

Oocyst Excretion in Dogs Fed Mouse Brains Containing Tissue Cysts of a Cloned Line of *Neospora Caninum*

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malmbergi, which shifted from *O. niloticus* to *C. callolepis* in Santa Anita Lagoon. The prevalence and abundance of *C. sclerosus* and *C. tilapiae* were higher in CINVESTAV-Mérida than in Lake Catemaco. This is probably a consequence of direct parasite transmission in a crowded environment. The presence of these monogeneans in the CINVESTAV aquaculture facilities highlights the increased mortality risk these parasites pose for cultured fish, as has been recorded for cichlid fish in Florida (Noga and Flowers, 1995).

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Oocyst Excretion in Dogs Fed Mouse Brains Containing Tissue Cysts of a Cloned Line of Neospora Caninum

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ABSTRACT: *Neospora caninum* is an apicomplexan parasite that causes neonatal neuromuscular disease in dogs and abortions in cattle. Bovine neosporosis is a major production problem worldwide. The parasite is transmitted to cattle via oocysts excreted by dogs or by transplacental transmission. Dogs are the only proven definitive host for *N. caninum*. One of 3 dogs fed mouse brains containing tissue cysts of a wild-type *N. caninum* strain CK0160SC3B (CKO) excreted oocysts in its feces. Two of 3 dogs fed mouse brains containing tissue cysts from a cloned line of the CKO strain excreted *N. caninum* oocysts in their feces. The results indicate that a single *N. caninum* tachyzoite contains all the genetic information needed to produce the asexual and sexual cycles in the canine intestine.

Neospora caninum is an apicomplexan parasite that causes neonatal neuromuscular disease in dogs and abortions in cattle

(reviewed by Dubey and Lindsay, 1996). Dogs are a definitive host of *N. caninum* (McAllister et al., 1998; Lindsay, Dubey, and Duncan, 1999; Lindsay, Upton, and Dubey, 1999). They are essential in the initial transmission of this parasite to cattle, and large abortion storms seen on farms are probably due to feed or water contaminated with *N. caninum* oocysts (i.e., post-natal transmission; McAllister et al., 1996; Thurmond et al., 1997; Schares et al., 1999). Before dogs were shown to be a definitive host, they were associated with neosporosis on farms in epidemiological studies (Pare et al., 1998), and this positive association has been verified by recent studies (Mainar-Jaime et al., 1999; Ould-Amrouche et al., 1999; Wouda et al., 1999). Neosporosis is an economically important cause of abortion

worldwide in dairy and beef cattle (Dubey and Lindsay, 1996); however, little is known about the life cycle of this parasite in the canine definitive host.

The present study was done to determine if dogs would excrete oocysts after ingesting tissue cysts of a wild type of *N. caninum* isolated from beef cattle and to determine if dogs fed tissue cysts resulting from infections with cloned tachyzoites would excrete oocysts. If dogs fed tissue cysts resulting from infections with cloned tachyzoites excreted oocysts, this would indicate that a single tachyzoite contained all the genetic information needed to produce the male and female sexual cycles in the canine definitive host.

Tachyzoites of the CK0160SC3B (CKO) strain of *N. caninum* isolated from an aborted bovine fetus were used in the present study. The CKO strain was cloned twice by limiting dilution and the resulting clone of *N. caninum* called the PB-1-2C clone. Eighty CD-1 mice were injected intramuscularly with 4 mg methylprednisolone acetate (MPA) on days -7 and 0 postinoculation (PI) of 2×10^5 tachyzoites. Forty mice received tachyzoites of the CKO strain (14th passage in cell culture) and 40 received tachyzoites of the PB-1-2C line (7th passage in cell culture).

Mice had been infected with the CKO strain for 58 days (experiment 1) or 92 days (experiment 2) before being fed to dogs. Mice had been infected with the PB-1-2C strain for 72 days (experiment 1) or 86 days (experiment 2) before being fed to dogs. Brains from a mouse in each group were examined for *N. caninum* tissue cysts by squash preparation and light microscopy. Tissue cysts were observed in squash preparations in mice from all groups. Dogs were fed mouse brains that had been removed from mice for less than 30 min.

