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Detection of *Hammondia heydorni* DNA in feces collected in and around an Ohio Wildlife Conservation Center

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Abstract

This study aimed to identify DNA attributed to *Hammondia heydorni* oocysts in the feces of wild canids in and around an Ohio wildlife conservation center. Two hundred and eighty-five wild canid fecal samples were analyzed using PCR with melting curve analysis to detect coccidian DNA. Coccidia-positive samples were further subjected to *H. heydorni*-specific and *N. caninum*-specific PCR assays. Samples positive by the *H. heydorni*-specific assay were additionally analyzed with a PCR assay to distinguish *H. heydorni* from *Hammondia triffittae*. Coccidian DNA was detected in 51 of the 285 (17.9%) wildlife samples. *H. heydorni* DNA was detected in three of the coccidia-positive wildlife samples (1.1%) and *N. caninum* was detected in none. Determining the presence of *H. heydorni* in wild canids will contribute to a greater understanding of the role these hosts play in the ecology of this parasite.

Keywords

Hammondia; fecal; coccidia; wild canids; PCR

1. Introduction

Hammondia heydorni and *Neospora caninum* are closely related, tissue-cyst forming members of the Sarcocystidae family. *Neospora caninum* is an important cause of abortion in cattle worldwide (Dubey and Schares, 2011) and can cause severe neuromuscular disease in young dogs (Reichel et al., 2007). However, *H. heydorni* generally does not produce clinical signs in either definitive or intermediate hosts (Dubey et al., 2002). Both parasites use domestic and wild canids as definitive hosts and a wide variety of herbivore species as intermediate hosts (Dubey et al., 2002, 2007). Oocysts of these parasites are indistinguishable, with both species producing nearly spherical, thin-walled unsporulated

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Conflict of interest

The authors declare that there is no conflict of interest.

oocysts approximately 11μm in diameter (Lindsay et al., 1999). Therefore, light microscopy cannot be used to distinguish *N. caninum* and *H. heydorni* oocysts shed by canids.

Because of the important economic impact of *N. caninum* on the cattle industry, much work has been done to understand the transmission dynamics of this disease. Comparatively little is known about the ecology of *H. heydorni* in wildlife. Surveys of free-ranging wild canids that have detected *H. heydorni* were unable to genetically distinguish this parasite from *N. caninum* (Gompper et al., 2003; Elmore et al., 2013). The goal of this study was to use a series of PCR assays to determine the presence of DNA attributed to coccidia, *N. caninum* or *H. heydorni* oocysts in wild canid fecal samples (Fig. 1). Assessing the presence of *H. heydorni* DNA contained in feces from wild canids will provide baseline information for better understanding the ecology of this parasite in free-ranging wildlife.

2. Materials and methods

Two hundred and eighty-five wild canid fecal samples were collected in southeastern Ohio in and around a 10,000-acre private conservation center with a variety of semi-free ranging herbivores from May – August, 2014. Fecal samples were identified as wild canid feces by size, shape, and content (hair, bones, etc.). Samples were kept at 4°C until processing.

Duplicate tubes of five grams of each sample were processed by standard double centrifugation fecal flotation using Sheather's sugar solution. One tube was used microscopically examination for the presence of oocysts and the other tube had the top 2 mL of the flotation solution removed into 6 mL of 2.5% potassium dichromate to inhibit fungal and bacterial growth for storage at 4°C (7-13 months) until processed for DNA extraction (Fig. 1). To extract DNA, the flotation material was washed with phosphate buffered saline and centrifuged to remove the potassium dichromate. The resulting pellet was homogenized in 1.5 mL tubes containing ceramic beads using a Precellys® 24 tissue lyser/homogenizer (Bertin Technologies, USA). DNA was extracted using a Qiagen DNeasy® Blood & Tissue kit (Qiagen, Germany). Control oocysts of *Hammondia heydorni* (UCD 2011 isolate provided by P. Conrad) underwent similar processing. The Qiagen DNeasy® Blood & Tissue kit was also used to extract DNA from cell cultures of *Neospora caninum* (VMDL isolate, Hyun et al. 2003). Other controls for the PCR assays included DNA from the following parasites: Neospora hughesi, Besnoitia, Caryospora, Frenkelia, or *S. neurona*

