



Crab-eating fox (*Cerdocyon thous*), a South American canid, as a definitive host for *Hammondia heydorni*

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ABSTRACT

Hammondia heydorni is a cyst forming coccidia closely related to other apicomplexans, such as *Toxoplasma gondii*, *Neospora caninum* and *Hammondia hammondi* with a two-host life cycle. Dogs and other canids as red foxes (*Vulpes vulpes*) and coyotes (*Canis latrans*) may serve as definitive hosts for *H. heydorni*. Sporulated oocysts are infective for cattle, sheep and goats, which may serve as intermediate hosts. Herein, we describe the ability of crab-eating fox (*Cerdocyon thous*), a wild carnivore that is commonly found from northern Argentina to northern South America, to serve as definitive host of *H. heydorni*. The whole masseter muscle and brain from two 2-year-old bovines were collected, minced and pooled together for the fox infection. The bovine pooled tissues were equally administered to four foxes, in two consecutive days. Two foxes shed subspherical unsporulated oocysts measuring 10–15 µm, after 8 and 9 days post-infection, respectively. One of the foxes eliminated oocysts for 5 days, while the other fox shed oocysts for 9 days. A DNA sample of oocysts detected at each day of oocyst elimination was tested by two PCRs, one of them carried out employing primers directed to the common toxoplasmatid 18S and 5.8S ribosomal RNA coding genes (PCR-ITS1) and the other based on heat-shock protein 70 kDa coding gene (PCR-HSP70). These samples were also submitted to a *N. caninum* specific nested-PCR protocol based on a *N. caninum* specific gene (Nc5-nPCR). All of them were positive by PCR-ITS1 and PCR-HSP70 but negative by Nc5-nPCR. The PCR-ITS1 and PCR-HSP70 nucleotide sequences amplified from the oocysts shed by the foxes revealed 100% identity with homologous sequences of *H. heydorni*. In conclusion, it is clear that *H. heydorni* also uses the crab-eating fox as a definitive host. The crab-eating fox is usually reported to live in close contact with livestock in several regions of Brazil. Therefore, it is reasonable to infer that such carnivores may play an important role in the sylvatic and domestic cycles of *H. heydorni* infection.

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1. Introduction

Hammondia heydorni is a cyst forming coccidia closely related to other apicomplexans, such as *Toxoplasma gondii*, *Neospora caninum* and *Hammondia hammondi* (Frenkel and Dubey, 1975; McAllister et al., 1998; Lindsay et al., 1999,

2001). Little is known about its life cycle except that it has an obligatory two-host life cycle (Dubey et al., 2002). Dogs fed sporulated oocysts did not shed oocysts in their feces, whereas sporulated oocysts were infective for cattle, sheep and goats (Heydorn, 1973; Dubey and Fayer, 1976; Blagburn et al., 1988). Dogs that were fed extra-intestinal tissues and intestine from infected animals shed unsporulated oocysts in their feces (Matsui et al., 1981, 1986). Shedding of *H. heydorni* oocysts by non-dog canids fed tissues of herbivores have already been described. The

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other canids that serve as definitive host for *H. heydorni* are red foxes (*Vulpes vulpes*) (Schaes et al., 2002) and coyotes (*Canis latrans*) (Dubey and Williams, 1980).

The sporulated oocysts of non-pathogenic *H. heydorni* morphologically resemble those of the clinically important *N. caninum*. Thus, oocysts of these species are morphologically indistinguishable and the coprological diagnosis in definitive host is difficult (Dubey et al., 2002; Heydorn and Mehlhorn, 2002). Oocysts of *H. heydorni* and *N. caninum* may, otherwise, be differentiated by molecular techniques (Šlapeta et al., 2002; Monteiro et al., 2008).

Herein, we describe the ability of crab-eating fox (*Cerdocyon thous*), a wild carnivore that is commonly found from northern Argentina to northern South America, to serve as a definitive host of *H. heydorni*.

