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IMPROVED PRODUCTION OF *NEOSPORA CANINUM* OOCYSTS, CYCLICAL ORAL TRANSMISSION BETWEEN DOGS AND CATTLE, AND IN VITRO ISOLATION FROM OOCYSTS

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ABSTRACT: Scarce information is available about *Neospora caninum* oocysts and sporozoites, in part because only small numbers of oocysts have typically been produced by experimentally infected dogs. We hypothesized that 1 reason for low experimental production of oocysts is that dogs have been fed tissues from experimentally infected mice instead of tissues from cattle (which are natural intermediate hosts of *N. caninum*). In this study, 9 dogs were fed tissues from *N. caninum*–infected calves, and oocyst production was compared with 6 dogs that were fed infected mouse carcasses. The number of oocysts produced by dogs that ingested infected calf tissues (mean = 160,700) was significantly greater ($P = 0.03$) than the number of oocysts shed by dogs that ingested infected mice (mean = 5,400). The second goal of our experiment was to demonstrate cyclical oral transmission of *N. caninum* between dogs and cattle. As few as 300 oocysts were used to successfully infect calves, and tissues from these calves induced patent infections in 2 of 3 dogs; oocysts from 1 of these dogs were administered to another calf, and tissues from this calf subsequently induced a third dog to shed oocysts. Oocysts were confirmed to be *N. caninum* using a species-specific polymerase chain reaction technique. In addition, sporulated oocysts were used to recover *N. caninum* in vitro after digestion in an acid–pepsin solution and inoculation of cell monolayers.

Neospora caninum is a protozoan parasite that is closely related to *Hammondia heydorni* and *Toxoplasma gondii* (Ellis et al., 1999). Neosporosis causes neuromuscular infection in dogs and is a common cause of bovine abortion (Dubey, 1999). Natural infections of the parasite have been identified in several other species of ruminants, including goats, sheep, deer (Dubey and Lindsay, 1996), and water buffaloes (Guarino et al., 2000). It is not clear whether original descriptions of neosporosis in horses were caused by *N. caninum* or the more recently described *N. hughesi* (Marsh et al., 1998). Dogs were demonstrated to be definitive hosts of *N. caninum* when they shed oocysts after consuming infected mice (McAllister et al., 1998; Lindsay et al., 1999). Since then, oocysts of the parasite have been found in naturally infected dogs (Basso et al., 2001; Schares et al., 2001a) and were also shed by dogs that consumed naturally infected bovine placenta (Dijkstra et al., 2001).

Although *N. caninum* has been extensively studied by a great number of researchers around the world, there are still many questions to be answered. Intestinal stages in the dog have not been described. There is little information regarding the potential for serologic cross-reactivity between *N. caninum* and the closely related *H. heydorni*. Recently, Schares et al. (2001a) reported that oocysts of *H. heydorni* and *N. caninum* cannot be differentiated solely by morphology. These authors mentioned that some past studies involving *H. heydorni* were perhaps inadvertently carried out with *N. caninum*.

The most likely cause of neosporosis abortion outbreaks in cattle is ingestion of oocysts in food or water (McAllister et al., 1996, 2000); however, there is no information about the effect of oocyst ingestion by pregnant cows. This information gap is in part a result of the low number of oocysts that are often produced by dogs in a laboratory setting (McAllister, 1999), thereby limiting the number of oocysts available for experimental transmission studies. In previous experiments, dogs have been induced to shed oocysts by ingestion of infected mice (McAllister et al., 1998; Lindsay et al., 1999); however, the number of oocysts is usually low, which has caused some investigators to question if dogs may be inefficient definitive hosts of *N. caninum* (Lindsay et al., 2001). We speculate that low numbers of oocysts have been produced in experiments because the bradyzoites in infected mouse carcasses that were fed to dogs have either been too few in number, immature, or attenuated by passage in an unnatural intermediate host. In the present study, we fed dogs *N. caninum*–infected bovine tissue and compared the shedding of oocysts with dogs that were fed infected mouse carcasses. We also investigated if *N. caninum* can be cyclically transmitted between dogs and calves, and we attempted to isolate *N. caninum* in vitro from oocysts.

