Detection of *Hammondia heydorni*–Like Organisms and Their Differentiation From *Neospora caninum* Using Random-Amplified Polymorphic DNA–Polymerase Chain Reaction

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Detection of *Hammondia heydorni*–Like Organisms and Their Differentiation From *Neospora caninum* Using Random-Amplified Polymorphic DNA–Polymerase Chain Reaction

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ABSTRACT: *Neospora caninum* and *Hammondia heydorni* are morphologically and phylogenetically related coccidians that are found in dogs. New diagnostic genetic loci, based on random-amplified polymorphic DNA–polymerase chain reaction (RAPD-PCR), were developed to aid in the detection of *H. heydorni*–like parasites and to discriminate them from *N. caninum* and other related coccidians of dogs. On the basis of the data obtained from 5 random decamers, *H. heydorni* (Manhattan-1) and *N. caninum* (NC1) were characterized by distinct banding patterns (similarity index = 0.068). High-stringency PCR assays were developed from the sequences of 2 cloned bands (GenBank BZ592549 and BZ592593), uniquely amplified from *H. heydorni*. Interestingly, using these primers, PCR amplification was achieved only from 2 of the 5 isolates presumed to represent *H. heydorni*. The same result was obtained from these 5 isolates using a recently described PCR assay directed to the *H. heydorni* internal transcribed spacer-1. It is concluded that *H. heydorni* and *N. caninum* are genetically distinct and that such tools may be useful for more detailed characterization of the diversity of related parasites occurring in dogs.

Neospora caninum is a parasite of livestock and companion animals and is an important cause of bovine abortion in dairy cattle worldwide (Dubey, 1999). 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 excrete only a few oocysts and the parasite has been isolated only twice from naturally infected dogs (Basso et al., 2001; Šlapeta, Modry et al., 2002). Furthermore, *N. caninum* oocysts resemble morphologically 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 occur that use dogs as their definitive host (Dubey et al., 2002; Schares et al., 2002; Šlapeta, Modry et al., 2002).

Until recently, the only genetic locus characterized for *H. heydorni* was the 18S ribosomal DNA (Dubey et al., 2002). More genetic data are needed to determine how distinct *H. heydorni* and related species are from *N. caninum* and to differentiate among them. Recently, *H. heydorni* was discriminated from *N. caninum* using primers based on

TABLE I. *Hammondia heydorni* isolates and their amplification results.

Date received	Host/isolate designation	Amplification with: CT-1/CT-2,* Common	NP6/NP21, *Neospora caninum*	JS4/JS5, *H. heydorni*	HhAP7F&R,† HhAP10 F&R†
4 February 1999	Dog/Manhattan-1	Yes	No	Yes	Yes
15 October 1998	Dog/Virginia-1	Yes	No	Yes	Yes
4 April 2001	Dog/Mississippi-1	Yes	No	No	No
1988‡	Dog/Alabama-1‡	Yes	No	No	No
17 April 2002	Dog/Brazil-1	Yes	No	No	No

* Newly designed from ITS-1 sequences, amplifying DNA from *Toxoplasma gondii*, *N. caninum*, and *Hammondia* sp.
† Newly designed from the sequences of the polymorphic RAPD bands unique to *H. heydorni*.
‡ Blagburn et al. (1988) and Dubey et al. (2002).

the first internal transcribed spacer (ITS-1) sequences of ribosomal DNA (Šlapeta, Koudela et al., 2002). The primers were designed from a region of the genome that is relatively conserved among many apicomplexans. Primers based on polymorphic sequences unique to *H. heydorni* may unequivocally differentiate it from *N. caninum* and provide an independent means of assessing genetic diversity among these related canine coccidia. An approach for studying DNA polymorphisms without the requirement of prior knowledge of the genome is the random-amplified polymorphic DNA–polymerase chain reaction (RAPD-PCR) (Welsh and McClelland, 1990; Williams et al., 1990). This technique uses single oligonucleotide primers at low stringency to produce polymorphic DNA. The patterns generated using this technique provide a rapid method for detecting genetic variation. Sequences obtained from individual polymorphic fragments thus generated can enable the design of primers for the PCR-based diagnosis and differentiation of closely related species under more stringent conditions, yielding reproducible results (Cere et al., 1996). In this study, we used RAPD-generated polymorphic fragments to design diagnostic primers to differentiate *H. heydorni* from *N. caninum* and used them to explore the genetic diversity among several canine coccidian isolates.