Six mixed-breed dogs about 12-wk old were used. Two experiments were conducted. Dogs in both experiments received 100 mg MPA intramuscularly on days -7 and 0 PI. In experiment 1, dogs 8 and 9 were each fed 12 mouse brains containing the CKO strain, and dogs 12 and 13 were each fed 12 mouse brains containing the PB-1-2C line. In experiment 2, dog 16 was fed 12 mouse brains, each containing the CKO strain, and dog 17 was fed 15 mouse brains, each containing the PB-1-2C line. Dogs were infected separately by placing the infected mouse brains in the dogs' feed bowls. This was done in the morning before the dogs were fed, but they were not fasted overnight. Each dog was watched to ensure it has ingested all the brains present. The dogs were then given their morning ration. Dogs were re-examined at 30 min and 1 hr to determine if they had vomited the inoculum.

Feces of each dog were examined for *N. caninum* oocyst excretion on days 4–15 PI by flotation in Sheather's sugar solution (Ernst and Benz, 1981; Table I). Feces containing unsporulated oocysts were collected, mixed in 2% (v/v) sulfuric acid solution (1 part feces to 9 parts sulfuric acid solution), strained with a tea strainer, and placed in 1-L erlenmeyer flasks to a depth of about 40 cm, and the suspension was mixed on a rotating mixer for 48–72 hr. Sporulated oocysts were purified by flotation in Sheather's sugar solution. Briefly, the oocyst sulfuric acid solution was concentrated in 50-ml tubes by centrifugation for 10 min at 1,500 rpm. The pellet was mixed with 40 ml Sheather's sugar solution and centrifuged again for 10 min at 1,500 rpm. The top layer of the suspension containing the sporulated oocysts was collected, mixed in tap water, and

TABLE I. Direct agglutination titers (expressed as reciprocals) of dogs fed mouse brains containing the CK0160SC3B (dogs 8, 9, 16) or PB-1-2C (dogs 12, 13, and 17) strain of *Neospora caninum*.

Day PI*	Dogs fed CK0160SC3B strain			Dogs fed PB-1-2C strain		
	8	9	16†	12	13†	17†
-22	Nd‡	Nd	—	—	—	—
-14	—	—	Nd	—	—	Nd
-7	—	—	Nd	—	—	Nd
0	—	—	—	—	—	—
6	—	—	Nd	Nd	Nd	Nd
7	Nd	Nd	—	100	100	—
11	—	—	Nd	Nd	Nd	Nd
14	Nd	Nd	—	200	50	—
15	—	25	Nd	Nd	Nd	Nd
20	—	25	Nd	Nd	Nd	Nd
21	Nd	Nd	50	Nd	Nd	—
28	800	100	100	Nd	Nd	—
36	800	100	800	Nd	Nd	—

* Day postinoculation (PI) sera sample collected.

† Dog excreted oocysts.

‡ Nd, no test conducted that day PI.

repelleted by centrifugation for 10 min. The pellet containing concentrated sporulated oocysts was stored at 4 °C in 2% sulfuric acid solution until used.

Concentrated fecal samples were treated with 50% (v/v) commercial bleach for 10 min to digest contaminating fecal material still present. The chlorine from the bleach was removed by repeated centrifugation in Hanks' balanced salt solution. The estimated numbers of total oocysts present from each dog that was visibly positive was determined by counting the numbers of oocysts present in the final pool of bleach-treated oocysts from that dog using a hemocytometer.

Blood samples were collected from each dog on days -7 and 0 before feeding oocysts and on days 14 (dogs 12 and 13) or 36 (dogs 8, 9, 16, and 17) after feeding infected mouse brains. The anti-*N. caninum* antibody titers were determined by the modified direct agglutination test (NAT) (Packham et al., 1998) on serum. Sera were examined at dilutions of 1:25 and endpoint titrated.

Three female gerbils were used to test infectivity of PB-1-2C strain oocysts as described by Dubey and Lindsay (2000). Two were fed 6,000 oocysts each, and 1 was fed 20,000 oocysts of the PB-1-2C strain. The 2 gerbils fed 6,000 oocysts were both killed 93 days PI. Blood was collected from the retro-orbital plexus and used for antibody determination in the NAT. Their brains were removed and ½ was fixed in 10% neutral buffered formalin and processed for histological examination. The remaining ½ was homogenized and inoculated onto cell cultures. The gerbil fed 20,000 oocysts died 25 days PI, and its brain was removed and processed for histology.

Six female γ -INF gene knockout mice were used to test the infectivity of a second batch of PB-1-2C oocysts. Three mice were subcutaneously inoculated with 5,000 oocysts each and 3 mice were fed 5,000 oocysts each. They were killed 30 days PI, and sera were collected for NAT testing. Their brains were removed and processed for histological examination.