MATERIALS AND METHODS

Infection of mice with *N. caninum* tachyzoites

Seventy-nine outbred ICR mice were purchased from Harlan Sprague Dawley (Indianapolis, Indiana). The mice were immunosuppressed with corticosteroids and inoculated with 3 different strains of *N. caninum* tachyzoites according to methods described by McGuire et al. (1997). Tachyzoites of NC-2 (Hay et al., 1990) were inoculated in 24 mice, NC-Illinois (isolated in our laboratory from a dairy calf in Illinois) in 31 mice, and NC-beef (isolated from a beef calf in Nebraska, McAllister et al., 1998, 2000) in 24 mice. The mice were killed between 6 wk and 2 mo after infection.

Infection of calves with *N. caninum* tachyzoites or oocysts

Eight newborn dairy bull calves were purchased from the University of Illinois Dairy. Each calf and its mother were confirmed to be seronegative for *N. caninum* using minor modifications of a previously described immunofluorescence antibody technique (IFAT) (Dubey et al., 1988); the sera were screened at a 1:50 dilution, and a commercial fluorescein isothiocyanate–labeled anti-bovine serum was employed as the secondary antibody. The calves were then infected with oocysts or tachyzoites of *N. caninum* (Table I) and housed indoors on a slatted floor, without bedding. After receiving colostrum, calves were exclusively fed milk replacer until they were killed by captive bolt at 6–8 wk of age.

Blood samples were collected from the calves 1 mo after infection and screened by IFAT for *N. caninum* antibodies. Calves with titers ≥1:400 were killed between 6 wk and 2 mo after infection.

Infection of dogs

Thirteen female mixed-breed hound puppies, 10–14 wk old, were purchased from a commercial class A breeder (Covance, Inc., Madison,

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TABLE I. Infection of calves with tachyzoites or oocysts of *Neospora caninum*.

Calf ID	Number of oocysts (oral)	Number of tachyzoites (i.v.)	Strain
A	0	1×10^7	NC-beef
B	0	1×10^7	NC-Illinois
C	500	0	NC-beef
D	300	0	NC-Illinois
E	400	0	NC-2
F	1,400	0	NC-Illinois
G	0	2×10^6	NC-beef
H	0	1×10^7	NC-beef

Wisconsin) after pretreatment to remove helminths and vaccination for canine distemper, parvovirus, adenovirus type-2, and parainfluenza. The dogs were housed individually in indoor kennels and were fed dry dog food. Each dog was tested for antibodies against *N. caninum* by an IFAT (Dubey et al., 1988). The dogs had never consumed raw meat or meat products.

Fecal samples from each dog were examined for 5 days before infection and served as negative controls. Six dogs consumed infected mouse carcasses (4–16 mice/dog). Nine other dogs each consumed approximately 3 kg of tissues from infected calves, including brain, spinal cord, heart, liver, kidney, tongue, diaphragm, and other skeletal muscles. The tissues were cut into pieces of about 3 cm³ and refrigerated (4 C) 1 day before consumption. Each dog consumed tissues from 1 calf (Fig. 1). Dogs 11 and 12 shared tissues from calf B. Fecal samples were examined daily for oocysts, starting on the fourth day postinfection.

