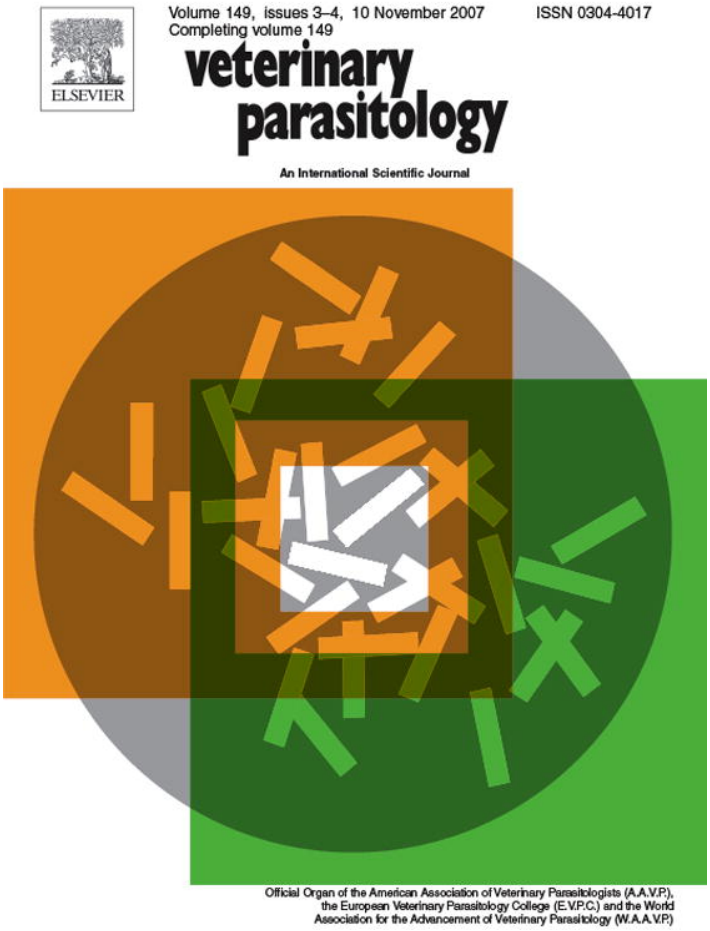

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Short communication

Fecal and serological survey of *Neospora caninum* in farm dogs in Costa Rica

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Abstract

To detect oocysts of *Neospora caninum* in dog feces and to determine the excretion pattern in dogs from specialized dairy farms in Costa Rica, a total of 265 fecal samples from 34 dogs were collected at intervals from February to August 2005. Fecal samples were examined for *N. caninum*-like oocysts microscopically, by DNA detection using the polymerase chain reaction (PCR), and by bioassay. *N. caninum* DNA was detected by PCR in four fecal samples, twice from one dog, but oocysts were not detected microscopically in these dogs. Sera of 31 of 34 dogs were tested for antibodies to *N. caninum* by a competitive-inhibition ELISA (VMRD®). Fifteen (48.4%) of 31 dogs had antibodies to *N. caninum* by ELISA. Seroconversion was not found in 28 dogs that were bled twice, 4 months apart (March and July 2005). Only one dog tested positive to *N. caninum* by both ELISA and PCR. This is the first report of finding *N. caninum* DNA in feces of naturally infected dogs in Costa Rican dairy farms.
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Keywords: *Neospora caninum*; Oocysts; Fecal samples; PCR; Dogs; Costa Rica