As an initial screening tool for the presence of small coccidian oocysts, PCR with melting curve analysis was performed using the universal coccidian primer cocktail developed by Lalonde and Gajadhar (2011) to amplify a ~315 bp region of 18S rDNA. Originally, this assay was published and applied to detect Cryptosporidium parvum, Toxoplasma gondii, Cyclospora cayetanensis, and several species of *Eimeria, Isospora,* and *Sarcocystis* in food sources and clinical samples. Based on the target area and DNA sequence alignment analysis, this assay was hypothesized to be applicable to a wide range of coccidia that might be found in wild canid fecal samples. The PCR mixture contained 2X Power SYBR® green PCR Master Mix (Applied Biosystems, USA), the universal coccidian primer cocktail (500 nM Crypto-F, Crypto-R, Cyclo-F, Cyclo-R, Sarco-R, Toxo-F, and Toxo-R), 1μl template DNA, and dH_2O. Assays were performed using a StepOne™ Real-Time PCR system

(Applied Biosystems). The cycling conditions consisted of initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 sec and annealing and elongation at 60°C for 1 min. Melting curve analysis began immediately after the final cycle and consisted of a 15 sec hold at 95°C followed by a gradual increase from 55°C to 95°C in 0.1°C increments. DNA extracted from control *H. heydorni* oocysts and culture-derived control *N. caninum* tachyzoites were used as positive controls; distilled water served as a negative control. To evaluate the application and analytic sensitivity of the universal coccidian PCR and melting curve analysis to *Hammondia* and *Neospora*, ten-fold dilutions of 2 - 2 × 10^4 *H. heydorni* control oocysts and 2 - 2 × 10^5 cultured-derived *N. caninum* control tachyzoites underwent DNA extraction and universal coccidian amplification as described above.

The *H. heydorni*-specific and *N. caninum*-specific PCR assays were applied to the samples considered positive by the universal coccidian PCR assay, showing amplicons with a distinct melting curve and threshold peak values (melting temperature of 76°-86°C with -Rn' values >0.1). The JS4/JS5 primers target the ITS-1 region of *H. heydorni* (Slapeta et al., 2002) whereas the Np6+/Np21+ primers target the Nc5 gene of *N. caninum* (Müller et al., 1996; Spencer et al., 2000). Both assays were performed concurrently using a StepOne™ Real-Time PCR system (Applied Biosystems). The final reaction mixtures contained 2X iQ™ SYBR® green Supermix (Bio-Rad, USA), 1μl template DNA, dH_2O, and 500nM JS4 and JS5 for the *H. heydorni*-specific assay and 500nM Np6+ and Np21+ for the *N. caninum*-specific assay. The cycling conditions consisted of initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 30 sec and annealing and elongation at 60°C for 1 min. All assays included positive controls (*H. heydorni* and *N. caninum* DNA for the JS4/JS5 and Np6+/Np21+ respectively), dH_2O and other parasite DNA as used in the universal coccidia PCR assay as a negative controls. Amplification products (7 μl) for both assays were visualized in 4% agarose gels stained with SYBR® Safe DNA gel stain (Thermo Fisher Scientific) using a Typhoon™ 9410 imaging system (Amersham Biosciences, USA) and compared to the positive controls for proper size amplification asscociated with either *Neospora caninum* or *Hammondia* identification.