Fecal examinations and processing of oocysts

Fecal samples from each dog were collected daily and examined using a standard flotation procedure. Each day, the total volume of feces collected in 24 hr was homogenized. Five grams of feces were diluted in water, filtered using gauze, centrifuged at 1,200 *g* for 10 min, and the sediment was mixed in Sheather's solution in a 15-ml plastic tube. This suspension was centrifuged at 1,200 *g* for 10 min with a coverslip touching the surface of the solution; then, the coverslip was examined on a glass slide using a phase contrast microscope at 200× magnification. Oocysts were counted. If more than 4 oocysts were observed, then the same 5-g sample was recentrifuged and oocysts were counted on a second (and sometimes a third) coverslip. The total number of oocysts produced was extrapolated from the weight of the dog's feces. The fecal samples containing oocysts were mixed with 5 volumes of 2% H_2SO_4 and aerated for 3 days, as described previously (McAllister et al., 1998), to induce sporulation.

Because cattle are intermediate hosts of both *N. caninum* and *H. heydorni*, it was necessary to confirm the species identity of oocysts that were shed by the dogs that consumed calf tissues. Therefore, oocysts from these dogs were examined using a *N. caninum*–specific polymerase chain reaction (PCR) procedure (described below), which was performed directly on oocysts, indirectly on tachyzoites cultured from oocysts, or on gerbils infected with oocysts.

Bioassay in Mongolian gerbils

Weaned Mongolian gerbils were purchased from Harlan Sprague Dawley. Sporulated oocysts of *N. canium* were washed with water and concentrated by Sheather's sugar solution as described above, and ap-

Dog ID	Patent period (days after infection)																											Intermediate host and organism strain *	Total oocyst production
	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30		
1																												mouse/NC-2	700
2																												mouse/NC-beef	500
3																												mouse/NC-IL	300
4																												mouse/NC-IL	100
5																												mouse/NC-beef	1,200
6																												mouse/NC-2	29,900
7																												calf E/NC-2	23,500
8																												calf D/NC-IL	25,100
9																												calf C/NC-beef	0
10																												calf A/NC-beef	54,100
11																												calf B/NC-IL	5,700
12																												calf B/NC-IL	345,900
13																												calf F/NC-IL	95,700
14																												calf G/NC-beef	392,800
15																												calf H/NC-beef	503,300

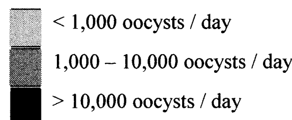

FIGURE 1. Oocyst production, prepatent period, and duration of oocyst shedding in dogs fed *Neospora caninum*–infected mouse carcasses or calf tissues. *Calf ID corresponds to Table I.

proximately 30 oocysts in 1 ml of water were administered by gavage to each gerbil. One month after infection the gerbils were killed and samples of blood and brain were collected. In 1 instance, the bioassay was performed by oral administration of 1,500 oocysts to a seronegative newborn calf, instead of using gerbils.

Direct culture of sporulated oocysts

Sporulated oocysts from dogs 7, 12, and 15 were washed in water and concentrated as described above. Numbers ranging from 60 to 1,100 sporulated oocysts in phosphate-buffered saline (PBS) were suspended in 2 parts pepsin–HCl (0.5 g NaCl, 0.52 g pepsin, 98.7 ml water, 1.3 ml concentrated HCl, pH 1.3) and 1 part PBS. The suspension was incubated for 5 min at 37 C and washed in PBS 3 times (1,200 *g,* 10 min); the sediment was suspended in Roswell Park Memorial Institute medium (RPMI) with 10% horse serum and cultured in a 25-cm^2 flask containing a monolayer of Vero cells. The medium in the flask was changed after 4 hr.

PCR of sporulated oocysts, tachyzoites, and gerbil brain

PCR of sporulated oocysts was performed with slight modifications of the method described by Hill et al. (2001). After microscopic examination of fecal floats, slides were rinsed to recover oocysts, and the solutions were centrifuged several times in distilled water (1,200 *g*/10 min) until the supernatant was clear. One volume of the sediment was then mixed with 10 volumes of Sheather's solution and centrifuged at 1,200 *g* for 10 min. The top of the supernatant containing oocysts was aspirated with a pipette and washed 2 times with water. Approximately 300–2,500 oocysts were mixed with 600 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 a trypsinized and washed monolayer of Vero cells from a 25-cm^2 flask. The suspension was incubated at 50 C overnight. Because of the small number of oocysts that were used, Vero cells were added to serve as a carrier and facilitate the precipitation of DNA. The solution was mixed in equal volumes with phenol, phenol–chloroform–isoamyl alcohol (25:24:1). DNA amplification was performed as described by Yamage et al. (1996) using the primer pair Np21/Np6. PCR assays using DNA from gerbil brain and from cultured tachyzoites were carried out as described above.