None of the dogs fed infected mouse brains developed diarrhea or any other clinical sign of intestinal disease. All dogs

ate the inoculum within 2 min. Most consumed the brains within 30 sec. None of the dogs vomited the inoculum.

One of the 3 dogs (dog 16) fed the CKO strain excreted oocysts in its feces for a single day on day 13 PI. Two of 3 dogs (dogs 13 and 17) fed the PB-1-2C strain excreted *N. caninum* oocysts. Dog 13 excreted oocysts on days 7, 8, 9, 10, 11, 12, 13, and 14 PI. Dog 17 excreted oocysts on days 8, 9, 10, 12, 13, and 15 PI. Oocyst production was minimal in dogs that excreted oocysts. Total oocysts of the PB-1-2C strain collected and purified from dogs 13 and 17 were approximately 810,000 and 161,000, respectively. No oocysts of the CKO strain were observed in the processed feces of dog 16.

None of the 6 dogs had antibodies to *N. caninum* prior to being fed mouse brains (Table I). Five of 6 dogs seroconverted to *N. caninum* during the study. Dogs 8 and 9 fed the CKO strain had titers of 1:800 and 1:100, respectively, on day 36 PI despite not excreting visible *N. caninum* oocysts in their feces, whereas dog 16 fed the CKO strain did excrete oocysts and had a titer of 1:800 on day 36 PI. Dog 12 fed the PB-1-2C strain did not excrete oocysts but had a titer of 1:200 on day 14, and dog 13 fed PB-1-2C strain did excrete oocysts and had a titer of 1:50 on day 14 PI. Dog 17 fed the PB-1-2C strain excreted oocysts but did not seroconvert by day 36 PI.

The gerbil fed 20,000 PB-1-2C oocysts of *N. caninum* collected from dog 13 died (apparently of a seizure) 25 days PI. No serum was obtained. Minimal lesions were present in its brain, and they consisted of focal areas of gliosis. No tissue cysts were seen in this gerbil. Neither of the 2 gerbils fed 6,000 oocysts of the PB-1-2C strain of *N. caninum* collected from dog 13 died. Both gerbils had agglutinating titers that were $\geq 1:800$ in their sera 93 days PI. *Neospora caninum* was not isolated in cell cultures from the brain of either gerbil during the 53 days the cultures were kept for examination. No lesions were present in the brains of these 2 gerbils. None of the 3 γ -INF gene knockout mice subcutaneously inoculated with PB-1-2C oocysts from dog 17 or the 3 γ -INF gene knockout mice fed oocysts from dog 17 died. No antibodies to *N. caninum* were present in a 1:25 dilution of sera from any of these 6 γ -INF gene knockout mice. No lesions were present in the brains of these mice.

None of the dogs fed *N. caninum*-infected mouse brains developed clinical signs of disease in the present study. This is consistent with previous reports (McAllister et al., 1998; Lindsay et al., 1999). It is also interesting to note that not all dogs seroconverted and that dog 17 excreted oocysts and did not seroconvert by day 36 PI. The low numbers of oocysts excreted by dogs may indicate that they are not the best definitive host for this parasite. It is possible that some other carnivore is the true definitive host in nature.

The oocysts of the PB-1-2C strain collected from dog 13 were infectious for gerbils as measured by lesions in the gerbil fed 20,000 oocysts and in the titers of $\geq 1:800$ in the 2 fed 6,000 oocysts. Oocysts of the PB-1-2C strain do not appear to be pathogenic for gerbils as the NC-Liverpool strain. The NC-Liverpool strain of *N. caninum* will cause fatal infections in gerbils fed as few as 1,000 oocysts (Dubey and Lindsay, 2000). The oocysts of the PB-1-2C strain collected from dog 17 were not infectious for γ -INF gene knockout mice. Oocysts of the NC-beef strain of *N. caninum* were also not very infectious for

γ -INF gene knockout mice because only 1 of 8 became infected (Lindsay et al., 1999).

The finding that the cloned line, PB-1-2C, could induce oocyst production in dogs indicates that all the genetic material needed is present in a single tachyzoite. Our results are similar to those of Pfefferkorn et al. (1977), who demonstrated that a cloned strain of *Toxoplasma gondii* could induce oocyst excretion in cats.

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