufficient DNA could not be extracted from oocysts produced in low numbers by the other control animals to permit PCR testing.

The number of oocysts shed by the three puppies used in the present study, combined with previous results in nine puppies (Gondim et al., 2002) was significantly higher (mean: 166,400) than the number of oocysts shed by the five control adult dogs (mean: 2900) ($P = 0.02$).

3.4. Serologic response

N. caninum antibody titers in the five dogs that consumed *N. caninum* twice, and in the matched control dogs and puppies are presented in Table 3.

Six of eight control animals tested positive for *N. caninum* antibodies by IFAT, 4 weeks after ingestion of *N. caninum*-infected tissues, with the titers ranging between 200 and 800. The two control animals (one

pup and one adult) that remained seronegative for *N. caninum* shed 504,400 and 11,400 oocysts, respectively. The two control dogs that did not shed oocysts (both adults) became seropositive for *N. caninum*.

4. Discussion

In weekly fecal exams, two dogs spontaneously re-shed small numbers of oocysts 2 months after a primary challenge with *N. caninum*-infected calf tissues. However, re-shedding was brief and production minor compared to the initial patent period.

When re-exposed after an 8 month period, two of two dogs did not shed *N. caninum* oocysts. Thus, it is possible that immunity resulting from a single exposure may last for more than 8 months. In contrast, two of three dogs did shed *N. caninum* oocysts when they were re-challenged between 18 and 20 months after the first infection, suggesting that if protective immunity does occur, it may lapse before this time. However, the number of dogs that could be tested in this experiment was too small to permit statistical confirmation, and further testing is needed. It is interesting to note that one of the dogs that re-shed oocysts had an antibody titer of 1:1600 at the time of the second challenge with infected tissues, so a high antibody titer, or a humoral immune response by itself, does not ensure that a dog will be refractory to the production of oocysts.

Data from three puppies in the present study were combined with data from nine puppies from a previous study (Gondim et al., 2002); oocyst production by puppies was significantly higher (total of 12 puppies, mean: 166,400) than oocyst shedding by five adult dogs following primary exposure to *N. caninum* (mean: 2900). This indicates that a dog's age can influence oocyst production. However, two control adult dogs only consumed brain and spinal cord tissues, so it is possible that the type of tissue may also influence oocyst production.

Oocysts shed by three of the control animals were tested for *N. caninum* and *H. heydorni*, and were positive for *N. caninum* and negative for *H. heydorni*. Sufficient DNA could not be extracted from oocysts produced in low numbers by three dogs. It is highly unlikely that these three dogs were concurrently infected with *H. heydorni*; the *N. caninum* experimentally infected calves used to feed the dogs were reared in isolation and fed milk replacer. It has not been demonstrated that *H. heydorni* can cause congenital infections in cattle.

The concept that a vaccine could be developed to prevent farm dogs from shedding *N. caninum* oocysts appears feasible, based on the possibility that dogs may become refractory to shedding oocysts after a primary infection. As an example, a prototype oral vaccine successfully prevented cats from shedding *Toxoplasma gondii* oocysts, although it was impractical for mass-manufacturing (Frenkel et al., 1991; Freyre et al., 1993). This *T. gondii* vaccine, which consisted of live attenuated bradyzoites extracted from murine brains and stored in liquid nitrogen, prevented 84% or 100% of cats from shedding oocysts after one or two vaccinations, respectively (Frenkel et al., 1991; Freyre et al., 1993). A possible limiting factor for use of a similar live *N. caninum* bradyzoite vaccine for dogs would be the delivery and storage of frozen bradyzoites. However, the same liquid nitrogen dewars that are used to store bovine semen for artificial insemination of cows could be used to transport and store a frozen vaccine for farm dogs. In vitro production of *N. caninum* bradyzoites (Weiss et al., 1999; Tunev et al., 2002; Vonlaufen et al., 2002, 2004; Risco-Castillo et al., 2004) could possibly lead to the development of an oral vaccine for farm dogs, to reduce the risk that they would transmit *N. caninum* infections to cattle.

Acknowledgements

The authors thank Eric Leisner and Christopher Learned for animal care. This study was supported by the Research Board of the University of Illinois. Luis F.P. Gondim was supported by a fellowship from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) of Brazil.

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