1. Introduction

Neospora caninum is an important cause of abortion in dairy cattle worldwide (Dubey, 2003). Dogs, coyotes, and possibly foxes are its definitive hosts (McAllister et al., 1998; Gondim et al., 2004; Wapenaar et al., 2006). Although *N. caninum* is transplacentally transmitted very efficiently in cattle, dogs are considered essential in the life cycle of this parasite (Dubey et al., 2007). However, unlike other coccidian parasites, relatively few oocysts are excreted in canine feces. Furthermore, the detection of *N. caninum* oocysts in feces is problematic because *N. caninum* oocysts morphologically resemble oocysts of three other coccidians (*Hammondia heydorni*, *Hammondia hammondi*, *Toxoplasma gondii*) that might be present in canine feces (Schares et al., 2005). Although molecular methods to detect *N. caninum*-like oocysts have been described (Hill et al., 2001; Slapeta et al., 2002; Sreekumar et al., 2004) the sensitivity is not high because of the low numbers of oocysts in canine feces. A comprehensive survey of *N. caninum* infection in feces of dogs from Germany by Schares et al. (2005) highlighted the difficulties of identification of *N. caninum* oocysts in canine feces. *N. caninum*-like oocysts were found in the feces of 47 of 24,089 fecal samples. Oocysts could be isolated from 29 of these 47 dogs and 28 of the 29 fecal samples were bioassayed in gerbils. Based on seroconversion in bioassayed gerbils,

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seven samples were considered to be *N. caninum*; five samples were definitively identified as *N. caninum* based on successful in vitro cultivation. *N. caninum* DNA was detected in four dogs by PCR directly from the dog feces. Among the other isolates, 12 were considered to be *H. heydorni*, two *T. gondii*, and two *H. hammondi*; *T. gondii* and *H. hammondi* are pseudo-parasites in dog feces and result from the ingestion of cat feces by dogs.

Detection of antibodies to *N. caninum* in canine serum does not provide epidemiologically important information because most dogs that excrete *N. caninum* oocysts do not have serum antibodies (McAllister et al., 1998; Dubey et al., 2007).

Previous to the detection of *N. caninum* oocysts in dog feces in Germany (Schares et al., 2005), *N. caninum* oocysts were reported in dogs from Argentina (Basso et al., 2001), United Kingdom (McGarry et al., 2003), Czech Republic (Slapeta et al., 2002), and New Zealand (McInnes et al., 2006). Basso et al. (2001) found a few *N. caninum* oocysts in the feces of a 45-day-old Rottweiler from La Plata, Argentina. Viable *N. caninum* was recovered from the gerbils that were fed these oocysts and the strain was successfully cultured in vitro. Slapeta et al. (2002) found oocysts in a 1-year-old German shepherd from Czech Republic; these oocysts were considered *N. caninum* based on PCR, and bioassay was not reported. McGarry et al. (2003) examined a total of 15 fecal samples from two foxhound kennels in U.K. (10 from one kennel of 80 and 5 from the second kennel of 60 dogs) and found *N. caninum* oocysts in two samples. One of these samples was identified as *N. caninum* based on PCR; a second fecal sample from this dog taken 4 months later revealed a few oocysts that were identified *N. caninum* based on PCR. McInnes et al. (2006) in New Zealand detected *N. caninum* DNA in the feces of a dog 2.5 years after they had isolated viable *N. caninum* from the skin of the dog.

Cattle are important for the economy of Costa Rica. In a cross-sectional study of 94 dairies in Costa Rica, 94.7% of the farms surveyed had *N. caninum* seropositive cows with an overall seroprevalence of 43.3% (Romero et al., 2005). Nothing is known of the epidemiology of *N. caninum* in Costa Rica. There are three reports of fatal neosporosis in Costa Rica, one in a dog (Morales et al., 1995), one in a goat (Dubey et al., 1996), and one in cattle (Pérez et al., 1998). The objective of the present study was to detect oocysts of *N. caninum* in farm dogs from specialized dairy farms with a high seroprevalence of *N. caninum* in Costa Rica.

2. Materials and methods

2.1. Study population

Twelve specialized dairy farms from the northern zones of Alajuela and Heredia were used in this study. On the selected farms, the within-herd seroprevalence of *N. caninum* ranged between 25.0% and 70.5% (Romero et al., 2002). Fecal samples from 34 dogs were analyzed and serum samples were collected from 31 of these dogs.

2.2. Sampling of dogs

In order to detect *N. caninum* oocysts and determine the excretion pattern, procedures were established to collect fresh feces of 34 dogs every 15 days during 7 months from February to August 2005. Feces from seven dogs from three farms were collected as planned, but feces from other dogs were collected inconsistently, depending on the cooperation of the owners. The dogs were isolated for 1 day before the farms were visited. Approximately 10 g of feces were picked up directly from the ground, and transported in a cooler at 4 °C to the Laboratory of Parasitology, School of Veterinary Medicine, Universidad Nacional of Costa Rica (UNA), where they were kept at 4 °C until processed (48 h maximum). A total of 265 feces were collected.