2. Materials and methods

2.1. Experimental infection

The whole masseter muscle and brain from two 2-year-old bovines were collected, minced and pooled together for the fox infection. The bovines were previously tested for the detection of serum antibodies to *N. caninum* by using the indirect fluorescent antibody test (IFAT), as described elsewhere (Paré et al., 1995). Four sibling crab-eating foxes (numbered from 1 to 4) that were born in a conservational park and kept in captivity were used in this study. The animals have been raised in captivity in the conservational park and were fed raw meat (beef and chicken) and commercial dog food until the age of 6 months. Then, the foxes were caged separately in steel cages and fed only commercial dog food and tap water *ad libitum* during the entire experiment (for 75 days), except in the days of infection. The foxes were also tested for the detection of serum antibodies to *N. caninum* by using IFAT and anti-dog IgG conjugated to fluorescein isothiocyanate as secondary antibody (anti-dog IgG-FITC conjugate from rabbit, Sigma, F7884, St. Louis, MO). Result was considered positive when titer was equal or greater than 25. The foxes were bled by puncture of jugular vein and the sera of the foxes were tested at the beginning (day 0, when the foxes were caged) and the end (day 75, when the foxes were sacrificed) of the experiment.

Before the experimental infection, daily examination of feces (from day 0 to day 30) of each caged animal was carried out by the concentration flotation technique using sucrose solution (sp. gr. 1.205) for the detection of coccidian oocysts (Ogassawara et al., 1980). The foxes were then fed with bovine tissues. The bovine pooled tissues were divided into two portions and were equally administered for the four animals in two consecutive days. After experimental infection, the feces excreted by each dog were daily examined again for 30 days. After that, the animals remained in steel cages for more 15 days receiving commercial dog food and tap water *ad libitum*. The foxes were then euthanatized and necropsied (on day 75 or 45 days after experimental infection).

Bovine (pool of brain and masseter) and fox tissues (brain and masseter, separately) were inspected for the presence of DNA of *H. heydorni* and *N. caninum* by using

PCR and direct sequencing of PCR products, using the protocols described below. The tissues were not inspected for the presence of cysts.

2.2. Oocyst examination

The number of oocysts per gram for the positive samples was counted in triplicate using the Neubauer chamber. Briefly, 1 g of previously homogenized feces from positive samples from each day was weighed and homogenized in water. Fecal suspensions were washed through a series of metallic sieves (65, 100, 200, final exclusion 400 μm). The oocysts and debris were concentrated by centrifugation (500 $\times g$ for 10 min). Then, for each day, sediment was resuspended in water to a final volume of 5 mL. Four aliquots were used for counting using a Neubauer chamber. An average counting was obtained and the total number of oocysts produced per gram per day was extrapolated from the 5 mL from each stool sediment. Unsporulated oocysts in concentrated sucrose solution were examined by light microscopy at a magnification of 1000 using an Olympus BX40 microscope connected to the Olympus DP70 microscope digital camera. Images were analyzed using the software Image-Pro[®] Plus version 5.1 (Media Cybernetics, Inc., Silver Spring, MD). Floated material was transferred to a slide and examined by light microscopy. When subspherical 10–15 μm size oocysts were observed, the slide was washed with 1 mL TE (10 mM Tris-HCl pH 8.0; 1 mM EDTA pH 8.0) in sterile Petri dishes. The oocysts were then transferred to 1.5 mL microtubes and washed twice in TE by centrifugation at 12,000 $\times g$ for 5 min.

2.3. DNA extraction

After the last wash, the supernatant was discarded and the pellet was resuspended in 500 μL of lysis buffer (10 mM Tris-HCl pH 8.0; 25 mM EDTA pH 8.0; 100 mM NaCl, 1% SDS). The oocyst suspension was submitted to three freeze thaw cycles and then proteinase K was added to 10 $\mu\text{g}/\text{mL}$. The suspension was incubated at 37 °C. After overnight incubation the DNA was extracted using a mixture of phenol, chlorophorm, isoamyl-alcohol (25:24:1) and precipitated with ethanol as described elsewhere (Sambrook et al., 1989). DNA isolation from bovine (brain and masseter pool) and fox tissues (brain and masseter, separately) was based on protocols described elsewhere (Pena et al., 2007).

2.4. Polymerase chain reaction, sequencing and sequence analysis

The PCRs for oocyst characterization were carried out employing primers directed to the common toxoplasmatid 18S and 5.8S ribosomal RNA coding genes (PCR-ITS1), to the Heat-shock protein 70 kDa coding gene (PCR-HSP70) and to the *N. caninum* Nc-5 coding sequences (Nc5-nPCR). The Nc5-nPCR was performed in a heminested format. For the detection and identification of DNA of toxoplasmatinae in tissue homogenates, only PCR-ITS1 and Nc5-nPCR were employed. The PCR protocols were performed as

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