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Coprodiagnosis of *Hammondia heydorni* in Dogs by PCR Based Amplification of ITS 1 rRNA: Differentiation from Morphologically Indistinguishable Oocysts of *Neospora caninum*

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SUMMARY

Hammondia heydorni is thought to be a non-pathogenic coccidian parasite of dogs that is closely related to *Neospora caninum*, an important parasite of cattle and dogs. Oocysts of these two species are morphologically indistinguishable from each other. A population of 2240 dogs in the Czech Republic was screened for the presence of *H. heydorni/N. caninum* oocysts and five (0.22%), represented by five of 3135 faecal samples (0.16%), were positive. The internal transcribed spacer 1 region of the rRNA gene (ITS1) from two isolates were cloned and the DNA sequences were identical with those of the ITS1 of *H. heydorni*. Based on the rRNA sequences available for *H. heydorni* and related coccidia, the primer pair JS4-JS5 was designed to amplify the 3′ end of the small subunit (SSU) rRNA gene and ITS1 of *H. heydorni*. When tested on DNA extracted from a variety of parasites, the primers amplified a specific 267 bp fragment in our isolates only. The presence of DNA equivalent to 10 oocysts was sufficient for the amplification of the ITS1. We present a PCR-based diagnostic method as the only fast and reliable method for the diagnosis of *H. heydorni* in dogs.

KEYWORDS: *Hammondia heydorni*; *Neospora caninum*; coprodiagnosis; PCR assay; epidemiology.

INTRODUCTION

Hammondia heydorni (Apicomplexa; Sarcocystidae; Toxoplasmatinae) and *Neospora caninum*, both have canids as definitive hosts and form a sister group to *Toxoplasma gondii* and *Hammondia hammondi*, for which felids serve as definitive hosts (Ellis *et al.*, 1999a; Mugridge *et al.*, 1999). *Neospora caninum* is an important cause of abortions in cows and paralysis in domestic dogs, in contrast to nonpathogenic *H. heydorni*. Oocysts of all the coccidian species grouped as Toxoplasmatinae are morphologically indistinguishable from each other, being thin-walled and almost spherical, only 11 to 14 μm in diameter (Lindsay *et al.*, 1999; McAllister, 1999). Therefore, a diagnosis based on light microscopy of oocysts in faeces is not a method of choice for species identification of these important parasites.

Diagnostic molecular techniques, such as PCR, offer a highly sensitive and specific alternative to morphological methods (Singh, 1997; Dinkel *et al.*, 1998; Schnitzler *et al.*, 1998; Adam *et al.*, 2000; Orlandi & Lampel, 2000). Assays for PCR-based detection of related coccidia have already been developed for *T. gondii*, *N. caninum*, and several *Sarcocystis* spp. (Ellis, 1998; Baszler *et al.*, 1999; Ellis

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et al., 1999b; Heckeroth & Tenter, 1999). The ITS1 rRNA region has been sequenced and is available in the GenBank for very closely related coccidia: *T. gondii, N. caninum, N. hughesi, H. hammondi* and *H. heydorni*. The ITS1 represents a promising target for species diagnosis, since it is a high-copy number element of the genome, is conserved within species, and has been shown to vary in sequence between mentioned coccidia, while the SSU rRNA genes are virtually identical (Homan *et al.*, 1997; Marsh *et al.*, 1998; Ellis *et al.*, 1999a; Jenkins *et al.*, 1999).

The epizootiology of *H. heydorni, N. caninum* and *N. hughesi* remains largely unknown. Recent experiments proved the validity of the separation of isolates from horses as *N. hughesi*, since dogs fed its tissue stages did not shed *N. caninum*-like oocysts (Walsh *et al.*, 2000). *H. heydorni* has attracted relatively little attention since its discovery (Blagburn *et al.*, 1988; Matsui, 1991), but, with the description of *Neospora*, it recently became necessary to diagnose oocysts shed by dogs (McAllister *et al.*, 1998; Lindsay *et al.*, 1999; Mehlhorn & Heydorn, 2000). Epidemiological studies have revealed strong associations between *N. caninum*-infected dairy herds and the presence of dogs on farms (Parte *et al.*, 1998; Bartels *et al.*, 1999), although the exact transmission route is yet to be established (McAllister, 1999; Mehlhorn & Heydorn, 2000; McAllister, 2001). Recently, a serosurvey suggested exposure or infection of humans with *N. caninum* (Tranas *et al.*, 1999).

