

NEOSPORA CANINUM-LIKE OOCYSTS OBSERVED IN FECES OF FREE-RANGING RED FOXES (*VULPES VULPES*) AND COYOTES (*CANIS LATRANS*)

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ABSTRACT: The aim of this study was to examine the feces of free-ranging foxes and coyotes for the presence of *Neospora caninum* oocysts. Feces were collected from 271 foxes and 185 coyotes in the Canadian province of Prince Edward Island, processed by sucrose flotation, and examined by light microscopy for the presence of coccidian oocysts. In 2 fox and 2 coyote samples, oocysts morphologically and morphometrically similar to oocysts of *N. caninum* were observed. DNA was extracted from these samples and subjected to nested polymerase chain reaction (PCR) using primers to the *N. caninum*-specific Nc5 genomic sequence. Through DNA sequencing, alignment of the sequences of at least 3 clones from each isolate to sequences deposited in GenBank revealed 95–99% similarity to the Nc5 sequence of *N. caninum*. PCR using primers specific for *Hammondia heydorni* failed to yield an amplification product from these DNA samples.

Neosporosis caused by the coccidian parasite *Neospora caninum* is a frequent cause of bovine abortion worldwide (Dubey, 2003). The domestic dog was identified as a definitive host for the parasite in 1998 (McAllister et al., 1998). Epidemiological evidence has suggested wild canids, specifically foxes and coyotes, may also play a role in the epidemiology of bovine neosporosis. Spatial associations have been found among the density of cattle, seropositivity for *N. caninum*, and abundance of coyotes (*Canis latrans*) and grey foxes (*Urocyon cinereoargenteus*) (Barling et al., 2000). In addition, there is some evidence for a sylvatic life cycle. *Neospora caninum* was isolated from a naturally infected white-tailed deer (*Odocoileus virginianus*) (Vianna et al., 2005) and a fatal case of neosporosis in a 2-month-old black-tailed deer fawn (*Odocoileus hemionus columbianus*) was observed (Woods et al., 1994). One dog was shedding *N. caninum* oocysts after being fed brains of naturally infected white-tailed deer (*Odocoileus virginianus*) (Gondim, McAllister, Mateus-Pinilla et al., 2004). Recently, the coyote was experimentally confirmed as a definitive host for *N. caninum*, providing the most convincing evidence thus far that wild canids may play a role in the epidemiology of bovine neosporosis (Gondim, McAllister, Pitt, and Zemlicka, 2004). However, despite the recent finding that coyotes can act as a definitive host for *N. caninum*, the occurrence of *N. caninum* oocysts in feces of wild canids in nature has yet to be reported. In addition, although red foxes (*Vulpes vulpes*) are a natural intermediate host for *N. caninum* (Almeria et al., 2002), no evidence for red foxes being a definitive host for *N. caninum* has been reported (Schaes et al., 2002).

The Canadian Province of Prince Edward Island (PEI) consists primarily of agricultural and forested land. The coyote population on PEI has increased rapidly since it was first encountered in 1983, and it occurs throughout the island. The red fox is also very common in the province, and many foxes have lost their fear of humans because they are often fed in campgrounds and urban areas. In addition, the estimated herd prevalence of *N. caninum* on dairy farms in PEI is 63% (Keefe and VanLeeuwen, 2000). Due to the density of foxes, coyotes, and cattle in the province, foxes and coyotes are often sighted close to farms and have frequent access to carcasses and placentas. The aim of this study was to examine the feces of free-ranging foxes and coyotes for the presence of *N. caninum* oocysts to gain a better understanding of their role in the epidemiology of bovine neosporosis.