To detect *N. caninum* antibodies, dogs were bled twice, 4 months apart (March and July 2005). The blood was taken from the cephalic vein with a 20-guage needle fitted to a Vacutainer® blood collection tube. The samples were centrifuged at 2500 × *g* for 5 min and the serum stored at −20 °C until processed. Fifty-nine blood samples were collected, 31 in the first sampling and 28 in the second sampling.

2.3. Coprological examination

The direct examination and sporulation of the fecal samples were done in the Laboratory of Parasitology, UNA. Feces (10 g) were mixed with 10 ml of distilled water and 50 ml of sucrose solution (specific gravity 1.28) was added. The mixture was sieved through gauze, and centrifuged in a 50 ml conical tube at 2500 × *g* for 10 min. A few drops were taken from the meniscus and transferred to a slide for microscopic examination to detect *N. caninum*-like oocysts. After microscopic examination 5 ml of the supernatant from the very top of the tube were removed and mixed with 45 ml of distilled water; the mixture was centrifuged at 2500 × *g* for 10 min. After discarding the supernatant,

10 ml of 2% H_2SO_4 were added to the sediment and incubated for 8 days at room temperature to achieve oocyst sporulation. After sporulation, samples were examined by light microscopy and stored at 4 °C until examination for *N. caninum* DNA. Coccidian oocysts were characterized by the size of the oocysts and the number of sporocysts contained within; those ranging between 11 and 14 μm with two sporocysts were considered *N. caninum*-like.

2.4. Detection of N. caninum DNA in canine feces by polymerase chain reaction (PCR)

The fecal floats stored in 2% H_2SO_4 were centrifuged for 10 min at 5000 × *g* and the sediment was transported to the Animal Parasitic Diseases Laboratory (APDL), Beltsville, MD, USA, in a cooler for DNA detection as described by Hill et al. (2001). After removing H_2SO_4 by dilution and centrifugation, the sediment was suspended in 0.5 ml Hank's balanced saline solution (HBSS); this procedure was repeated until the sample turned pink, indicating a neutral pH.

The neutralized fecal floats were suspended in DNAzol® and placed in a 1.5 ml microcentrifuge tube containing 2-mm-diameter glass beads. The samples were stirred with the beads for 10 min at room temperature, and DNA was extracted from the sample in DNAzol® as per the manufacturer's instructions. The extracted genomic DNA was stored frozen until used in PCR assays.

One microliter of each DNA sample was transferred into a PCR reaction tube, along with 50 μl of 1 mM $MgCl_2$, 1× PCR buffer (10 mM Tris–HCl, 50 mM of KCl, pH 8.3), 200 μM of dNTPs, and 2.5 units of amplitaq polymerase. The primer pairs Np21 [gtgcgtccaatcctgaac] and Np6 [cagtcaacctacgtcttct], which amplify the Nc5 genomic sequence of *N. caninum* (Yamage et al., 1996) were used in batches of 200 pg each. To avoid the inhibition of PCR amplification due to fecal contaminants in the DNA, bovine albumin (0.8 mg/ml) was added to each reaction. PCR for *N. caninum* was accomplished with an initiation cycle of 94 °C for 5 min, followed by 40 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 45 s, followed by a 4 °C hold prior to electrophoretic separation. Electrophoresis of the PCR-amplified product was carried out in 2% agarose gels (Invitrogen® E-gel). A sample was considered positive when amplification yielded a band of 328 base pairs. Controls in each run included a water control, as well as a separate PCR assay containing the unknown DNA sample and a competitor molecule of the targeted *N. caninum*-specific Nc5 genomic sequence (Liddell et al., 1999; Hill et al., 2001) which produced a 444 bp amplicon using the Np6/21 primer set and the same PCR conditions as a check against false-negative reactions. In addition, each unknown DNA sample was analyzed using primer pairs designed to amplify *H. heydorni* (Sreekumar et al., 2004), *H. hammondi*, and *T. gondii* genomic DNA (Sreekumar et al., 2005) to assess the specificity of the assay.