There is evidence that the *Hammondia* organisms shed by foxes are genetically distinct from *H. heydorni* and constitute a separate species: *H. triffittae*. Gjerde and Dahlgren (2011) found the ITS-1 sequence of *H. triffittae* to be identical to that of *H. heydorni*. Therefore, the JS4/JS5 primers used in this study to detect *H. heydorni* are inadequate to differentiate these species. To distinguish them, the AT9/AT264 primers developed by Abel et al. (2006) were applied to the samples that tested positive with the JS4/JS5 primer set. These primers target the intron 1 region of the alpha tubulin gene which distinguishes these species on the basis of a 9 bp repetitive insertion present in *H. triffittae* but lacking in *H. heydorni* (Abel et al. 2006). The PCR mixture contained 2X Power SYBR® green PCR Master Mix (Applied Biosystems), 500 nM AT9 and AT264 primers, 1μl template DNA, and dH_2O. DNA from *H. heydorni* control oocysts served as a positive control and dH_2O served as a negative control in each assay. Assays were performed using a StepOne™ Real-Time PCR system (Applied Biosystems). The cycling conditions consisted of initial denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 15 sec and annealing and elongation at 56°C for 30 sec.

To confirm the identity of the amplicons, DNA sequencing was performed on the following amplified products: three JS4/JS5-positive amplicons, three AT9/AT264-positive amplicons, and three universal coccidia-positive but *Neospora*- and *Hammondia*-negative amplicons. The templates and applicable primers were submitted to The Ohio State University Plant-Microbe Genomics Facility for BigDye Terminator Cycle Sequencing on a 3730 DNA Analyzer (Applied Biosystems). The resulting sequences were aligned using Vector NTI® software (Thermo Fisher Scientific). The DNA sequences were compared to published sequences using BLAST analysis available through the NCBI database.

3. Results

Of the 285 samples examined, 59 (20.7%) showed the presence of small oocysts-like structures (10-15 um) by fecal flotation. Other parasite ova seen included *Eimeria*, *Sarcocystis*, *Cystoisospora*, *Capillaria*, *Stongyle*-type ova, *Trichuris*, and *Giardia*. To better identify the small oocyst-like structures, initial screening with the universal coccidia PCR assay yielded 51 coccidia-positive samples (Fig. 1). This assay was sensitive enough to detect DNA from 20 *N. caninum* tachyzoites and two *H. heydorni* oocysts, and consistently detected both *H. heydorni* and *N. caninum* controls. *Hammondia heydorni* and *N. caninum* both produced melting curve peaks at approximately 82.9°C (82.91°-82.96°C).

The *H. heydorni*-specific assay was performed on the 51 coccidia-positive samples. This assay was able to detect DNA from two *H. heydorni* oocysts and did not amplify the *N. caninum* control. Three of the samples (1.1%) had positive amplification above threshold (Fig. 1). Sequencing results showed 99-100% identity to the ITS-1 region of *H. heydorni* (GenBank GQ984218) for these three samples. These three samples also showed above threshold amplification using the AT9/AT264 primers. Sequences derived from the AT9/AT264 amplicons confirmed the presence of *H. heydorni* rather than *H. triffittae*. Comparing the sequences of the samples to *H. heydorni* and *H. triffittae* illustrates the lack of the *H. triffittae*-specific 9 bp repetitive insertion in these samples (Table 1).

The *N. caninum*-specific assay was also performed on the 51 coccidia-positive samples. All samples were negative for *N. caninum* (Fig. 1). This assay was able to detect DNA from two *N. caninum* tachyzoites and did not amplify the *H. heydorni* control.

To further confirm the lack of *Neospora* and *Hammondia* in the universal PCR positive sample group, three samples were selected for sequencing with the DNA sequencing results indicating the presence of a *Sarcocystis* sp., an *Eimeria* sp., and a *Cystoisospora* sp. (Table 2), thereby confirming the broad application of the universal primers to the coccidia group of parasites.

4. Discussion

The low detection of *H. heydorni* DNA in wild canid feces in this study is similar to previous surveys. Gompper et al. (2003) reported a prevalence of 2% in coyote feces in New York, and Elmore et al. (2013) found a single *Neospora/Hammondia*-like positive sample out of 95 arctic fox feces in Canada (1.1%). However, neither of these studies confirmed the identity of these oocysts using molecular methods. Evaluation of dogs also show very low

prevalence: 0.8% in dogs in China (Jie et al., 2013), 0.05% in dogs in Germany (Schares et al., 2005), and 0.2% in dogs in the Czech Republic (Slapeta et al., 2002). Li et al. (2014) reported a seemingly unusual high prevalence in dogs in rural China (17.5%).