Information about the *H. heydorni*–like isolates used is given in Table I. These isolates were considered as *H. heydorni* based on the morphology of the oocysts (10.7 by 13.8 μm, n = 50) and their inability to amplify the target fragment with *N. caninum*–specific primers (Table II). The NC1 isolate of *N. caninum* (Dubey et al., 1988) and the VEG strain of *Toxoplasma gondii* (Dubey et al., 1996) were maintained as tachyzoites in vitro in HCT-8 (ATCC CCL-244) cells. Isolates of *Isospora canis*, Nemeséri, 1959, *Sarcocystis tenella* (Railliet, 1886), Moule, 1886, and *H. hammondi*, Frenkel and Dubey, 1975 were available as sporocysts or sporulated oocysts.

To obtain DNA from *H. heydorni*, *H. hammondi*, *S. tenella*, and *I. canis*, sporulated oocyst or sporocyst suspensions were washed by repeated centrifugation in distilled water to remove the potassium dichromate. Each pellet was treated with 10–15 ml of 5.25% sodium hypochlorite (on ice) to remove organic debris and then washed in water. The oocysts were 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. *Toxoplasma gondii* and *N. caninum* DNA were isolated using DNAzol from culture-derived tachyzoites. Canine DNA was isolated from the blood of an uninfected dog using the same procedure. The DNA preparations were suspended in distilled water and quantified spectrophotometrically (DU 640, Beckman, Fullerton, California).

Five random decamers (Table II) were used to investigate microhet-

TABLE II. Primers and PCR conditions.

Primer name, sequence (5′–3′)	Target DNA (band size [bp])	Cycling conditions*; (reference)
AP7, gtgatcgcag	DNA	45 (94 C/1 min, 36 C/45 sec, 72 C/1 min); (Dubey et al., 2003)
AP10, ccggtgtggg	DNA	
AP15, cggacgtcgc	DNA	
AP17, tcacgatgca	DNA	
AP22, ctgagacgga	DNA	
HhAP7F, ggcagtgggcacatacag	*Hammondia heydorni* DNA (517)	10 (94 C/1 min, 65 C/1 min, 72 C/1 min), 20 (94 C/1 min, 60 C/1 min, 72 C/1 min); (this study)
HhAP7R, gcagtgcttcgagaatgc		
HhAP10F, ccggagtacaatagcgctgcc	*H. heydorni* DNA (369)	
HhAP10R, ccacgaccgccaaatgatacaac		
CT-1, tgaatcccaagcaaaaca	Toxoplasmatiid DNA (~400)	40 (94 C/1 min, 60 C/1 min, 72 C/1 min); (this study)
CT-2, gcgcgagccaagacatccat		
JS4, cgaaatgggaagttttgtgaac	*H. heydorni* DNA (~270)	35 (95 C/1 min, 65 C/1 min, 72 C/1.5 min); (Šlapeta, Koudela et al., 2002)
JS5, cagcagctacatacgtaga		
COC-1, aagtataagcttttatacggct	Apicomplexan DNA (298–350)	40 (94 C/1 min, 60 C/1 min, 72 C/1 min); (Ho et al., 1996†)
COC-2, cactgccacggtagtccaatac		
NP6, cagtcaacctacgtcttct	*Neospora caninum* DNA (328)	40 (94 C/1 min, 50 C/1 min, 72 C/2 min); (Yamage et al., 1996)
NP21, gtgcgtccaatcctgtaac		

* An initial denaturation at 95 C for 5 min and final extension at 72 C for 5 min added to all protocols.
† Original PCR conditions modified.