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MATERIALS AND METHODS

Sample collection

Registered trappers and hunters trap foxes and coyotes on PEI primarily for their furs. Thirty-two trappers and hunters were contacted and agreed to participate in this study; no foxes or coyotes were killed deliberately for this study. Fox and coyote carcasses were collected from 19 October 2004 until 24 March 2005. All carcasses were sampled by W.W. and 2 senior veterinary students as soon as possible after each reported death, varying from a few hours to 5 days. Most carcasses were sampled within 24 hr at the location where they were stored and skinned. Fecal samples were extruded from the rectum using a disposable latex glove and stored at 4 C in individually labeled jars. If no feces were readily available at the distal end of the rectum, the rectum was stripped manually to collect a minimum of 5 g of feces. Additionally, the canine tooth of the lower jaw and a blood sample from the heart or femoral artery were collected. Sex, date, and location where the animal was killed were recorded. Location was recorded on a map using community names. Latitude and longitude of these locations were recorded using an interactive mapping system (<http://www.gov.pe.ca/mapguide>). To differentiate between a juvenile (<12 mo) and an adult animal, radiographs were taken of individual canine teeth to assess the width of the pulp cavity. The age of adult animals was determined by counts of the annual growth zones in canine tooth cementum (Johnston Biotech, Sarnia, Ontario, Canada) (Grue and Jensen, 1976; Johnston et al., 1999).

Fecal examination

Fecal examination was performed within 48 hr after sample collection using a standard sucrose flotation method as described by Gondim et al. (2002) with slight modifications. Approximately 5 g of feces were used for the fecal flotation, with the remainder of the sample set aside for molecular analysis. Slides were examined for the presence of coccidian oocysts matching the morphologic and morphometric characteristics of *N. caninum* (Gondim, McAllister, Pitt, and Zemlicka, 2004), and the number of oocysts on each slide was recorded.

Serology

Sera of foxes and coyotes from which *N. caninum*-like oocysts were identified were tested for the presence of *N. caninum* antibodies by an indirect fluorescent antibody test (IFAT) (Dubey et al., 1988) performed

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at the U.S. Department of Agriculture, Animal Parasitic Diseases Laboratory in Beltsville, Maryland, using a 1:50 and a 1:100 dilution.

Molecular techniques (polymerase chain reaction, cloning, sequencing)

DNA was extracted from fecal samples in which *N. caninum*-like oocysts had been observed, and from 4 fecal samples (2 foxes and 2 coyotes) with no observed *N. caninum*-like oocysts as negative controls, using a commercially available DNA extraction kit (QIAamp DNA Stool Mini Kit, Mississauga, Ontario, Canada), according to the manufacturer's protocol. The amount of fecal sample used for DNA extraction was approximately 5 g, with no purification or concentration of oocysts performed before DNA extraction. Following DNA extraction, samples were shipped by air to the U.S. Department of Agriculture, Animal Parasitic Diseases Laboratory in Beltsville, Maryland, for molecular analysis. The genomic Nc5 region was selected as the target sequence for DNA amplification by nested polymerase chain reaction (PCR) using the *N. caninum*-specific primer pair Np21/Np6 (Yamaga et al., 1996) in the primary reaction, followed by amplification with internal primers Np7/Np10. The Np7 primer sequence was 5'-GGGT GAACCGAGGGAGTTG-3' and the Np10 primer sequence was 5'-TCGTCGGCTTGCTCCCTATGAAT-3', and these primers amplified a 197-base pair (bp) sequence internal to the 337 bp Np6/21 PCR product. To ensure that the nested Nc5 PCR assay was specific for *N. caninum*, amplifications with Np21/6 followed by Np7/10 were also performed on *H. heydorni* DNA. In addition, two different PCRs utilizing primers HhAP7F, HhAP7R, HhAP10F, and HhAP10R (Sreekumar et al., 2003) specific for *H. heydorni* were conducted on DNA extracted from fecal samples containing *N. caninum*-like oocysts, as well as from fecal samples containing slightly larger oocysts (measuring 12–14 μm) than what has been described for *N. caninum*. A positive *N. caninum* control sample was DNA extracted from the brain tissue of *N. caninum*-infected mice. A positive *H. heydorni* control sample was DNA-extracted from *H. heydorni* oocysts. A no-template control (diluent) and DNA extracted from 4 fecal samples (2 foxes and 2 coyotes with no *N. caninum*-like oocysts observed) were included in both the primary and nested assays. PCR products were analyzed by acrylamide gel electrophoresis as described (Liddell et al., 1999). The PCR products were cloned using a pGEM-T Easy kit (Novagen, Madison, Wisconsin). Both strands of a minimum of 3 individual clones from at least 2 independent PCR amplifications were subjected to DNA sequencing using M13 forward and reverse primers. DNA sequencing reactions were analyzed on an ABI377 DNA sequencer using a Big Dye Terminators kit (Applied Biosystems, Foster City, California). Base calling was confirmed by visual inspection of the DNA sequence electropherogram. The DNA sequences were aligned using the DNA Sequencer program (GeneCodes Corporation, Ann Arbor, Michigan), and the consensus sequence was used to compare DNA sequences deposited in GenBank.

