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Molecular and Biological Characterization of *Hammondia heydorni*–Like Oocysts From a Dog Fed Hearts From Naturally Infected White-Tailed Deer (*Odocoileus virginianus*)

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ABSTRACT: *Neospora caninum* and *Hammondia heydorni* are morphologically and phylogenetically related coccidians that are found in dogs. Although there is serological evidence of *N. caninum* infection in the white-tailed deer (*Odocoileus virginianus*), the parasite has not been yet isolated from the tissues of this host. In an attempt to isolate *N. caninum* from deer, hearts from 4 deer with antibodies to *N. caninum* were fed to 2 dogs. One of these dogs shed unsporulated oocysts 12–14 μm in diameter. Sporulated oocysts were not infective to Mongolian gerbils (*Meriones ungulatus*), and DNA isolated from these oocysts was not amplified using *N. caninum*–specific primers. However, positive amplification with the *H. heydorni*–specific first internal transcribed spacer (ITS-1) primers and common toxoplasmatiid ITS-1 primers confirmed the presence of *H. heydorni* DNA in the samples. The oocysts were considered to be *H. heydorni* on the basis of their morphology, biology, and molecular characteristics. This is the first record of a *H. heydorni*–like parasite in the white-tailed deer.

Neospora caninum is a parasite of livestock and companion animals and is an important cause of bovine abortion in dairy cattle worldwide (Dubey, 2003). It is transmitted transplacentally, by the ingestion of infected tissues and by the ingestion of food and water contaminated with oocysts excreted in the feces of dogs. The domestic dog is the only known definitive host for *N. caninum* (McAllister et al., 1998). The role of the dog in the epidemiology of *N. caninum* is currently unclear because experimentally infected dogs usually excrete only a few oocysts, and the parasite has been isolated only a few times from naturally infected dogs (Basso et al., 2001; Gondim et al., 2002; Šlapeta, Modry et al., 2002; McGarry et al., 2003). Furthermore, *N. caninum* oocysts morphologically resemble the oocysts of a related coccidian, *Hammondia heydorni*, and there is no simple method to distinguish them.

Little is known about the life cycle of *H. heydorni* or whether additional *Hammondia* species that use dogs as their definitive host occur (Dubey et al., 2002; Schares et al., 2002; Šlapeta, Modry et al., 2002). *Hammondia heydorni*–like oocysts were found in the feces of dogs that were fed naturally infected tissues from cattle (*Bos taurus*), water buffalo (*Bubalus bubalis*), sheep (*Ovis aries*), goats (*Capra hircus*), moose (*Alces alces*), and camels (*Camelus dromedarius*) (reviewed in Dubey et al., 2002). In addition, red foxes (*Vulpes vulpes*) fed tissues from sheep, cattle, roe deer (*Capreolus capreolus*), mountain gazelle (*Gazella gazella*), and reindeer (*Rangifer tarandus*) shed *H. heydorni*–like oocysts in their feces (Dubey et al., 2002). Until recently, all these oocysts excreted in feces of dogs and foxes were considered 1 species. However, studies of Schares et al. (2002, 2003) indicated the parasite in foxes is different morphologically and biologically from the parasite in dog feces. Furthermore, molecular studies indicate that there are more than 1 genetic variant at present designated as *H. heydorni* (Sreekumar et al., 2003). We report isolation of another *H. heydorni*–like parasite from the white-tailed deer (*Odocoileus virginianus*) for the first time from this host.