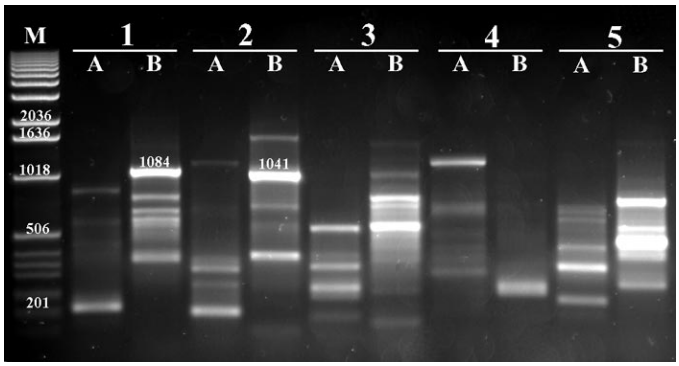

FIGURE 1. RAPD-PCR fingerprints of *Neospora caninum* (**A**) and *Hammondia heydorni* (**B**) with random primers AP7 (1), AP10 (2), AP15 (3), AP17 (4), and AP22 (5). Polymorphic fragments of 1,084 bp (1B) and 1,041 bp (2B) were cloned and sequenced. M = 1 kb DNA ladder (GIBCO BRL), Gaithersburg, Maryland.

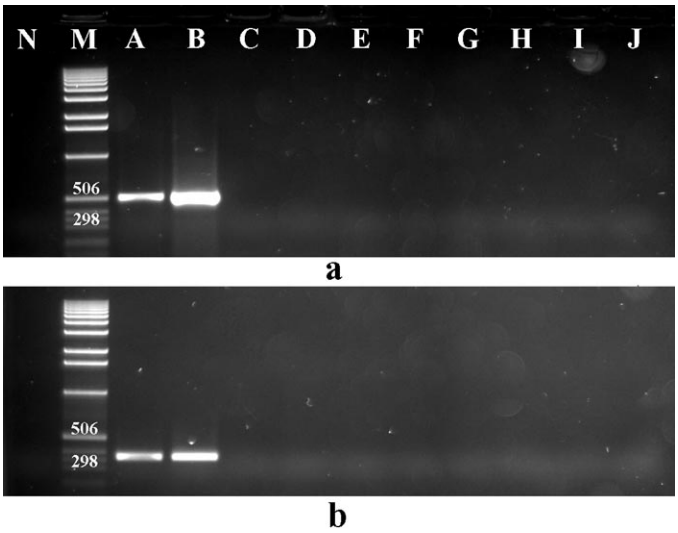

FIGURE 2. PCR with de novo primers HhAP7 (**a**) and HhAP10 (**b**). Specific amplification of 517-bp (a) and 369-bp (b) fragments noticed only with *Hammondia heydorni* DNA (lanes A and B). No amplification was noticed with negative control (N), *Neospora caninum* (C, D, and E), *Sarcocystis tenella* (F), *Isospora canis* (G), *Toxoplasma gondii* (H), *H. hammondi* (I), and canine DNA (J). M = 1 kb DNA ladder (GIBCO BRL).

erogenity between the isolates of *N. caninum* (NC1) and *H. heydorni* (Manhattan-1). The Manhattan-1 isolate was chosen because of high oocyst count and apparent absence of other parasites. The RAPD-PCR reactions were set up in 25-μl volumes in 0.2-ml thin-walled PCR tubes as described by Dubey et al. (2003). The PCR assays were run 3 times to ensure reproducibility. Electrophoresis was performed at 5 V/cm (90–100 V) and documented using ProExpress Gel Documentation system (Perkin Elmer, Wellesley, Massachusetts). Data from each isolate–primer combination were combined, and the similarity coefficient between the isolates was calculated (Nei and Li, 1979).