To evaluate the role of naturally infected dogs in the transmission of *N. caninum* to cattle, a fast and reliable method for its detection and differentiation from similar species occurring in faeces of dogs must be developed. The purposes of this study were: (i) to obtain 'wild' *H. heydorni/N. caninum* oocysts and to characterize further the isolates; (ii) to determine the ITS1 sequences of the *H. heydorni/N. caninum* isolates, and (iii) to design diagnostically relevant primers for a specific and sensitive PCR protocol for the identification of oocysts.

In this paper we present a novel approach to coprodiagnosis by PCR, specifically faeces samples containing oocysts of *N. caninum* and/or *H. heydorni*.

MATERIALS AND METHODS

Faecal specimens and morphologic evaluation of oocysts

A total of 3135 individual faecal samples from 2240 dogs was examined in the Czech Republic in 1999. The majority of the dogs examined were German shepherds. Coccidian infections were detected initially by microscopic examination of faecal samples after the fresh stool flotation in the modified Sheather's sugar solution (SG 1.30). Faeces positive for oocysts (Table I) were mixed with 2.5% (w/v) potassium dichromate solution and allowed to sporulate in Petri dishes in a thin layer at room temperature. Sporulated oocysts were measured with a calibrated ocular micrometer using bright-field microscopy and photographed using an Olympus BX 60 microscope equipped for Nomarski interference contrast microscopy. Oocysts were further

Table I
Morphology and history of oocyst recovered from the faeces of the dogs[a]

	Oocyst		*Sporocyst*			
No.	*Mean oocyst proportions (μm) ± range*	*Shape index ± range*	*Mean sporocyst proportions (μm) ± range*	*Shape index ± range*	*Locality of the dog in the Czech Republic*	*History of eating raw meat*
CZ-1	11.9 × 11.0 (11–13.0 × 10–12.0)	1.08 (1–1.25)	8.9 × 6.1 (8–9.5 × 6–7.0)	1.45 (1.23–1.58)	Cheb	*b*
CZ-2	11.9 × 10.3 (11–13.0 × 8–11.5)	1.16 (1–1.63)	8.6 × 5.8 (8–9.0 × 5.5–6.0)	1.47 (1.33–1.64)	Domažlice	+ beef
CZ-3	12.2 × 11.1 (11.5–14.0 × 10–12.0)	1.1 (1–1.4)	8.4 × 5.9 (8–9.0 × 5–6.5)	1.43 (1.33–1.7)	Kladno	*b*

[a] Thirty oocysts from each sample were measured.
[b] Not available.

purified by centrifugation, counted with a haemocytometer, and stored at 4°C for up to 12 months prior to the DNA extraction.

DNA extraction

An aliquot of 500 µL of each sample was suspended in 1.5 mL of phosphate-buffered saline (PBS; 0.01 M, pH 7.2) and centrifuged at 14 000*g* for 3 min. The pellet was washed two more times in PBS, and finally homogenized by grinding with 0.5 mm glass beads (Sigma) in 1.5 mL microtubes for 30 min with an occasional dip in liquid nitrogen. Total cellular DNA was extracted with 2% sarcosyl (Sigma) and 0.5 mg mL^{-1} pronase E (Sigma) followed by the phenol/chloroform extraction and ethanol precipitation as described previously (Maslov *et al.*, 1996). DNA was dissolved in 50 µL distilled H_2O and stored at −20°C for further analysis.