Since each fecal sample in this study was collected from the environment and not from individual animals, the proportion of *H. heydorni*-positive samples reported here does not represent a true prevalence but rather estimates environmental contamination of this parasite within the study area. All three *H. heydorni*-positive samples had *Neospora/Hammondia*-like oocysts visualized microscopically, so the positive DNA amplification seen likely derives from oocysts rather than tissue cyst stages present in intermediate host prey passing through the intestines of the wild canids.

The limited sequencing results from the three coccidia-positive but *H. heydorni*- and *N. caninum*-negative samples suggest that the 48 fecal samples that were coccidia-positive but negative for *H. heydorni* and *N. caninum* could harbor a number of other coccidia species that use canids as definitive hosts such as *Sarcocystis* or *Cystoisospora*. It is also possible that these coccidia-positive samples represent felid-specific coccidian oocysts such as *T. gondii* and *Hammondia hammondi* which contaminated the samples through consumption of felid feces or were domestic cat feces that were visually similar to canid feces and mis-identified. Other coccidian parasites not known to infect canids likely were detected as a result of tissue cyst stages or gastrointestinal coccidia present in the prey animals, passing through the gut of the predator and into the collected samples as several of the microscopy results found sporulated *Eimeria*. These results highlight that universal primers although good for amplifying a number of coccidia for screening purposes, this assay should be followed up with specific DNA detection methods if species identification is needed. This study also highlights the limitations of visual microscopy for field collected samples and the need for confirmatory testing by another testing modality such as PCR followed by DNA sequencing of the amplicon.

Although it is to-date considered non-pathogenic and low in prevalence, more fully understanding the ecology of *H. heydorni* may provide useful information in the future. For example, its interactions with other gastrointestinal parasites could potentially impact the transmission of these pathogens. Rynkiewicz et al. (2015) suggest parasites that share space and resources and alter the immune environment that other parasites experience. Rynkiewicz et al. (2015) also suggest that these co-habiting parasites will likely face a high level of competition, citing the relationship between the nematode *Heligmosomoides polygyrus* and the coccidian *Eimeria hungaryensis* in the intestines of wild wood mice as an example. Because of their similarities in life cycles and biological niches, it is possible that *H. heydorni* could be acting as a competitor with other more pathogenic endoparasites, thereby negatively affecting their transmission. However, Moreno et al. (2013) found a synergistic effect on oocyst shedding in wild capybaras and guanacos co-infected with multiple *Eimeria* species. Further studies are needed to better understand the potential effects of multi-parasite infections and competition on transmission.

Additionally, *H. heydorni* may serve as a sentinel of climate change and habitat loss, with alterations in precipitation and water runoff impacting the spread of oocysts and thereby the

transmission and prevalence of this parasite. Transmission via surface water has been previously well documented for other gastrointestinal protozoan parasites. Miller et al. (2002) identified freshwater runoff as a likely cause of movement of *T. gondii* oocysts from inland felid feces to coastal waters in California. Surface water runoff from melting snow has also been implicated in the spread of *T. gondii* oocysts from land to sea in arctic habitats (Simon et al. 2013). The salinity and vegetation of wetlands has been shown to significantly remove fecal pathogens such as *T. gondii, C. parvum*, and *Giardia duodenalis* and prevent their spread via surface water (Hogan et al., 2013), but loss of these habitats removes this barrier to transmission. *Hammondia heydorni* likely behaves in a manner similar to these other protozoal parasites and too may serve as an indicator of environmental changes, especially in areas with large populations of dogs or wild canids and susceptible prey animals.

