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Rapid communication

Coyotes (*Canis latrans*) are definitive hosts of *Neospora caninum*

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Abstract

Four captive-raised coyote pups consumed tissues from *Neospora caninum*-infected calves. Faeces were examined from 4 days before to 28 days after infection. One pup shed *N. caninum*-like oocysts, which tested positive for *N. caninum* and negative for *Hammondia heydorni* using PCR tests. Coyotes are the second discovered definitive host of *N. caninum*, after dogs. In North America, the expanding coyote ranges and population increase the probability of contact with domestic livestock. To reduce the risk of transmission of *N. caninum* to intensively farmed cattle, we recommend protection of feedstuffs using canid-proof fences, and careful disposal of dead stock.

Keywords: *Neospora caninum*; Coyote; *Canis latrans*; Canid; Oocyst; Definitive host

Neosporosis, caused by the protozoan parasite *Neospora caninum*, is a frequent cause of bovine abortion worldwide (Dubey, 2003). Dogs are a definitive host of the parasite (McAllister et al., 1998). When identifying definitive hosts of *N. caninum*, it is essential to avoid confusion with the closely related organism *Hammondia heydorni* (Ellis et al., 1999). Besides dogs, other canids have been considered to be potential definitive hosts of *N. caninum* (McAllister, 1999). Antibodies to *N. caninum* have been found in North American coyotes (*Canis latrans*), British red foxes (*Vulpes vulpes*), and Australian dingoes (*Canis familiaris dingo*; Lindsay et al., 1996; Barber et al., 1997; Buxton et al., 1997). In Texas, Barling et al. (2000) performed a spatial analysis study and found statistical associations among the density of cattle, seropositivity for *N. caninum*, and abundance of coyotes and grey foxes (*Urocyon cinereoargenteus*).

The aim of our study was to determine if coyotes are a definitive host of *N. caninum*, by feeding them infected bovine tissues and then examining their faeces.

Three newborn dairy bull calves (1, 2, and 3) seronegative for *N. caninum* at 1:25 dilution by indirect fluorescent antibody test (IFAT), were acquired from the University of Illinois Dairy. The calves were kept indoors and were exclusively fed milk replacer without antibiotics. The three calves were infected during the first week of life with *N. caninum*. Calf 1 was injected intravenously with 5×10^5 tachyzoites (NC-Illinois strain; Gondim et al., 2002), calf 2 was injected intravenously with a mixture of 1×10^6 tachyzoites of NC-beef strain (McAllister et al., 1998, 2000) and NC-Illinois strain (5×10^5 tachyzoites from each strain), and calf 3 was orally administered 29,000 oocysts of the NC-beef strain via an oesophageal feeder. Calves were euthanised 6–12 weeks after infection. At the time of euthanasia the three calves had *N. caninum* antibody titres $\geq$1:800 by IFAT. The entire brain and spinal cord, as well as a portion of the heart, tongue, diaphragm, other skeletal muscles and kidney, were cut into pieces of approximately 3 cm^3, mixed, and shipped on ice to the Logan Field Station of the National Wildlife Research Center, in Logan, UT.

Four female coyote puppies were used in the experiment. Two littermates (A and B) were infected when they were 8 weeks old, and the other two pups (C and D), from different litters, were infected when they were 12 weeks old. They were born and raised in captivity at the Logan Field Station. The puppies were housed in outdoor pens with their littermates and mothers, prior to study initiation, and were fed a commercially prepared carnivore diet (Fur Breeders Agriculture Cooperative, Sandy, UT). The pups were negative for antibodies against *N. caninum* at a 1:25 dilution, as determined using an IFAT (Dubey et al., 1988). During the study, animals were housed individually; coyotes A and B

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in indoor kennels, and C and D in outdoor pens. Pups A and B each consumed 1 kg of tissue from calf 1, within a 2-day period (time of first exposure, day 0). Pups C and D refused to eat the infected tissue and were difficult to handle; therefore it was necessary to induce general anaesthesia (using sodium thiopental) on two successive days, in order to administer blended tissues mixed with water, via an oesophageal tube. Pup C was administered a total of 1 kg of mixed tissues from calf 3, and pup D was administered a total of 0.8 kg of mixed tissues from calf 2.