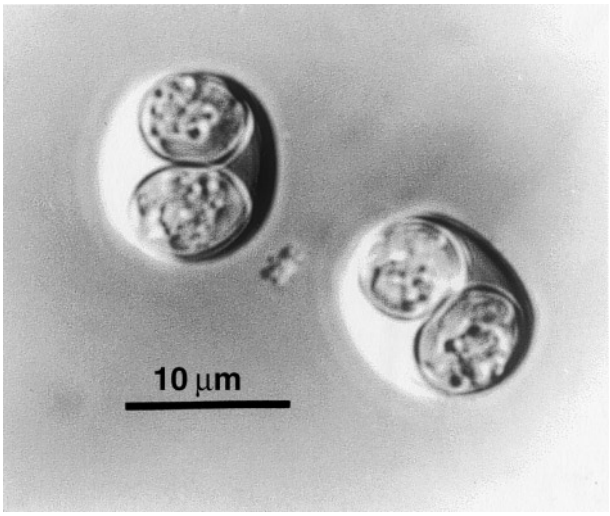

Fig. 1. Oocysts of the isolate CZ-1. Morphologically indistinguishable oocysts of *N. caninum/H. heydorni*. Finally determined by PCR as *H. heydorni*.

PCR amplification

The ITS1 region was amplified using the modified ITS-1 and ITS-4 primers of White *et al.* (1990). The PCR conditions were: 94°C for 5 min, 30 cycles of 94°C for 1 min, 55°C for 1.5 min, and 72°C for 2 min, followed by the extension at 72°C for 10 min.

Species-specific PCR amplification

Species-specific primers were designed for *H. heydorni* based on known ITS1 sequences. The primers were: JS4 (5′-CGA AAT GGG AAG TTT TGT GAA C-3′) that anneals to the 3′ conserved region of the SSU rRNA gene and JS5 (5′-CAG CAG CTA CAT ACG TAG A-3′) that anneals to the species-specific region of ITS1 (Generi-Biotech) (Fig. 2). The amplification reactions were performed in 25 µL reaction mixtures containing 25–100 pmol of each primer, reaction buffer, dNTPs, and 0.5–2 U of *Taq* DNA polymerase according to manufacturer's instructions (catalog no. R001, TaKaRa) and of 1 µL of tested DNA. Reactions were run in an Eppendorf Mastercycler, Techne (two types), Biometra T3, MJ Research and Quatro TC-40 thermocyclers. The PCR conditions were: 95°C for 5 min, 35 cycles of 95°C for 1 min, 65°C for 1 min, and 72°C for 1.5 min, followed by the extension at 72°C for 10 min. The expected size of the amplicon was about 270 bp. The PCR products were analysed by electrophoresis in a 1% agarose gel stained with ethidium bromide.

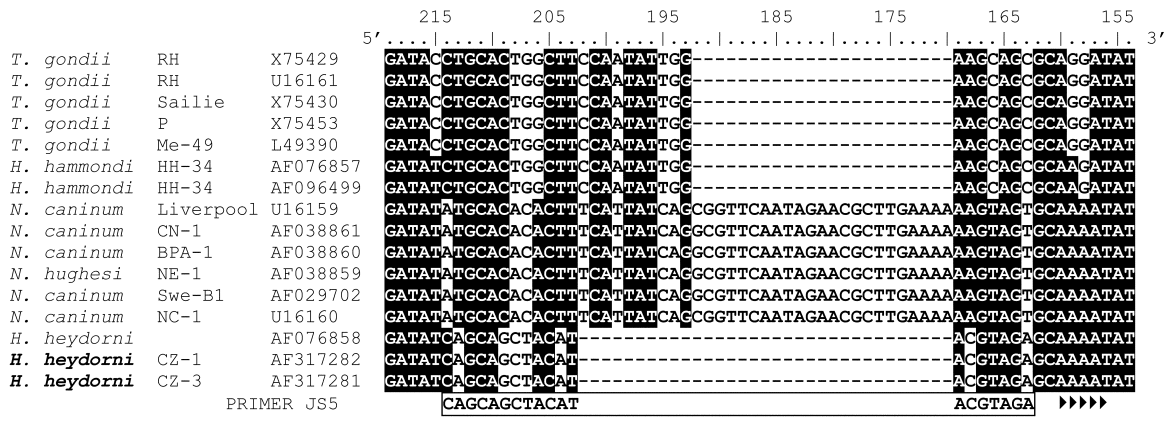

Fig. 2. Clustal X generated partial alignment of positions 154–219 of ITS1 sequences of closely related species; species, strain/isolate and GenBank accession nos. are given on the left; sequences obtained in this study are in bold; JS5 primer is marked below, arrows show the direction of the primer toward the SSU rRNA gene.