RESULTS

Prevalence, serology, and descriptive information

In total, fecal samples from 271 foxes and 185 coyotes were examined. Oocysts matching the morphological characteristics of *N. caninum* were observed in the feces from a juvenile female and a 2-yr-old female fox (0.7%), as well as a juvenile female and a 2-yr-old male coyote (1.1%) (Fig. 1). These oocysts were unsporulated, spherical to subspherical, and measured 10–11 μm in diameter, ranging in number from 3 to 6 oocysts per cover slip. The 2 foxes were trapped and collected on the same day in the same location. It was not known whether these foxes were related (belonging to the same family). In an additional fox and coyote, spherical to subspherical coccidian oocysts were observed that were larger (measuring 12–14 μm) than what has been described previously for *N. caninum*. All 4 animals containing *N. caninum*-like oocysts were observed to be negative for *N. caninum* antibodies on serological examination. The fox and coyote that contained larger (12–14 μm)

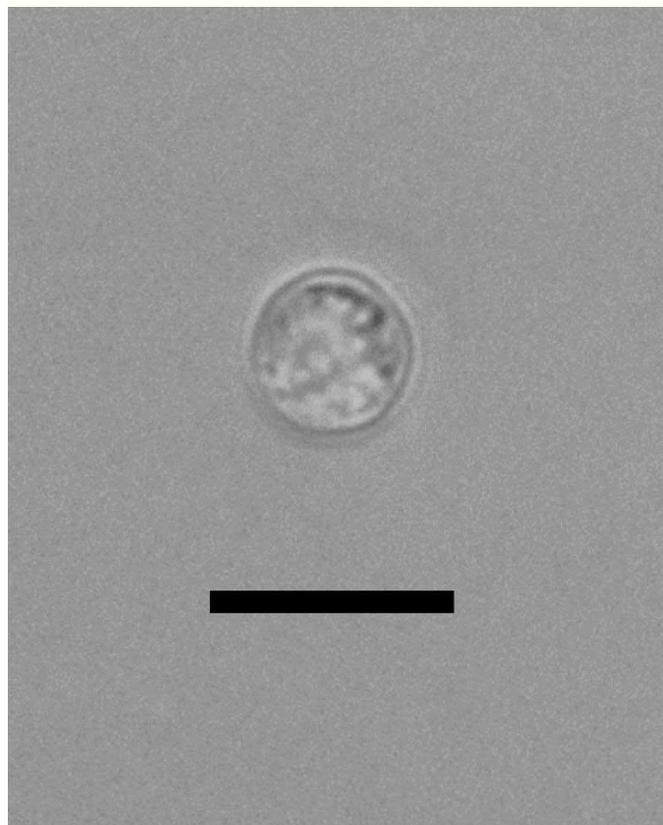


FIGURE 1. Unsporulated *N. caninum* oocyst observed in coyote 1. Bar = 14 μm .

oocysts in their feces were also negative for *N. caninum* antibodies on serological examination.

Polymerase chain reaction

Following amplification of DNA from foxes 1 and 2, and from coyotes 1 and 2 using the *N. caninum* nested PCR, a ~200-bp product was observed (Fig. 2). Two PCRs using primers specific for *H. heydorni* failed to yield an amplification product from these 4 DNA samples as well as from the 2 samples with larger oocysts. The *N. caninum* PCR failed to amplify DNA extracted from the 2 samples with larger oocysts and from 4 additional fecal samples included as negative controls (data not shown). A statistically significant difference ($P < 0.03$) in PCR outcome between 4 samples with *N. caninum*-like oocysts and 4 negative control fecal samples was calculated using a Fisher exact test.