During studies on the genetic characterization of *Toxoplasma gondii* isolates from wildlife (Dubey et al., 2004) in March 2003, tissues from 4 white-tailed deer from Mississippi were also examined for antibodies to *N. caninum* using the *N. caninum*–agglutination test (NAT; Romand et al., 1998). The NAT titers were 1:25 in 2 and 1:50 in 2 deer. Heart tissue from these 4 deer were pooled and fed to 2 laboratory-raised dogs. The dogs had not ingested uncooked meat products before feeding on deer tissues. Feces of these dogs were examined daily for 3 wk for coccidian oocysts by floatation in sugar solution. Oocysts were collected from feces, sporulated in 2.5% potassium dichromate aqueous solution at room temperature for 7 days, and then stored at 4 C until used. Sporulated oocysts were washed with water to remove potassium dichromate by centrifugation, treated with 5.25% sodium hypochlorite solution (Clorox), washed, and divided into aliquots for bioassay, in vitro cultivation, and polymerase chain reaction (PCR) studies. Aliquots were fed to 2 gerbils (*Meriones ungulatus*) and to 5 interferon gamma gene knockout (KO) mice (Dubey and Lindsay, 1998). For in vitro cultivation, 1 aliquot was vortexed for 5 min with 500-μm glass beads (Microbeads, Ferro Corporation, Cleveland, Ohio) and subsequent incubation in an excystation medium (sodium taurocholate 250 mg, sodium deoxycholic acid 400 mg, trypsin [1:250] 25 mg in 100 ml saline, pH 7.5) at 37 C. After excystation, the suspension was washed with growth medium and layered over each of the 2 CV1 (African Green monkey [*Cercopithecus aethiops*] kidney cells) and equine dermal cell monolayers grown over coverslips in multiwell plates. The coverslips were removed at intervals, fixed with Bouin fixative and stained with Giemsa.

For obtaining DNA, the oocyst suspension was ruptured by 2–3 freeze–thaw cycles, followed by grinding of the pellet in small volumes (about 30 μl) in a 0.2-ml microtissue grinder (Wheaton, Fischer Scientific, Pittsburgh, Pennsylvania). The DNA was extracted from the homogenized suspensions using DNAzol (MRC, Cincinnati, Ohio) according to the manufacturer's instructions. Five sets of primers, *Neospora*-specific NP6/NP21 (Yamage et al., 1996), *H. heydorni*–specific first internal transcribed spacer (ITS-1) RAPD primers JS4/JS5 (Šlapeta, Koudela et al., 2002), *H. heydorni*–specific HhAP7 and HhAP10 primers, and common toxoplasmatiid ITS-1 primers CT1/CT2 (Sreekumar et al., 2003), were used for PCR amplification of the DNA, according to previously described protocols. The PCR products were electrophoresed in a 2% agarose gel, and the gel-cleaned PCR products were directly sequenced in both directions using the Big Dye terminator system, version 3.1 (Applied Biosystems, Foster City, California) using an ABI 377 sequencer. The sequence chromatograms were edited using Sequencher 4.1 software (Genecodes Corp., Ann Arbor, Michigan).

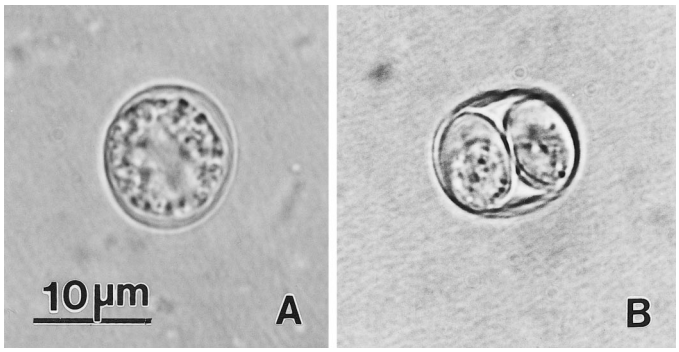

FIGURE 1. Unsporulated (**A**) and sporulated (**B**) oocysts of the *Hammondia heydorni*–like parasite shed in the feces of a dog fed naturally infected deer tissues.

Searches were performed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) to determine whether the sequences were similar to any of the previously published sequences.

One dog shed unsporulated oocysts in feces 10–12 days after ingesting deer tissue. Unsporulated oocysts were 12–14 × 10–12 µm (n = 100) in size, and sporulated oocysts contained 2 sporocysts, 6–8 × 4–6 µm in size (Fig. 1A, B). There were 4 sporozoites in each sporocyst. The gerbils and KO mice fed sporocysts remained asymptomatic, and antibodies to *N. caninum* were not found in 1:25 dilution of their serum tested 30 days after inoculation. Protozoans were not found in histological sections of rodents fed oocysts.