The DNA extraction, amplification, and detection steps were physically separated and were performed in different rooms. Positive controls were performed for primers JS4-JS5 (*H. heydorni* isolate CZ-1); for primers NS3-SR1 and JV1-JV2 (*N. caninum* strain NC-1) and a negative control was also included (reaction mixtures without DNA). An amplification experiment was considered invalid when a failure in any of the controls occurred. For specificity screening, the following DNAs were used: DNA of *N. caninum* (strain NC-1), *T. gondii* (strains K1 and P(CZ)), DNAs extracted from *H. heydorni/N. caninum* negative excrements containing various canine intestinal parasites (*Toxocara canis, Toxascaris leonina, Trichuris vulpis, Sarcocystis sp., Isospora ohioensis*-group, *Isospora canis, Cryptosporidium parvum*), from excrements free of parasites that contained only bacteria and fungi, and DNA extracted from dog whole blood. All samples were obtained from this study and the presence of parasites was microscopically determined, DNA was extracted according to described procedure and the concentration of DNA was estimated on gel.

Co-infection with N. caninum

To exclude the possibility of co-occurrence of both morphologically indistinguishable coccidia (*N. caninum* and *T. gondii*), a second PCR was performed with primers NS3 (5′-GTG GAT ATT TTG CAC TA-3′) and SR1 that anneal to the ITS1 of *N. caninum*. Primer NS3 is a modification of the primer NS2 described previously by Ellis *et al.* (1999b), relative to which NS3 was shifted three bases towards the 5′ end of the ITS1. The strain NC-1 was used as a positive control. A correct amplification yielded a 149 bp product.

Control for inhibition

Due to possible unknown factors present in some samples, reactions may be inhibited and may, therefore, give false-negative results. To control such potential inhibition, the DNA of *H. heydorni* was added into each negative DNA sample and the first PCR was repeated. In samples containing *H. heydorni* and closely related species *T. gondii* and *N. caninum*, the SSU rRNA gene was amplified using the specific primer pair JV1-JV2 of Votý pka *et al.* (1998).

PCR sensitivity

The sensitivity of a standard PCR procedure was determined using freshly isolated oocysts of the CZ-1 isolate (Table I). DNA extracted from 1000, 5000 and 10 000 oocysts and resuspended in 200 μL, respectively, were used. The isolations were always performed at least twice in parallel. The DNA pellets were dissolved in 50 μL of distilled water and 0.5 μL or 1 μL of the sample was used for PCR. The volume of extracted DNA was equivalent to the number of oocysts using a formula = [number of oocysts used for the extraction/amount of dilution volume at the end of the DNA extraction]; e.g. the DNA extraction from 1000 oocysts diluted 1 : 50 means that 1 μL contains the DNA of 20 oocysts. The PCR protocol was tested down to 0.1 μL of DNA (= DNA of 2 oocysts).

DNA cloning, sequencing and comparative analysis

Amplicons were gel-purified and cloned into the TA TOPO II cloning vector (catalog no. 180714 25-0184 version E, Invitrogen). Sequencing reactions were performed using the Perkin-Elmer DNA sequencing Kit (catalog no. 4303152; PE Biosystems) and analysed on the ABI 377 automatic DNA sequencer. Sequences were assembled using the program SeqMan II (DNASTAR Inc.). The obtained sequences and all known ITS1 of related coccidia retrieved from the GenBank were aligned using the program Clustal X (Thompson *et al.*, 1997) and analysed using the program PAUP* (Swofford, 1998). Sequences retrieved from the database for considered species were as follows (GenBank accession numbers are given in parenthesis): *N. hughesi* NE1 strain (AF038859); *N. caninum* strains BPA1 (AF038860), Swe-B1 (AF029702), CN1 (AF038861), N.C.-1 (U16160), N.C.-Liverpool (U16159); *H. hammondi* H.H-34 strain (AF076857, AF096499); *H. heydorni* (AF076858) and *T. gondii* strains RH (U16161, X75429), P (X75453), Me49 (L49390) and Sailie (X75430).