Cloning and sequencing

DNA sequencing of at least 3 clones (6 clones from fox 1, 11 clones from fox 2, 3 clones from coyote 1, and 8 clones from coyote 2) for each isolate and alignment of Nc5 sequences revealed 95–99% similarity to the Nc5 sequence of *N. caninum*. A consistent observation was the single nucleotide gap at nucleotide 690 of the Nc5 sequence derived from *N. caninum* oocysts recovered from fox 1 (Fig. 3). Although PCR amplification of *N. caninum* oocyst DNA from fox 1 and coyote 1 revealed a single clonal population, 2 distinct populations were

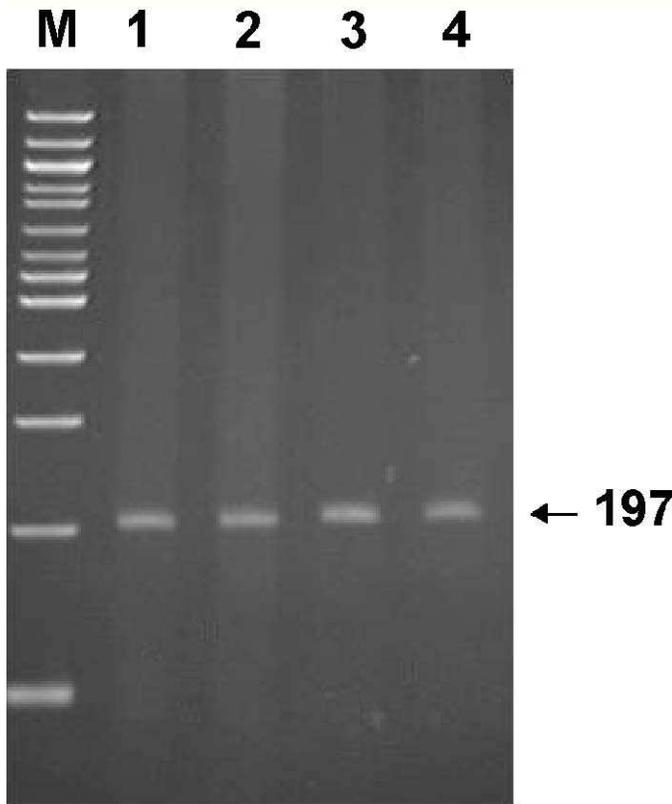


FIGURE 2. Acrylamide gel electrophoresis of amplification products from nested Np7/10 PCR of oocyst DNA extracted from *N. caninum* oocysts in feces of fox 1 (lane 1), fox 2 (lane 2), coyote 1 (lane 3), and coyote 2 (lane 4). M, 100-bp molecular size markers.

evident by DNA sequence analysis of 11 clones derived from fox 2 oocyst DNA and 8 clones derived from coyote 2 oocyst DNA (Fig. 3). Although none of the 6 sequences was identical to each other, BLAST-N similarity searching of the DNA database revealed nearly complete identity to the *N. caninum* Nc5 sequence derived from a variety of hosts. Nucleotide sequence data reported are available in the GenBank database under the accession numbers DQ132435 (NcFox1), DQ132436 (NcFox2a), DQ132437 (NcFox2b), DQ132438 (NcCoyote1), DQ132439 (NcCoyote2a), and DQ132440 (NcCoyote2b).