Statistics

Oocyst production was compared between groups of dogs that consumed infected mouse carcasses or tissues from infected calves using a directional Mann–Whitney test.

RESULTS

Infection of dogs, and oocyst production

The range of *N. caninum* oocysts produced by the 6 dogs that consumed infected mice was 100–29,900 (mean 5,400). The 9 dogs that were fed infected calf tissues produced between 0 and 503,300 oocysts (mean 160,700). These differences were statistically significant ($P = 0.03$). Oocyst production, prepatent period, and duration of oocyst shedding are reported in Figure 1. Small numbers of *Isospora canis* oocysts and *Giardia* sp. cysts were shed by a few dogs, both before and after infection with *N. caninum*. No other type of protozoal cysts, sporocysts, or oocysts was observed throughout the study.

The 8 calves used to infect the dogs had antibody titers for *N. caninum* of 1:400 (1 calf), 1:800 (1 calf), 1:1,600 (3 calves), and ≥1:3,200 (3 calves). Before infection, the dogs were seronegative by *N. caninum* IFAT at a 1:25 dilution; 25 days after infection, 6 out of 14 dogs had titers ≥1:50. Two dogs that were seronegative 25 days after infection were tested again 8 mo later; at that time, they both had *N. caninum* antibody titers of 1:400. All dogs remained clinically healthy during the study.

TABLE II. Confirmation of the species identity of oocysts that were shed by dogs after consuming tissues of calves infected with *Neospora caninum*.

Dog ID	Culture of gerbil brain*	PCR of gerbil brain	Culture of oocysts*	PCR of oocysts
7	+†	+	+	ND
8	+	+	−	ND
10	ND	+‡	−	ND
11§	−	ND	ND	ND
12	ND	ND	+	ND
13	ND	ND	ND	+
14	ND	ND	−	+
15	ND	ND	+	+

* Tachyzoites isolated from gerbil brain or from oocysts were confirmed to be *N. caninum* by PCR.
† +, positive; −, negative; ND, not determined.
‡ In this instance, the bioassay was performed in a seronegative calf instead of in a gerbil.
§ The concentration of oocysts shed by dog 11 was below the sensitivity of these procedures.

Direct culture of sporulated oocysts

In 3 out of 6 attempts, tachyzoites were observed in Vero cells 6–12 days after the flasks were inoculated with sporulated oocysts. The tachyzoites were confirmed to be *N. caninum* by PCR assay.

Confirmation of the identity of oocysts shed by dogs that consumed calves

Eight of 9 dogs shed oocysts after consuming tissues of infected calves. In 7 of the 8 cases, the presence of *N. caninum* was confirmed by PCR, directly on oocysts, on tachyzoites cultured from oocysts, or on animals infected with the oocysts (Table II). The protozoan species could not be confirmed by PCR or bioassay from the dog that produced only 5,700 oocysts, presumably because the concentration of oocysts was below the sensitivity threshold for these procedures.

Cyclical transmission of *N. caninum* using calves and dogs

Three of 4 dogs produced *N. caninum* oocysts after consuming calves that had ingested *N. caninum* oocysts. The following discussion may be aided by referring to Table I and Figure 1. Dog 3 produced 300 oocysts after consuming mice infected with the NC-Illinois strain of *N. caninum*. Calf D was administered these 300 oocysts by gavage. Tissues of calf D were consumed by dog 8, which produced 25,100 oocysts. Calf F was administered 1,400 of these oocysts. Dog 13 consumed tissues from calf F and subsequently shed 95,700 oocysts.