Nucleotide sequence accession numbers

The ITS1 sequences of the isolates CZ-1 and CZ-3 and the diagnostic fragment JS4-JS5 were assigned the accession numbers AF317282, AF317281 and AF317280, respectively.

RESULTS

Prevalence and morphometry of detected oocysts

Microscopical examination of 3135 faecal samples from 2240 dogs revealed the presence of oocysts with the *Neospora caninum/Hammondia heydorni* morphology in only two adult dogs. The parasites derived

from these dogs are referred to as isolates CZ-1 and CZ-3 (Fig.1). In addition three puppies of the same litter were also positive for oocysts and yielded isolate CZ-2; (0.16% samples, 0.22% dogs). The morphology and history of each isolate is summarized in Table I.

Amplification, sequencing and analysis of the ITS1 rRNA region

The ITS1 regions of isolates CZ-1 and CZ-3 was amplified (442 bp), cloned and sequenced. The final alignment was based on a 421 bp region for which sequence data were available for all sequences under consideration. A BLAST search revealed that the sequences were almost identical with each other, (two different positions) and were very similar to the *H. heydorni* ITS1 sequence (five and seven different positions, respectively). ITS1 sequences from morphologically related *Neospora* spp., *T. gondii* and *H. hammondi*, differed in 81 to 93 different positions, including insertions and deletions. Sequence alignment enabled us to design the species-specific primer JS5 (Fig. 2) and primer JS4 annealing to the SSU rRNA conserved region characteristic for coccidia. The sequence alignment in NEXUS format is available on request or at ftp://vfu-www.vfu.cz/slapeta/alignments.

Species-specific PCR for N. caninum

The primer pair NS3-SR1 selectively amplifies the ITS1 region in *N. caninum* (NC-1) only, giving a characteristic 149 bp band. This band was never amplified by PCR with these primers or DNA from the isolates CZ-1, 2, 3 or any other tested controls (data not shown).

Species-specific PCR for H. heydorni

The primer pair JS4-JS5 was found to selectively amplify *H. heydorni* (Fig. 2), and the amplification of a predicted band 267 bp fragment in all three isolates was considered as a proof of the presence of *H. heydorni* infection.

All samples, that did not contain oocysts of *N. caninum/H. heydorni* from microscopical examinations did not produce PCR product with primers JS4 and JS5 (Fig. 3, and data not shown). To exclude the possibility that DNA of an organism other than *H. heydorni* could give rise to a PCR amplification fragment of 267 bp, closely related coccidia, a whole blood sample from a dog, and faecal samples containing variety of canine parasites were tested under various conditions, and all gave negative results. Samples free of parasites but abundant with bacteria and fungi did not produce an amplicon (data not shown).

To determine the lower number of *H. heydorni* oocysts that revealed a detectable PCR product,

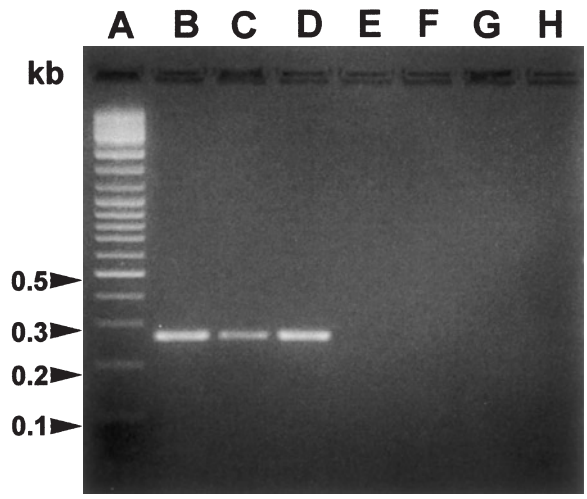

Fig. 3. Species-specific PCR amplification of the ITS1 region using the JS4-JS5 primers. Lanes: DNA ladder, A; isolate CZ-1, B; isolate CZ-2, C; isolate CZ-3, D; *N. caninum* (NC-1), E; *T. gondii* (K-1), F; *Isospora ohiohensis* – group, G; *Sarcocystis* sp., H. Controls not shown.