Faecal samples were collected for 4 days before the coyotes were infected with calf tissues, and for 28 days after infection. Faeces were examined by a standard sucrose flotation technique as described earlier (Gondim et al., 2002). No *N. caninum*-like oocysts were observed in any specimens prior to consuming the infected tissues, although *Isospora* spp. oocysts were observed in the faeces of 2/4 coyotes before and after infection. *Isospora* spp. have a direct faecal-oral life cycle (Baek et al., 1993) and were presumed to have been transmitted from the pups' mothers. One of the four coyotes (C) shed approximately 500 *Neospora*-like oocysts between 8 and 10 days after infection (Fig. 1A). The unsporulated oocysts were spherical to subspherical and measured 10 μm in diameter ($n = 10$). They were aerated in 2% H_2SO_4 as previously described (Gondim et al., 2002). After aeration, sporulated oocysts contained two sporocysts, each with sporozoites (Fig. 1B).

Approximately 150 oocysts were concentrated by sucrose, washed thrice in water, and a final volume of 200 μl of sediment containing oocysts was obtained. The sediment was suspended with 500 μl of PBS and ground for 10 min by vortexing in a 1.5 ml tube with 500 μl of glass beads (500 μm in diameter). The solution was transferred to a new tube, mixed with 600 μl of a digestion buffer [100 mM NaCl, 10 mM Tris–HCl (pH 8.0), 25 mM ethylenediaminetetraacetic acid, 0.5% sodium dodecyl sulphate], 5 μl of proteinase K (20 mg/ml), and incubated at 65 °C for 2 h. DNA was extracted by standard phenol–chloroform and 2-propanol precipitation. The DNA was suspended in water, but the final solution was yellow as a consequence of the large amount of sediment in the sample. This DNA sample was further purified using a DNA extraction kit (SoilMaster™ DNA Extraction Kit, Epicentre, Madison, WI), according to the manufacturer's protocol.

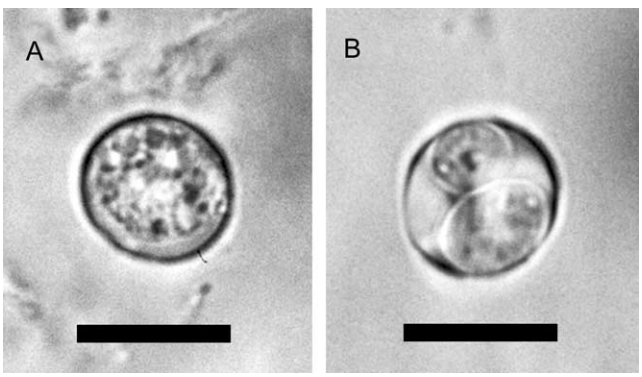

Fig. 1. *Neospora caninum* oocysts shed by a coyote (*Canis latrans*). Bars 10 μm. (A) Unsporulated oocyst. (B) Sporulated oocyst containing two sporocysts.

DNA extracted from these oocysts were tested by PCR using the *N. caninum*-specific primer pair Np6/Np21 (Yamage et al., 1996); 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 *H. heydorni* using the coyote oocysts was conducted using the specific primers (JS4/JS5) and conditions reported by Slapeta et al. (2002). DNA from *H. heydorni* (kindly provided by Dr John Ellis) and Vero cell DNA were used as positive and negative controls, respectively. The oocysts shed by coyote C tested positively for *N. caninum* by PCR; in contrast, PCR for *H. heydorni* yielded negative results (Fig. 2).

Blood samples were collected from the coyotes 28 days p.i. All developed antibody titres for *N. caninum* (A, 1:800; B, 1:800; C, 1:800; D, 1:1600). Their sera were also tested for *Toxoplasma gondii* by IFAT, and were negative at 1:50.

This study demonstrates that coyotes are a definitive host of *N. caninum*. One coyote pup clearly produced *N. caninum* oocysts, as demonstrated by morphologic and genetic techniques. This is the second discovered definitive host of *N. caninum*, following dogs (*C. familiaris*). It is pertinent to note that wolves (*Canis lupus*) are more closely related to dogs than are coyotes (*C. latrans*; Vilà et al., 1999), and thus probably are also definitive hosts of this protozoan.