DISCUSSION

Neospora caninum-like oocysts were observed in the feces of 2 of 271 (0.7%) and 2 of 185 (1.1%) free-ranging foxes and coyotes, respectively, in PEI Province. Molecular techniques and DNA sequencing methods employed in this study verified the similarity of these oocysts to *N. caninum*. Both the prevalence and the number of *N. caninum*-like oocysts excreted in the feces of foxes and coyotes were low. Although our understanding of *N. caninum* oocyst excretion is limited, the low prevalence of oocyst excretion and the low numbers of oocysts observed in the feces of these foxes and coyotes suggest that *N. caninum* oocyst excretion is not common among wild canids on PEI. In previous experimental infections, dogs fed tissues from *N. caninum*-infected calves excreted more than 10,000 oocysts per day, with a total production of up to 500,000 oocysts (Gondim et al., 2002). In contrast, only 1 of 4 coyotes

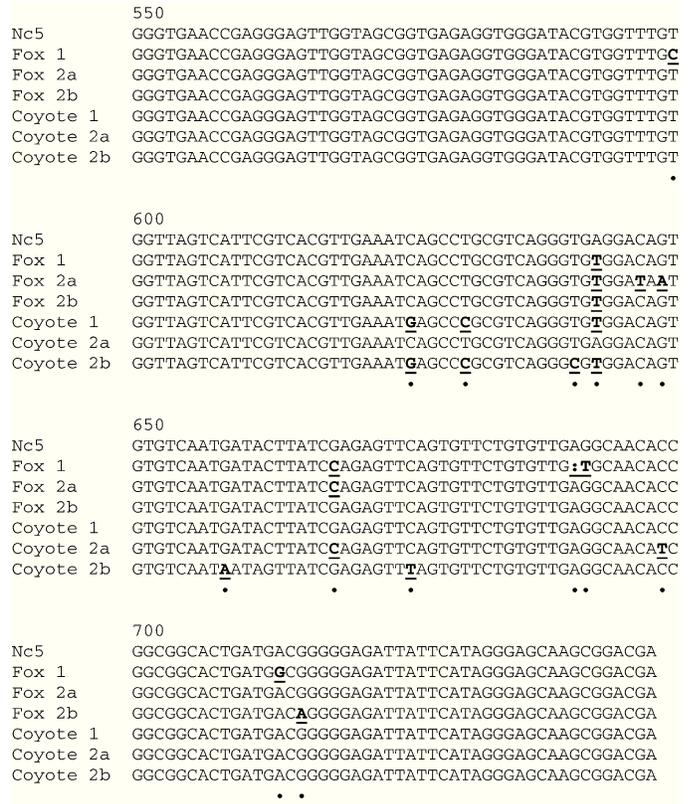


FIGURE 3. Alignment of Np7/10 sequences derived from nested PCR amplification of DNA from the *N. caninum* Nc-1 strain Nc5 sequence and *N. caninum* oocysts in feces of fox 1, fox 2 (a, b), coyote 1, and coyote 2 (a, b). The symbol • and bold underlined nucleotides indicate a nucleotide difference between at least 1 of the fox or coyote Np7/10 sequences and the *N. caninum* Nc-1 strain Nc5 sequence according to Yamage et al. (1996).

fed tissues from *N. caninum*-infected calves excreted oocysts and total production over 2 days was approximately 500 oocysts (Gondim, McAllister, Pitt, and Zemlicka, 2004). Infrequent excretion in wild canids would make it difficult to find a high prevalence of fecal-positive samples when sampling a limited number of samples during a short time period. Similar epidemiology has been described for *Toxoplasma gondii* (Pena et al., 2006), for which the percentage of cats shedding oocysts was much lower than cats seropositive to *T. gondii*. In general, the low numbers of oocysts shed in a sample would suggest inefficiency as a definitive host; however, excretion of *N. caninum* oocysts is influenced by a number of variables such as the intermediate host that was ingested (Gondim et al., 2002). In a previous experiment, foxes did not excrete oocysts after experimental infection, and only a few oocysts were shed by the positive control dogs following ingestion of goat and sheep tissues (Schaes et al., 2002). In this experiment, adequacy of infectious challenge or other experimental or field conditions may have contributed to the lack of oocyst excretion in foxes. Further experiments are needed to study oocyst shedding by wild canids.

