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Short communication

Experimental evidence against transmission of *Hepatozoon canis* by *Ixodes ricinus*

Alessio Giannelli[a], Rafael Antonio Nascimento Ramos[a], Filipe Dantas-Torres[a,b], Norbert Mencke[c], Gad Baneth[d], Domenico Otranto[a,*]

[a] *Dipartimento di Medicina Veterinaria, Università degli Studi di Bari, 70010 Valenzano, Bari, Italy*
[b] *Centro de Pesquisas Aggeu Magalhães (Fiocruz-PE), 50670-420 Recife, Pernambuco, Brazil*
[c] *Bayer Animal Health, Global Veterinary Services – CAP, Leverkusen, Germany*
[d] *School of Veterinary Medicine, Hebrew University, Rehovot, Israel*

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ABSTRACT

Hepatozoon canis is among the most widespread tick-borne protozoa infecting domestic and wild carnivores. Its distribution is related to the occurrence of its major vector, the brown dog tick *Rhipicephalus sanguineus*. However, the role of *Ixodes ricinus* as a vector of *H. canis* has been hypothesized. In the present study, the development of *H. canis* was investigated in *I. ricinus* and *R. sanguineus* nymphs collected from a naturally infested dog. All *I. ricinus* ticks examined ($n = 133$) were negative by cytological examination at days 20, 30, and 90 post collection, although *H. canis* DNA was detected in one nymph at day 20 and in 2 nymphs at day 30 post collection. On the other hand, *H. canis* sporogony was documented by cytology, and *H. canis* DNA was detected by PCR in *R. sanguineus* at day 30 post collection. These results indicate that *H. canis* sporogony does not occur in *I. ricinus*, but in *R. sanguineus*, suggesting that *I. ricinus* does not act as a vector of *H. canis*.

Introduction

Hepatozoon canis (Eucoccidiorida, Hepatozoidae) is among the most widespread tick-borne protozoa infecting domestic dogs and wild carnivores (Baneth, 2011). It usually causes subclinical infection, which may remain without clinical signs until reaching a high level of parasitaemia or upon infection with concomitant pathogens, such as *Babesia vogeli*, *Ehrlichia canis*, *Anaplasma platys*, and *Leishmania infantum* (Otranto et al., 2011). The life cycle of *H. canis* includes several developmental stages within the vertebrate and invertebrate hosts and, unlike other haemoprotozoa, its transmission occurs through the ingestion of infected ticks, which harbour mature oocysts (Baneth, 2011). Briefly, after the ingestion of a tick carrying mature oocysts, each one containing several sporocysts, infective sporozoites are released in the carnivore gastrointestinal tract, penetrate the gut wall, and reach blood or lymph circulations. Merogony takes place in haemolymphoid tissues (i.e., spleen and lymph nodes), followed by further replication in bone marrow, liver, and other organs (Baneth et al., 2007). As early as 13 days post infection, micromerozoites penetrate neutrophils and monocytes and may appear as mature gamonts in the blood about 28 days post infection (Baneth et al., 2007). When a blood-feeding tick ingests *H. canis* gamonts, gametogenesis occurs in its gut, followed by sporogony in the haemocoel (Baneth, 2011).

The brown dog tick *Rhipicephalus sanguineus* plays a major part in the epidemiology of *H. canis* worldwide (Baneth, 2011), and its role as a vector has been reinforced by the evidence of transstadial transmission from tick larvae to nymphs (Giannelli et al., 2013). However, the invertebrate host range of *H. canis* has not yet been fully elucidated. To date, only *Amblyomma ovale* has been regarded as a second suitable vector in South America (Rubini et al., 2009). Additionally, mature *H. canis* oocysts have been reported in *R. microplus* (formerly *Boophilus microplus*), *Haemaphysalis longicornis*, and *Haemaphysalis flava* (Murata et al., 1995; de Miranda et al., 2011). The finding of *H. canis* DNA in a single specimen of *Ixodes ricinus* adult tick (Gabrielli et al., 2010) suggested its potential role as a vector of *H. canis* among domestic and wild canids (e.g., dogs, jackals, foxes, and wolves) (Duscher et al., 2013). In addition, the detection of high prevalence rates of *H. canis* infection in foxes from Italy, Slovakia, Croatia, Israel, and Portugal, ranging from 13.4% to 48% in woodland settings or in *R. sanguineus*-free regions (Conceição-Silva et al., 1988; Fishman et al., 2004; Majláthová et al., 2007; Dezdek et al., 2010; Gabrielli et al., 2010), has been correlated with the occurrence of *I. ricinus* (Duscher et al., 2013). However, this possible association remains only circumstantial, since no study has demonstrated the presence of infective *H. canis* oocysts in *I. ricinus* or its capacity to transmit this pathogen.