The minimum infectious dose of *N. caninum* oocysts needed to infect cattle is not known, but it is no more than 300 oocysts (Gondim et al., 2002). In the present study using coyotes, one 12-week-old pup shed approximately 500 oocysts. If such slight shedding of oocysts is typical of coyotes, then they could not be an efficient definitive host of this organism. However, the first experimental protocols in

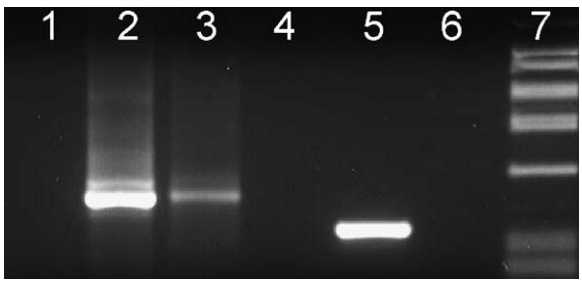

Fig. 2. PCR for *Neospora caninum* and *Hammondia heydorni* using DNA extracted from oocysts shed by a coyote (*Canis latrans*). (1) Vero cell DNA using specific primers to *N. caninum* (negative control), (2) *N. caninum* DNA (positive control) showing prominent band at approximately 328 bp, (3) DNA extracted from the coyote oocysts using specific primers to *N. caninum*, (4) Vero cell DNA using specific primers to *H. heydorni* (negative control), (5) *H. heydorni* DNA (positive control) showing prominent band at approximately 270 bp, (6) DNA extracted from the coyote oocysts using specific primers to *H. heydorni*, (7) $\Phi\times$ 174 DNA marker (Invitrogen).

dogs also induced scant production of oocysts (McAllister, 1999), similar to the present initial finding in coyotes. Using a more recently reported protocol, hound pups were induced to shed a mean of 160,000 oocysts (enough to infect >500 cattle per dog) after consuming tissues from freshly killed *N. caninum*-infected calves (Gondim et al., 2002). The current study was hampered by several technical problems that may have mitigated production of oocysts by the coyotes. The availability of captive-raised coyotes is highly limited. Unlike previous studies, in which dogs consumed infected tissues immediately after euthanasia of intermediate hosts, in the present experiment tissues were chilled and shipped by overnight courier from the University of Illinois to the Logan Field Station in Utah; it is unknown if this reduced the viability of the protozoan's tissue stages. Coyotes are seasonal breeders, which created scheduling difficulties and resulted in the use of pups that were younger than preferred. The coyote pups were approximately half the size of the previously mentioned hound pups, and they were only able to consume about one-third as much infected tissue. Further investigation is needed to determine factors that may potentiate oocyst production by coyotes, to compare the relative efficiency of dogs and coyotes as definitive hosts of *N. caninum*, and to estimate the potential risk of *N. caninum* transmission that coyotes pose to livestock.

Historically, coyotes were confined to the prairie areas of North America, but during the last 100 years the coyotes' range has expanded to include the entire continental US, much of Canada, and Central America (Moore and Parker, 1992). The expanding range and population of these animals increase the probability of contact with domestic animals, and this increases the risk of *N. caninum* transmission between coyotes and livestock.

Based upon the finding that coyotes are a definitive host of *N. caninum*, we suggest that reasonable steps be taken to reduce the risk of transmission of this organism between coyotes and ruminant livestock. For intensively farmed cattle, such measures could include erection of canid-proof fences around silage piles, baled hay, and other feedstuffs that are kept outdoors. Dead livestock and the offal from home slaughter should be disposed off in a manner that prevents consumption by canids. Coyotes and dogs should be discouraged from denning adjacent to barns or pens that house livestock or where feeds are stored. An interesting consideration is that, in some circumstances, dogs may help to repel coyotes, although dogs themselves can transmit *N. caninum* to cattle (De Marez et al., 1999; Gondim et al., 2002). We do not recommend a policy of coyote eradication, because of this animal's ability to adapt to changing conditions, to rapidly rebound from reduced population density, and also because of public sentiment.

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