Dog 1 produced 700 oocysts after consuming mice infected with the NC-2 strain of *N. caninum*. Calf E was administered 400 of these oocysts. Tissues of calf E were consumed by dog 7, which subsequently shed 23,500 oocysts.

Dog 2 produced 500 oocysts after consuming mice infected with the NC-beef strain of *N. caninum*. Calf C was administered these 500 oocysts and developed a *N. caninum* IFAT titer of 1:800. Tissues of calf C were consumed by dog 9, which failed

to produce oocysts despite developing a *N. caninum* IFAT titer of ≥1:50 on the 25th day after infection.

DISCUSSION

The number of oocysts produced by dogs that ingested tissues from infected calves was significantly greater ($P = 0.03$) than the number of oocysts produced by dogs that ingested infected mouse carcasses. Cattle are natural intermediate hosts of this parasite, and they provide dogs with an abundant meal. In contrast, experimentally infected mice are inefficient intermediate hosts of *N. caninum*.

Neospora caninum oocysts were administered orally to calves; the tissues from these calves induced shedding of oocysts by 2 out of 3 dogs. Oocysts from 1 of these dogs were administered to another calf, and tissues from this calf induced shedding of oocysts by a third dog. Oocysts were confirmed to be *N. caninum* by PCR. These results show that the parasite can be cyclically transmitted between calves and dogs. Previously, Dijkstra et al. (2001) induced oocyst-shedding in dogs by feeding them naturally infected bovine placenta. Schares et al. (2001a, 2001b) demonstrated cyclic heteroxenous transmission of *N. caninum* using goats, sheep, guinea pigs, multimammate rats, BALB/c mice, and dogs.

In vitro isolation of *N. caninum* directly from oocysts was an important ancillary achievement in the course of this investigation. This procedure for isolation into cell culture does not require the use of rodents (Dubey and Lindsay, 2000).

All dogs remained clinically healthy during the experiment, and 6 out of 14 did not develop a detectable antibody titer within the first 3–4 wk after infection. These findings are consistent with previous studies (McAllister et al., 1998; Lindsay et al., 1999; Dijkstra et al., 2001; Schares et al., 2001b). Basso et al. (2001) reported soft feces in a naturally infected dog that was shedding *N. caninum* oocysts; however, it is possible that other factors may have contributed to the diarrhea.

The presence of *N. caninum* was confirmed by a species-specific PCR procedure in 7 out of 8 cases in which dogs shed oocysts after consumption of infected bovine tissues. In the 1 case that could not be confirmed, the low concentration of oocysts that were produced appeared to be below the sensitivity of the assays.

Although their oocysts are similar to those of *N. caninum*, it is highly unlikely that these dogs could have been concurrently infected with *H. heydorni*, which has not been demonstrated to cause congenital infections. The calves were reared in isolation and fed milk replacer; they, therefore, had scarce opportunity to acquire secondary infections. For example, *Sarcocystis cruzi* infects most cattle in North America, but the dogs did not shed sporocysts during the experiment. Lastly, *H. heydorni* oocysts have been reported to measure up to 14 μm in diameter (Dubey and Fayer, 1976; Nassar et al., 1983); yet, we did not observe oocysts larger than 11.5 μm.

In summary, this article presents 3 new findings. First, dogs shed greater numbers of *N. caninum* oocysts after consuming tissues from infected calves than after consuming infected mice; this finding helps to explain poor oocyst shedding that has been reported in previous experiments and increases the plausibility that dogs are important in the transmission of this parasite to cattle. Second, cyclical oral transmission of *N. caninum* was demonstrated between dogs and cattle, as is expected to occur in nature; the low number of oocysts that caused infections in calves (as few as 300) also increases the plausibility that canine transmission of this parasite may be common. Third, *N. caninum* was cultured in vitro directly from oocysts.

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