* Corresponding author. Tel.: +39 080 4679839; fax: +39 080 4679839.
E-mail addresses: domenico.otranto@uniba.it, d.otranto@veterinaria.uniba.it (D. Otranto).

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The present study aimed to investigate if *H. canis* developed in *I. ricinus* nymphs collected from a naturally infested dog, which was also infested by *R. sanguineus* ticks.

Materials and methods

Tick source

Ticks were collected from a two-year-old, mixed-breed dog from the municipality of Putignano (province of Bari) that had visited the Gallipoli Cognato Forest (Matera province, Basilicata region, southern Italy) with its owner. The dog was heavily infested by engorged ixodids and diagnosed as infected with *H. canis* by detection of gamonts in the blood and by PCR (see below). Ticks were manually collected and placed in glass vials with cotton plugs and were transported to the Department of Veterinary Medicine of the University of Bari. In the laboratory, ticks were identified according to morphological features (Manilla, 1998; Walker et al., 2000) as *I. ricinus* ($n = 133$) and *R. sanguineus* ($n = 30$) nymphs. Finally, ticks were placed in an incubator under controlled conditions (i.e., 20 ± 3 °C, RH > 80% for *I. ricinus* and 27 ± 1 °C, RH > 70% for *R. sanguineus*).

Detection of H. canis gamonts in the blood of the infected dog

Blood smears were stained with the May-Grünwald Giemsa Quick Stain (Bio Optica, Milano, Italy) and examined under a light microscope for the presence of *H. canis* gamonts (100 microscopic fields) under an oil immersion objective. The level of parasitaemia, estimated as the percentage of peripheral blood neutrophils containing intracellular gamonts, was 80%.

Biological parameters assessed in collected ticks

Ticks were monitored daily, and the following biological parameters were recorded: overall mortality (i.e., number of ticks found dead at the end of the study) and the length of the premoult period (i.e., number of days from detachment to ecdysis).

Detection of H. canis oocysts in infected ticks

I. ricinus ticks were examined at 3 different time points: 39 specimens both on day 20 (T1) and day 30 (T2) post collection and 48 specimens on day 90 post collection (T3). *R. sanguineus* ticks were examined only at T1 and T2 (15 specimens per each dissection time). Ticks were placed on slides containing a drop of 0.9% NaCl solution and dissected with a sterile scalpel. They were incised through the spiracular plate to examine haemolymph smears under a light microscope using different magnifications. Oocysts were morphologically identified (Baneth et al., 2007) and measured with the aid of an image analyser programme (Leica®, LAS 4.1). Finally, all the dissected ticks were placed individually in sterile tubes with phosphate buffered saline (PBS) and stored at −20 °C until molecular analysis.