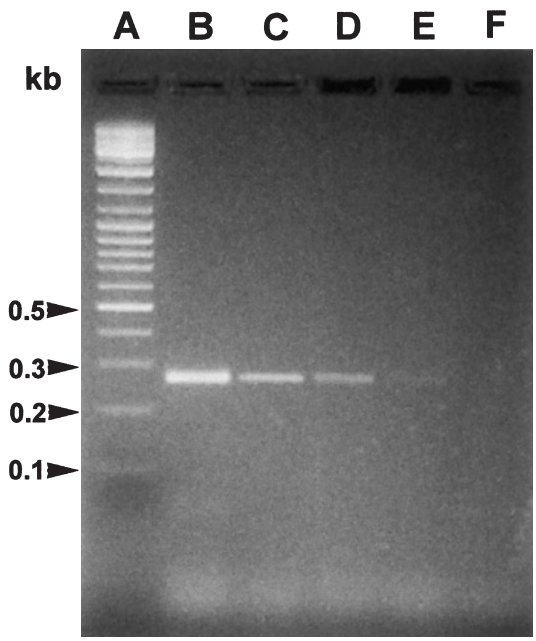

Fig. 4. Sensitivity of method. Lanes: DNA ladder, A; the DNA equivalent of 200 oocysts, B; 100 oocysts, C; 20 oocysts, D; 10 oocysts, E; 5 oocysts, F. Controls not shown.

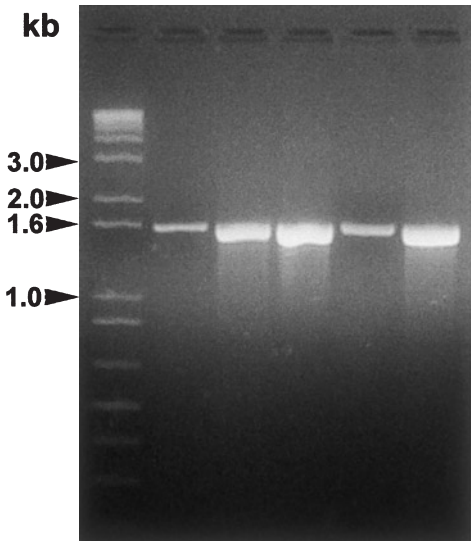

Fig. 5. PCR amplification with JV1-JV2. Lanes: DNA ladder, A; isolate CZ-1, B; *N. caninum* (NC-1), C; *T. gondii* (K-1), D; *Isospora ohiohensis* – group, E; *Sarcocystis* sp., F. Controls not shown.

suspensions containing 10 000, 5000 or 1000 of the CZ-1 oocysts were used for isolation of total DNA. DNA equivalent to 200, 100, 50, 25, 20, 10 oocysts gave a positive signal (Fig. 4, and data not shown). The equivalent of 10 oocysts or less was below the sensitivity limit for this method using the ethidium-bromide stained agarose gels.

Control for inhibition

All control samples that did not yield a product with the JS4 and JS5 primers did produce a specific band after a second round of PCR following the addition of DNA from the CZ-1 (data not shown). When primers JV1 and JV2 were used with all samples containing coccidia (*T. gondii, N. caninum, I. ohioensis*-group, *Sarcocystis* sp.), a 1.6 kb SSU rRNA gene was amplified (Fig. 5 and data not shown).