Sporozoites were found to excyst within 20 min of incubation of cleaned oocysts in the excystation media. The sporozoites did not infect the equine dermal cell monolayer. Dividing forms could be seen in the CV1 cells stained on day 14 after inoculation. However, no additional growth was seen, and the parasites gradually disappeared from the monolayers after 4 wk. A *H. heydomi*–like parasite from the feces of a dog was grown in vitro by Speer et al. (1988), but they were unable to infect new monolayers by passaging, and the parasite died out in culture. Schares et al. (2003) maintained a *H. hedorni*–like parasite from the feces of foxes in vitro and were able to demonstrate oocyst shedding in fox by feeding the infected culture. However, the parasite that Schares et al. (2003) studied was different from the parasite from dogs; its oocysts were larger and it was not transmissible to dogs through tissues of an intermediate host (sheep). In the present study, only a few oocysts were shed in dog feces, and we were unable to obtain sustainable growth in culture.

No amplification was observed with either the *N. caninum*–specific primers or the *H. heydorni*–specific RAPD–derived primers. The *H. heydorni*–specific ITS-1 primers resulted in the amplification of a product of the expected size (~270 bp). The sequences (GenBank AY531300) were found to be identical to those of *H. heydorni* isolates from dogs and fox. The common toxoplasmatiid ITS-1 primers resulted in the amplification of a product in the size range of 399 bp (GenBank AY530018). The results of the BLAST search of this fragment with the public database are shown in Table I. Polymorphism was noticed at 8 locations. The sequences of the deer isolate were identical to that of a *H. heydorni* isolate of dog from the United States. However, the sequences also had greater similarity to an isolate from fox than other canine isolates from Germany, Czech Republic, and Australia. Recent evidence has pointed to the presence of molecular differences among *H. heydorni* isolates from dogs and foxes. Mohammed et al. (2003) concluded that the isolates of *H. heydorni* from foxes and dogs constitute genetically different populations on the basis of the ribosomal DNA sequences. The deer isolate, which was excreted by a dog, was found to be closer to the fox isolate than the dog isolates from Germany, Czech Republic, and Australia.

The results of the present study indicate that dogs can excrete *H. heydorni*–like oocysts in their feces, and these must be distinguished from *N. caninum* oocysts. The results also point to a greater genetic diversity among the *H. heydorni* isolates irrespective of their host.

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TABLE I. Sequence comparison of a 399-bp region of ITS-1 from different isolates of *Hammondia heydorni*.

Isolate location/host(s)	GenBank accession no.	Polymorphic sites							
		165	209	293	317	331	356	361	368
United States/Deer, dog*	AY530018*	A	C	A	T	C	C	T	—†
USA?/Dog	AF096501	.‡	.	.	.	.	.	.	—
Germany/goat, fox	AF395867	.	G	.	.	.	.	.	—
Czech Republic/Dog	AF317282	G	G	G	C	.	A	.	T
Germany/Guinea pig, dog	AY189897	G	G	G	C	T	A	.	—
Australia/Dog	AF508030	G	G	G	C	T	A	.	—
Czech Republic/Dog	AF317281	G	G	G	C	T	A	C	T

* Present study.
† Alignment gaps are indicated by dashes.
‡ Sequences identical to the deer isolate are indicated with a period.

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Ingestion of *Cryptosporidium* Oocysts by *Caenorhabditis elegans*

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ABSTRACT: *Cryptosporidium parvum* has been associated with outbreaks of human illness by consumption of contaminated water, fresh fruits, and vegetables. Free-living nematodes may play a role in pathogen transmission in the environment. *Caenorhabditis elegans* is a free-living soil nematode that has been extensively studied and serves as a good model to study possible transmission of *C. parvum* oocysts that may come into contact with produce before harvest. The objective of this study was to determine whether *C. elegans* could serve as a potential mechanical vector for transport of infectious *C. parvum* and *Cyclospora cayetanensis* in agricultural settings and whether *C. elegans* could ingest, excrete, and protect oocysts from desiccation. Seventy to 85% of worms ingested between 0 and 500 oocysts after 1 and 2 hr incubation with oocysts. Most of the nematodes ingested between 101 and 200 oocysts after 2 hr. Intact oocysts and empty shells were excreted by nematodes. Infectivity was determined by the neonatal assay with different treatments of worms (intact or homogenized) or oocysts or both. Adult *C. elegans* containing *C. parvum* kept in water were infective for mice. In conclusion, *C. elegans* adults can ingest and excrete *C. parvum* oocysts. *Caenorhabditis elegans* containing *C. parvum* oocysts can infect mice but does not seem to protect oocysts from extreme desiccation at 23 C incubation of a day or longer. *Cyclospora* oocysts were not ingested by *C. elegans*. The role of free-living nematodes in produce contamination needs to be further examined.