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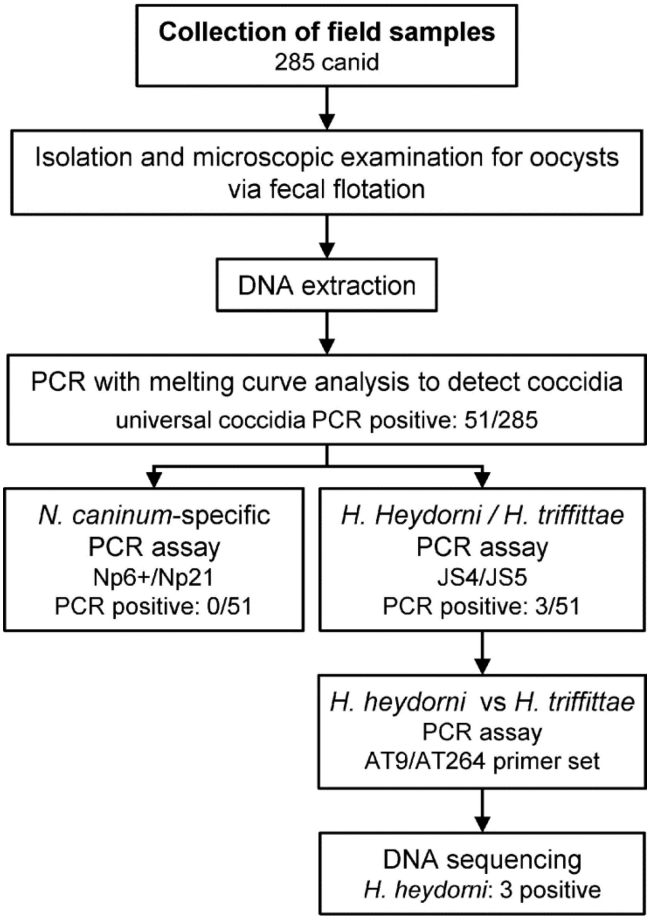

Fig. 1.

Study design flow chart. Collection of 285 canid fecal samples from the field, followed by fecal flotation for microscopic examination and isolation of parasite ova. DNA isolated from flotation material with subsequent PCR testing, first with a broad universal coccidia PCR assay whereby the 51 positive samples were further analyzed using *Neospora caninum* and *Hammondia* PCR assays with DNA sequencing of the three *Hammondia heydorni* positives.

Table 1

Comparison of the intron 1 region of the alpha tubulin published sequences of *H. triffittae* (GenBank GQ984228) and *H. heydorni* (GenBank GQ984230) to the sequenced samples, tentatively identified as *Hammondia* based on JS4/JS5 PCR results. The sample sequences lack the 9 bp repetitive insertion thereby confirming their identity as *H. heydorni* and not *H. triffittae.*

Sample	Nucleotides sequenced	Identity	Sequence (3'-5')
HtRtVulpes-1981-N (*H. triffittae*)	GenBank GQ984228	--	ATGT**GTGCATGG**A**GTGCATGG**TAAA
HhCanis-2000-1-N (*H. heydorni*)	GenBank GQ984230	--	ATGT**GTGCATGG**–––––––––TAAA
Wild canid 1	162	97% *H. heydorni*	ATGT**GTGCATGG**–––––––––TAAC
Wild canid 2	136	98% *H. heydorni*	ATGT**GTGCATGG**–––––––––TAAG
Wild canid 3	146	99% *H. heydorni*	ATGT**GTGCATGG**–––––––––TAAG

Table 2

Sequencing results of the coccidia-positive, *H. heydorni*- and *N. caninum*-negative wildlife samples (coccidia + samples 1-3) indicate the presence of a variety of other coccidia species which seen by microscopy and amplified using the coccidia universal primers yet no *Hammondia* spp or *Neospora caninum* DNA detected.

Sample	Nucleotides sequenced	Amplicon DNA identity
Coccidia + Sample 1	269	99% *Cystoisospora timoni* (GenBank EU200792)
Coccidia + Sample 2	264	88% *Eimeria subspherica* (GenBank AB769644)
Coccidia + Sample 3	263	96% *Sarcocystis oreamni* (GenBank KT225488)