Fragments unique in the RAPD fingerprint of *H. heydorni* DNA were selected for cloning and sequencing. The region of the gel containing the band was excised and placed in a microcentrifuge tube. The DNA was extracted from the gel piece using GFX PCR gel band purification kit (Amersham, Piscataway, New Jersey), reamplified using the original random primer, and cloned into TOPO-TA vectors (Invitrogen, Carlsbad, California). The vectors were inserted into competent DH5α cells (Invitrogen) and cultured overnight according to the manufacturer's instructions. Colonies with inserts were selected by blue–white differentiation, and white colonies were cultured overnight in Luria–Bertani broth at 37 C. Plasmids were extracted using QIAprep Spin Miniprep Kit (Qiagen, Valencia, California), and DNA was quantified. Sequencing reactions were performed using the Big Dye terminator system (Applied Biosystems, Foster City, California) and sequenced in an ABI 3100 sequencer. The sequence chromatograms were edited using Sequencher software (Genecodes Corp., Ann Arbor, Michigan). BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) searches were performed to determine whether the sequences were similar to any of the previously published sequences of *H. heydorni* or any other parasite.

De novo primers were designed from the cloned RAPD products using the Gene Tool software program (Bio Tools Inc., Edmonton, Alberta, Canada). Primer pairs were optimized for amplification of the target fragments from *H. heydorni* DNA. To ascertain the specificity of these primers for *H. heydorni*, amplification was attempted from DNA of other apicomplexans (*N. caninum*, *S. tenella*, *I. canis*, *T. gondii*, and *H. hammondi*), canine DNA, and negative controls without any DNA. The quality of the *H. heydorni* DNA samples was verified using newly designed primers complementary to portions of the ITS-1 sequences conserved among *T. gondii*, *N. caninum*, and *Hammondia* sp. (Table II).

Experiments were conducted to determine the minimum amount of parasite DNA required to produce a visible band, using the newly designed PCR assays. PCR reactions were run, using serial 10-fold dilutions of DNA (representing 10,000 oocysts to 0.001 oocyst). The lowest number of oocyst(s) yielding a detectable band was considered to be the threshold level. For comparative purposes, the ITS-1 primers (Šlapeta, Koudela et al., 2002) were used under the same PCR conditions.

All 5 random primers produced DNA fingerprint patterns with both *N. caninum* and *H. heydorni* DNA (Fig. 1). The reproducible banding patterns were distinct for each template, with monomorphic fragments being amplified using only 2 primers. Of the 44 fragments amplified by the 5 primers in total (21 for *N. caninum* and 23 for *H. heydorni*), only 3 (1 using primer AP7 and 2 using primer AP15) were shared. On the basis of the combined data from all the primers, a low similarity coefficient of 0.068 was established. The DNA fingerprint patterns generated by the 2 parasites in this study showed significant heterogeneity. The low similarity coefficient indicated that the parasites, although morphologically similar, were genetically distinct. It would be worthwhile to investigate the microheterogeneity among these parasites using a larger panel of random primers.

Two intense and reproducible bands of ~1,000 bp (designated henceforth as AP7 and AP10 fragments), amplified only from the *H. heydorni* DNA using primers AP7 and AP10, respectively, were selected for cloning and sequencing (GenBank BZ592549 [AP7] and BZ592593 [AP10]). The fragments bore no significant similarity to any published sequences for *H. heydorni*, *N. caninum*, or any other apicomplexan when the public database was queried with BLAST. Given the nature of distinct banding patterns between *N. caninum* and *H. heydorni*, it is