2.5. Detection of antibodies to N. caninum in sera of dogs

2.5.1. Enzyme-linked inmunosorbent assay (ELISA)

A commercial competitive ELISA kit (VMRD®, Pullman, WA) with a reported 72.0% sensitivity and 90.0% specificity for canine samples was used to detect *N. caninum* antibodies. Sera that presented inhibition percentages equal or higher than 30% were considered positive (Capelli et al., 2006).

2.5.2. Imunofluorescent antibody test (IFAT)

Dog sera stored at −20 °C were transported to APDL, Beltsville, MD, where an indirect IFAT was performed as described (Dubey et al., 1988). Samples with titers equal or higher than 1:50 were considered positive for *N. caninum* antibodies.

2.6. Isolation of viable N. caninum from dog feces by bioassay in gerbils

A total of 265 dog fecal floats that had been stored in H_2SO_4 at 4 °C for 5–10 months were bioassayed for *N. caninum* at the APDL. Samples were aerated for 1 week at room temperature to allow oocysts to sporulate. For this, each of the four samples that were positive for *N. caninum* by PCR were bioassayed separately into two gerbils and the remaining 261 samples were mixed into nine pools. The samples were diluted with distilled water, neutralized with sodium hydroxide, centrifuged, and the sediment was orally inoculated into two gerbils (*Meriones unguiculatus*) for each of the nine pooled and the four individual samples as described by Dubey and Lindsay (2000). Three months later each gerbil was bled and the sera were examined for antibodies to *N. caninum* using the *Neospora* agglutination test (NAT) (Romand et al., 1998). The gerbils were killed 2 weeks after serological examination and smears from the cerebrum of these animals were examined microscopically for protozoal tissue cysts.

Table 1
Comparative descriptive analysis of the different characteristics of *Neospora caninum* positive dogs when tested by PCR

Characteristic	Dog 2	Dog 10	Dog 24
Sex	Male	Female	Male
Age	1 year	8 years	10 years
Breed	Labrador	Undefined	Undefined
Feeding	Dog food	Leftovers	Dog food
Organ ingestion	Placentas, cow fetus	Placentas, cow fetus, calf carcasses	Placentas, cow fetus, calf carcasses
Free or restrained	Restrained, sometimes free	Restrained	Confined during the day, free at night
Other animals in farm	Rodents, coyotes	Rodents, coyotes, cats	Rodents, coyotes
Bovine abortion	Yes	Yes	Yes
Serological result	Negative	Negative	Positive
Within-herd seroprevalence to *N. caninum* (Romero et al., 2005)	32.8%	33.5%	36.6%

2.7. Record sheet to determine the presence of risk factors

A record sheet was filled for each of the 34 dogs that took part in the study. The sheet included information of the farm (name, seroprevalence) and the dog (breed, age, sex, feeding and housing). A descriptive analysis was done to compare environmental and handling conditions of dogs positive for *N. caninum* by PCR and ELISA.

3. Results

3.1. Microscopic examination

Coccidian oocysts were detected microscopically in seven fecal samples; these samples were from five dogs that belonged to four farms. Of these seven samples, oocysts measuring 14 μm and containing two sporocysts (*Neospora*-like) were found only in one dog.

3.2. N. caninum DNA detection

Four (1.5%) of 256 fecal samples analyzed by PCR were positive for *N. caninum* DNA. These samples belonged to three dogs from three different farms. One dog tested positive twice, at the beginning of the study and 2.5 months later; this dog tested negative at the other two sampling times. None of the samples that were positive in PCR were positive in the coprological analysis. The characteristics of these three dogs, based on the surveys applied, are presented in Table 1.

3.3. Serological examination

Fifteen (48.4%) of the 31 dog sera analyzed were positive by ELISA in the first sampling. Four months later, 13 of the previously seropositive dogs were again seropositive; it was not possible to analyze the other two previously seropositive dogs. The seronegative animals remained negative in the second sampling (one dog was not analyzed during the second sampling). Changes in the serological status of the dogs between samplings were not detected. From the serum samples that tested positive in ELISA, 78.6% (22 of 28) showed inhibition percentages higher than 50.0% (Table 2).