Although *N. caninum*-like oocysts were observed in the feces of 2 foxes, this result does not confirm foxes are a definitive host for *N. caninum*. It is possible that these foxes ingested freshly passed, unsporulated *N. caninum* oocysts through co-

prophagy or predation, and no sexual reproduction of the parasite took place in these foxes. Assessing the suitability of particular animals as definitive hosts is challenging, and in previous experimental infections foxes did not excrete *N. caninum* oocysts when fed tissues of infected sheep and goats. However, the foxes used for previous experimental infection were 4 mo and older, and infection was performed with a single *N. caninum* isolate obtained from a naturally infected dog (Schares et al., 2002). These factors could have contributed to the failure of foxes to excrete *N. caninum* oocysts.

The possibility that the *N. caninum*-like oocysts observed could be a different species of coccidian, with *N. caninum* DNA amplified by PCR coming from a recent meal, needs to be considered. Theoretically, DNA from *N. caninum* bradyzoites could have passed through the gastrointestinal tract after ingesting an infected intermediate host, excreted in the feces, and be detected by PCR. Recently, unsporulated *T. gondii* oocysts were observed in the feces of 2 dogs, probably due to coprophagy, because *Felis* spp. (i.e., cats) are the only known definitive hosts for *T. gondii* (Schares et al., 2005). Although foxes prey primarily upon rabbits, rodents, birds, and invertebrates, they also scavenge carcasses and consume vegetation that could act as possible sources for *N. caninum* oocysts (Richards et al., 1995). Due to the limited amount of DNA, it was not possible to perform a PCR to detect DNA from *T. gondii*. However, the Nc5 region used is known to be specific for *N. caninum* (Kaufmann et al., 1996).

In this study, both foxes and coyotes that excreted *N. caninum* oocysts were negative on serological examination. This is in agreement with previous studies where dogs did not seroconvert when shedding oocysts (Lindsay et al., 1999; Dijkstra et al., 2001).

Because *N. caninum* oocysts are morphologically similar to *H. heydorni* oocysts (Schares et al., 2005), for which foxes are a definitive host (Dubey et al., 2002), molecular confirmation of oocyst identity was carried out. Ideally, a bioassay to confirm that the oocysts observed in these foxes and coyotes were *N. caninum* would have been appropriate, but it was not feasible due to the low number of oocysts observed. However, the use of PCR assays and sequencing as described herein can specifically identify *N. caninum* oocysts (Hill et al., 2001) and was used previously in place of a bioassay to confirm *N. caninum* oocyst shedding in coyotes (Gondim, McAllister, Pitt, and Zemlicka, 2004). A PCR utilizing specific primers for *H. heydorni* failed to yield an amplification product, providing additional evidence that the oocysts observed microscopically were *N. caninum*. However, PCR used to identify *H. heydorni* was not a nested PCR, therefore negative *H. heydorni* results may have been caused by using a less sensitive protocol compared with that used for *N. caninum*. The genetic variation observed in the Nc5 sequence is consistent with that observed by others and may be due to the repetitive nature of Nc5 in the *N. caninum* genome (Muller et al., 2001). The reason for this variation to be present in oocysts recovered from 1 host (fox 2 or coyote 2) and not another (fox 1 or coyote 1) is unknown. It is unlikely due to mutation during PCR amplification of oocyst DNA because sequence analysis was performed on numerous clones derived from 2 different PCR amplifications. Genetic and biological variation among different isolates of *N. caninum* has been reported (Atkinson et al., 1999; Schock et al., 2001; Gon-

dim, Laski et al., 2004). The Nc5 region must be regarded as an anonymous sequence because translation of the sequence revealed a few short open reading frames. However, none demonstrated any significant homology to peptide sequences (Kaufmann et al., 1996).

Although other explanations for finding *N. caninum* DNA in fecal material must be considered, the most likely explanation is that foxes and coyotes are definitive hosts and were passing *N. caninum* oocysts formed after ingesting infected material. Despite the fact that epidemiological evidence indicates foxes and coyotes are associated with *N. caninum* infections in cattle (Barling et al., 2000), *N. caninum*-like oocysts were detected in very few foxes and coyotes in PEI, and oocysts were observed in very low numbers. Further work is required to better understand the dynamics of *N. caninum* oocyst excretion in definitive hosts and to determine their role in the epidemiology of bovine neosporosis.

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