Parasites, such as *Cryptosporidium parvum*, *Cyclospora cayetanensis*, and *Giardia lamblia* have been causative agents in outbreaks of human illness associated with consumption of contaminated water, fresh fruits, and vegetables (Orlandi et al., 2002).

The dissemination of pathogenic parasites onto raw produce has not been thoroughly studied (Wasilewska and Webster, 1975) but may be introduced during pre- and postharvest practices. Potential agricultural sources of produce contamination include farm workers, animals, irrigation water, soil, and insects. Flies have been shown to transport infectious *C. parvum* oocysts (Graczyk et al., 1999). If produce comes into contact with manure, then free-living soil nematode contamination could occur. Irrigation water can also be contaminated with feces of humans and wild animals, introducing large numbers of parasites into the environment, particularly in rivers and lakes. Rotifers can ingest *C. parvum* oocysts and *G. lamblia* cysts. The fate of the parasites once ingested by rotifers needs to be studied further (Fayer et al., 2000; Trout et al., 2002).

A well-studied and harmless free-living nematode, *Caenorhabditis elegans*, may serve as a model to study nematodes acting as carriers or transport hosts by ingesting harmful bacteria and then protecting such organisms from environmental conditions while in the gut (Chang et al., 1960). *Salmonella enterica* serotype Poona and *S. typhimurium* can be ingested by *C. elegans*, providing protection against inactivation by produce sanitizers (Labrousse et al., 2000; Caldwell et al., 2003). The objective of the present study was to determine whether *C. elegans* could serve as a potential mechanical vector for transport of infectious *Cryptosporidium* in agricultural settings and if the nematode could ingest, excrete, and protect oocysts from desiccation. *Caenorhabditis elegans* (N2, wild-type strain) was used in all experiments. The worms were grown on K-agar plates (pH 6.5) (2.36 g potassium chloride, 3 g sodium chloride, 2.5 g Bacto Peptone, and 17 g/L agar in deionized water). The agar was autoclaved, cooled, and supplemented with 1 g cholesterol (95%), 11.1 g calcium chloride, and 24.7 g magnesium sulfate. Medium was distributed in plastic petri plates. *Escherichia coli* was cultured in OP50 broth (5 g sodium chloride and 10 g Bacto Peptone) for 24 hr, plated in K agar, and incubated at 37 C for 24 hr until confluent growth was established. Nematode cultures were fed with confluent cultures of *E. coli* OP50.

Cryptosporidium parvum oocysts (Iowa isolate, bovine genotype) were obtained from the Parasitology Laboratory, University of Arizona. Oocysts were labeled with Merifluor® Cryptosporidium/Giardia Direct Immunofluorescent Detection Reagent (Meridian Bioscience Inc., Cincinnati, Ohio). The oocysts (approximately 3×10^7) were resuspended in 100 μl K-medium and 40 μl of the Detection Reagent was added. Incubation was at room temperature for 60 min, with mixing every 15 min. Detection Reagent was removed by microcentrifugation (8,000 rpm for 4 min) and oocysts were washed with fresh K-medium.

Unsporulated *C. cayetanensis* oocysts were obtained from naturally infected individuals with cyclosporiasis. Fecal samples containing oocysts were concentrated using the modified ethyl acetate method followed by a discontinuous sucrose gradient (Ortega et al., 1998).

Between 100 and 200 adult nematodes were placed on K-agar plates