Only one dog tested positive for *N. caninum* by ELISA and PCR. The other two dogs were positive in PCR and remained negative in ELISA throughout the study. The same serological results were obtained after examining the sera of the three dogs by IFAT. Overall, 30 samples were positive by both ELISA and IFAT, 14 sera were positive by ELISA alone, and four sera were positive by IFAT alone.

3.4. Bioassay for N. caninum

The gerbils fed dog feces remained healthy and antibodies to *N. caninum* were not found in 1:25 dilution of serum from any of the gerbils; *N. caninum*

Table 2
Distribution of seronegative and seropositive sera to *N. caninum* from farm dogs of Costa Rica in relation to the inhibition percentage (IP) values determined with ELISA (≤30: negative seroreactors, 31–50: low seroreactors, ≥51: high seroreactors)

	IP-values (%)			
	≤30	31–50	51–70	71–90
Sera analyzed in the first sampling period	16	4	2	9
Sera analyzed in the second sampling period	15	2	4	7
Total sera analyzed	31	6	6	16

tissue cysts were not found in the brains of these gerbils killed 2 weeks after serological examination.

3.5. Risk factors

Risk factors present on farms from which PCR or ELISA positive dogs were sampled were ingestion of bovine tissues, direct contact between dogs and cows, and the presence of other animals on the farm, such as coyotes and rodents.

4. Discussion

The difficulty in finding *N. caninum* oocysts in feces from naturally infected dogs was confirmed in the present investigation. *N. caninum* DNA was found in four fecal samples but these samples were negative by direct microscopical examination and by bioassay, perhaps related to the presence of few oocysts. Only a small amount of total feces was bioassayed in gerbils. Additionally the samples had been stored for approximately 8–10 months before inoculation into gerbils. The minimum infective dose for *N. caninum* oocysts for any host is unknown and there are no suitable animal models to compare the infectivity of *N. caninum* oocysts. Currently, gerbils are the best rodent models because outbred mice are not very susceptible and the immunosuppressed mice strains (e.g.) interferon gamma gene knockout (KO) mice, although susceptible to *N. caninum* oocysts (McAllister et al., 1998), are expensive and not all *N. caninum* strains can be grown in cell culture (Schares et al., 2005).

It is of interest that *N. caninum* DNA was found twice from one dog in samples taken 2 month apart, confirming the findings of McGarry et al. (2003) in a naturally infected dog from United Kingdom. Gondim et al. (2005) reported that three of the five dogs that had shed *N. caninum* oocysts after primary infection re-shed oocysts when re-exposed 18–20 months later. These results indicate that dogs may shed oocysts more than once during their lives.

In the present study, two of the three dogs found positive by PCR were seronegative, both in ELISA and IFAT. Experimentally infected dogs that had shed oocysts did not seroconvert in several instances and these data were recently summarized (Dubey et al., 2007). The failure to seroconvert after oocyst shedding may be related to the time of sampling and the life cycle of the parasite. There may not have been enough time between oocyst shedding and drawing of the blood samples. Alternatively, *N. caninum* infection may have been confined to the intestinal epithelium without invasion of the extra-intestinal organs; the locations of *N. caninum* schizonts and gamonts in canine intestine have not yet been identified (Dubey et al., 2004).

It is likely that the seropositive dogs have already shed *N. caninum* oocysts. In this respect serological surveys provide an estimate of the exposure to the parasite and seropositivity in dogs has been found to be a major risk factor for *N. caninum* infection in cattle (see Dubey et al., 2007). There are many reports of *N. caninum* infection in dogs worldwide based on seropositivity (Dubey et al., 2007). The present finding *N. caninum* antibodies in 48% of dogs is apparently the first report from Costa Rica.

Most dogs that tested positive in ELISA or in PCR, had a history of ingesting fetal tissues, placentas and calf carcasses. Dogs have been shown to excrete *N. caninum* oocysts after eating placentas of naturally infected cattle (Dijkstra et al., 2001) and tissues of experimentally infected calves (Gondim et al., 2002, 2005). To prevent *N. caninum* infection, dairy farmers need to be aware of the role of canids in spreading the parasite to their livestock in Costa Rica.

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