DISCUSSION

The goal of this study was to establish the prevalence of the pathogenic *N. caninum* and non-pathogenic *H. heydorni* coccidia in canine faeces (McAllister *et al.*, 1998; McAllister, 1999) and to design a diagnostic method that could distinguish between both morphologically identical parasites. Colourless, thin-walled, oocysts around 11–14 µm in size, each with two sporocysts containing four sporozoites but without a Stieda body, matching descriptions of oocysts of *N. caninum* and *H. heydorni* (Mugridge *et al.*, 1999) were found at a low prevalence in 0.16% ($n=5$) of 3135 faecal samples and 0.22% ($n=5$) of 2240 dogs. This prevalence falls within the ranges of infections with *H. heydorni* published previously (Svobodová *et al.*, 1995; Epe *et al.*, 1998). Although we did not study the life cycle of the coccidia, we found, that dogs infected naturally with the isolate CZ-2 had a documented history of eating raw meat, which supports a possible transmission from an intermediate host.

Since morphological features are insufficient for species-specific detection of these oocysts, molecular methods were thought to offer a solution. We thus amplified and sequenced the ITS1 of two isolates, CZ-1 and CZ-3, that were obtained from our study. The DNA sequences had a high similarity with the ITS1 region of *H. heydorni* (GenBank accession no. AF076858), which is derived from two different isolates (Ellis *et al.*, 1999a). The first was collected from a naturally infected dog originating from the USA (Blagburn *et al.*, 1988), while the second was isolated from a red fox (*Vulpes vulpes*) captured in Saudi Arabia (Ellis *et al.*, 1999a). Interestingly, despite their distant geographic origin the ITS1 sequences of both isolates were identical and are referred under the same accession number (Ellis *et al.*, 1999a).

The ITS1 region of *H. heydorni* provided the best match for the ITS1 sequences of the isolates CZ-1 and CZ-3, differing in two positions from each other and in five and seven positions, respectively, from the *H. heydorni* sequence. All three sequences shared exactly the same insertions and deletions which clearly distinguished them from the ITS1 sequences of other coccidian species. Only the ITS1 of genera *Toxoplasma, Neospora* and *Hammondia* can be aligned unambiguously. The most closely related genera *Besnoitia* ITS1 is too different to be robustly aligned (Ellis *et al.*, 1999a).

Within the ITS1 sequence of known coccidia, the intraspecific heterogenity is low (Homan *et al.*, 1997; Marsh *et al.*, 1998; Ellis *et al.*, 1999a). Therefore, the differences in ITS1 described herein for two geographically related isolates are of some interest.

The primer pair JS4-JS5 proved to be diagnostic for *H. heydorni*, since it amplified the ITS1 region in the studied isolates (CZ-1, 2, 3) that belong, based on their ITS1 sequences, to *H. heydorni*. These primers did not produce any amplicon with the DNA of *N. caninum*, other coccidia and all relevant controls. Moreover, the primers JS4 and JS5 produced a specific band with the DNA equivalent of 10 oocysts, and

thus proved to be a very sensitive diagnosis tool. Since the purpose of this method is to discern between pathogenic and non-pathogenic coccidia after they were diagnosed by microscopical examination, we assume that such a sensitivity is sufficient. It is also within the sensitivity limits of other PCR-based assays applied for the diagnostic of related Apicomplexan species (Schnitzler *et al.*, 1998; Orlandi & Lampel, 2000). The method is not designed for routine screening of fresh faecal samples but as an additional method following a microscopical finding of a suspected coccidium in dogs.

Another important outcome of this study is that we did not find dogs naturally infected and shedding oocysts of *N. caninum* and that only infections caused by *H. heydorni* were detected in this parasitological survey. This finding casts some doubt upon oocysts shedding of experimentally infected dogs and upon the possibility that dogs contaminate the environment with *N. caninum* (Wouda *et al.*, 1999; Bergeron *et al.*, 2000, Mehlhorn & Heydorn, 2000; McAllister, 2001). Nevertheless, infections caused by *N. caninum* have been detected in the Czech Republic (Koudela *et al.*, 1998).

In conclusion, the present study demonstrates the utility of PCR-based method to distinguish between two morphologically identical coccidia. The described method provides confirmatory tool for the diagnoses of *H. heydorni* and in combination with the PCR assay for *N. caninum* should provide definitive identification of these two parasites.

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