Detection of H. canis DNA

DNA was extracted from tick samples using the guanidine isothiocyanate-phenol technique (Sangioni et al., 2005) and from the dog blood sample using a commercial kit (Qiagen, DNeasy Blood & Tissue Kit, Milan, Italy), following the manufacturer's instructions. Samples were tested by a conventional PCR for the detection of *H. canis* as previously described (Inokuma et al., 2002). Amplicons were resolved in ethidium bromide-stained agarose (Gellyphor, EuroClone, Milan, Italy) gels (1.5%) and sized by comparison with Gene RulerTM 100-bp DNA Ladder (MBI Fermentas, Vilnius, Lithuania) as molecular marker, and finally gels were photographed using Gel Doc 2000 (Bio-Rad, Hercules, CA, USA). Amplicons were purified using Ultrafree-DA columns (Amicon, Millipore, Milan, Italy) and sequenced directly (Applied Biosystems, Monza, Milan, Italy) using the TaqDyeDeoxyTerminator Cycle Sequencing Kit (Applied Biosystems). Sequences were determined in both directions, using the same primers individually as for the PCR and compared with those available in GenBank using the Basic Local Alignment Search Tool (BLAST).

Table 1
Ixodes ricinus and *Rhipicephalus sanguineus* ticks positive for *Hepatozoon canis* by PCR at different time points (i.e., 20 (T1), 30 (T2), and 90 (T3) days post collection).

Time point	*Ixodes ricinus* (nymphs)	*Rhipicephalus sanguineus* (adults)
T1	1/39 (3%)	5/15 (33%)
T2	2/39 (5%)	5/15 (33%)
T3	0/48 (0%)	–

Results

Out of 133 *I. ricinus* nymphs collected, seven (5.2%) died during the observation period and none of the remaining ($n = 126$) moulted to adults. All *R. sanguineus* engorged nymphs ($n = 30$) moulted to adults in 16 ± 2 days post collection. All *I. ricinus* examined were negative for the detection of *H. canis* by microscopy at any tick dissection time point, but *H. canis* DNA was amplified from 3 nymphs (one at T1, 2 at T2) (Table 1). Conversely, different *H. canis* stages were found in four (27%) *R. sanguineus* adults at T2. Immature oocysts ($n = 49$; $188.3 \pm 34.7\ \mu m \times 171.1 \pm 40.0\ \mu m$) were subspherical in shape and displayed a condensed central matrix, enclosed by an evident outer membrane (Fig. 1A). Developed oocysts ($n = 2$; $237.1 \pm 27.1\ \mu m \times 226.9 \pm 24.0\ \mu m$) harboured oval-shaped sporocysts ($25.5 \pm 2.9\ \mu m \times 20.2 \pm 5.8\ \mu m$), which contained sporozoites (Fig. 1B). Sporocysts ($37.0 \pm 4.2\ \mu m \times 21.6 \pm 1.5\ \mu m$) were also found freely in the haemolymph of all positive ticks (Fig. 1C). *H. canis* DNA was successfully amplified from 10 (33%) *R. sanguineus* ticks (Table 1). The sequences obtained from the DNA amplicons matched (100% identity) with a GenBank *H. canis* reference sequence (accession number: KC138535).

Discussion

The present study failed to document *H. canis* development in *I. ricinus* nymphs. Conversely, different stages of *H. canis* were found in *R. sanguineus* adults that fed as nymphs on the same infected dog as early as 30 days post detachment. It means that the dog included in this study got infected in Putignano, where *H. canis* is highly prevalent in ticks and dogs (Otranto et al., 2011; Dantas-Torres et al., 2012). Conversely, the dog became infested by *I. ricinus* during its visit to Basilicata, where this tick is quite common (Falchi et al., 2012).

Comparing tick biological parameters recorded to those reported in the literature (Dantas-Torres et al., 2010), *R. sanguineus* fitness was not apparently affected by *H. canis* infection. The moulting period of *I. ricinus* was not evaluated, due to the longer times required for nymphs to moult (about 120 days) (Dusbábek, 1996). Although *I. ricinus* ingested *H. canis* gamonts during the blood meal, as inferred by 3 PCR-positive specimens, further development and sporogony did not occur. This finding might explain the detection of 2 gamonts in the haemolymph of *I. ricinus* soon after their collection from red foxes (Conceição-Silva et al., 1988) as well as the PCR positivity of a single tick collected from the environment in central Italy (Gabrielli et al., 2010). Accordingly, the detection of *I.*