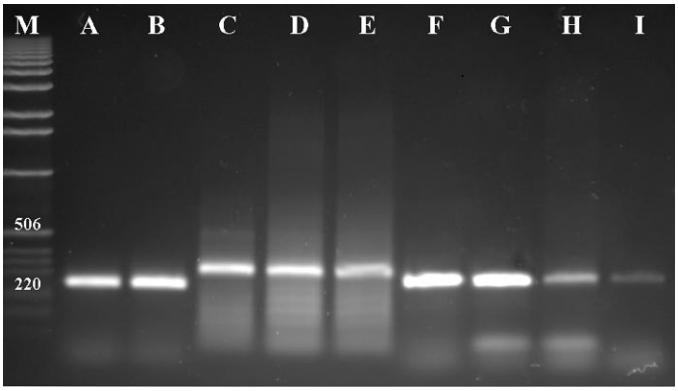

FIGURE 3. Amplification of ~270-bp fragment from *Hammondia heydorni* (**A**–**B**), 328-bp fragment from *Neospora caninum* (lanes C, D, and E) and ~300-bp fragments from *Sarcocystis tenella* (F), *Isospora canis* (G), *Toxoplasma gondii* (H), and *H. hammondi* (I) DNA using previously described primers (refer Table II). M = 1 kb DNA ladder (GIBCO BRL).

possible that these fragments are unique to *H. heydorni* and can be valuable genetic markers for the unequivocal differentiation of *H. heydorni* from *N. caninum*. Such polymorphic fragments have been successfully used in the design of specific primer sets capable of differentiating other coccidians (Cere et al., 1996).

Two sets of primers were designed, 1 each from the AP7 fragment (HhAP7 forward and reverse) and AP10 fragment (HhAP10 forward and reverse). Under optimal thermal cycling conditions (Table II), a single amplification product was obtained from each assay. As estimated by their migration in an agarose gel, the fragments appeared to correspond to the sizes expected (517 and 369 bp for AP7 and AP10, respectively), based on their design (Fig. 2). The optimal $MgCl_2$ concentration was determined to be 1 mM for AP7 fragment and 1.25 mM for the AP10 fragment.

The primers designed from the 2 fragments were found to specifically amplify the DNA from 2 *H. heydorni* isolates (Manhattan-1 and Virginia-1) obtained from dogs of 2 different localities in the United States (Fig. 2). No amplicon was produced from the templates of the other canine apicomplexans, the host DNA, or negative controls, providing evidence for specificity of the primers. The suitability of these other DNA samples as PCR templates was confirmed by the amplification of fragments of expected length using appropriate primer sets. Thus, whereas an amplicon of 328 bp was produced from the *N. caninum* DNA using the NP6/NP21 primer sets (Yamage et al., 1996), fragments of approximately 300 bp were amplified using the common apicomplexan ssrRNA primers (Ho et al., 1996) from *S. tenella* and *I. canis*, *T. gondii*, and *H. hammondi* DNA (Fig. 3).

The sensitivity assays revealed that whereas the new primers could detect 100 oocysts, the ITS-1 primers were able to produce a signal with 1 oocyst in a 25-μl reaction. It is possible that a large proportion of these oocysts was degraded, and thus the number of intact oocysts contributing to the template was much less than assumed. The threshold should be verified using high-quality DNA from fresh oocysts.

Effective amplification of the ~400-bp product was achieved from all the 5 *H. heydorni* DNA samples using the toxoplasmatiid ITS-1 primers, confirming the presence of an adequate amount of amplifiable template. The Manhattan-1 and Virginia-1 isolates, which gave positive results using the primers constructed herein, also tested positive using the previously published, *H. heydorni*–specific, ITS-1 primers (Fig. 3), confirming their identity as *H. heydorni*. However, no amplification was observed with the DNA of the other 3 *H. heydorni* isolates using the de novo primers, *H. heydorni* ITS-1 primers, or the *N. caninum*–specific primers. Thus, the evidence suggests that these 3 isolates are neither *N. caninum* nor *H. heydorni*.

The results of amplification with the 5 pairs of primers (common toxoplasmatiid ITS-1, *N. caninum*–specific, *H. heydorni*–specific ITS-1, and 2 *H. heydorni*–specific de novo primers) indicate that more than 1 *Hammondia*-like parasite might be present in dogs (Table I). It has been proposed that *H. heydorni* encompasses more than 1 species (Dubey et al., 2002). Results of PCR obtained using the 2 new primer pairs reported herein provide additional support for the genetic distinction between *H. heydorni* and *N. caninum* and suggest a greater diversity between the morphologically similar parasites presently grouped as *H. heydorni*. Further studies are warranted to elucidate the identity of these hitherto uncharacterized parasites.

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