Veterinary Parasitology 164 (2009) 324–327

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Short communication

Acquisition and transmission of *Hepatozoon canis* (Apicomplexa: Hepatozoidae) by the tick *Amblyomma ovale* (Acari: Ixodidae)

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ARTICLE INFO

Article history:
Received 17 February 2009
Received in revised form 26 March 2009
Accepted 6 May 2009

Keywords:
Hepatozoon canis
Transmission
Amblyomma ovale
Dogs

ABSTRACT

The present study aimed to evaluate under controlled conditions the acquisition of *Hepatozoon canis* by *Amblyomma ovale* after feeding on infected dogs, and the subsequent induction of infection in uninfected dogs that ingested the experimentally infected ticks. Two *H. canis* naturally infected dogs were infested with *A. ovale* adult ticks derived from an uninfected laboratory tick colony. After feeding, two *A. ovale* females presented *H. canis* oocysts in the hemolymph at the first and fourth days after removal of ticks from dogs. The oocysts had an average size of 244.34 μm × 255.46 μm. Three uninfected dogs were fed with ticks previously fed on the infected dogs. Only one dog became infected 32 days after oral inoculation, presenting circulating gametocytes, parasitemia less than 1%, and positive PCR confirmed to be *H. canis* by DNA sequencing. The results obtained indicated *A. ovale* ticks as potential vector of *H. canis* in rural areas of Brazil.

1. Introduction

Canine hepatozoonosis is a disease caused by protozoa of the genus *Hepatozoon*, transmitted by ticks. Since the first description, the occurrence of the parasite has been reported in several countries of the world (Baneth et al., 2000).

Currently there are two species of *Hepatozoon* that infect dogs, *Hepatozoon canis* and *Hepatozoon americanum* (Baneth et al., 2000). Genetic characterization of the species of *Hepatozoon* spp. that infects dogs in Brazil has revealed that *H. canis* or closely related genotypes are the etiologic agent of canine hepatozoonosis in this country (Rubini et al., 2005; Paludo et al., 2005). Thus, the distribution of *H. canis* has included Africa, Southeast Asia, Middle East, Europe, and South America, whereas *H. americanum* has been restricted to the United States (Baneth et al., 2003; Gavazza et al., 2003; Vincent-Johnson et al., 1997; Oyamada et al., 2005; Rubini et al., 2008).

Christophers (1907) identified the brown dog tick, *Rhipicephalus sanguineus*, as invertebrate host of *H. canis*, although other tick species have been identified as possible vectors of the protozoa, such as *Haemaphysalis longicornis* and *Haemaphysalis flava* in Japan (Murata et al., 1995) and *Amblyomma ovale* in Brazil (Forlano et al., 2005).

R. sanguineus is the main tick species that infest dogs in Brazil, mostly in urban areas and less abundantly in rural areas (Labruna and Campos Pereira, 2001; Szabó et al., 2001). The genus *Amblyomma* is the largest in Brazil, with 33 described species. Depending of the geographic region of Brazil, dogs from rural areas are infested by different *Amblyomma* species, including *A. ovale*, *Amblyomma aureolatum*, *Amblyomma tigrinum*, and *Amblyomma cajennense* (Freire, 1972; Labruna et al., 2000; Labruna & Campos Pereira, 2001; Labruna et al., 2005). Due to its broad distribution, *A. ovale* is frequently reported infesting wild carnivores and dogs in rural areas of Brazil (Aragão and Fonseca, 1961; Ribeiro et al., 1997; Barros and Baggio,

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doi:10.1016/j.vetpar.2009.05.009

1992; Labruna et al., 2005). The presence of *A. ovale* in dogs is probably linked to the sharing of habitat with wild animals that act as primary hosts of this species (Szabó et al., 2001). While it is well known that carnivores, including the domestic dog, are among the preferred hosts for the adult stage of *A. ovale*, a few literature reports suggest that small wild rodents are preferred hosts for immature stages (Jones et al., 1972; Labruna et al., 2005).

During a study of hepatozoonosis in two rural areas of Rio de Janeiro, Forlano et al. (2005) found *Hepatozoon*-infected dogs naturally infested by *A. cajennense*, *A. ovale*, *A. aureolatum* and *R. sanguineus*. These ticks were collected and searched for the presence of oocysts; only one specimen of *A. ovale* was shown to be infected by one oocyst similar to *H. canis*. A dog that was orally inoculated with macerate of *A. ovale* became infected by the hemoparasite, as demonstrated by gametocytes in the peripheral blood 63 days after inoculation. These procedures indicated that *A. ovale* is a potential vector of canine hepatozoonosis in rural areas of Brazil.

Considering the ability to infest domestic and wild animals, *A. ovale* may be an important carrier of *H. canis* in rural areas. The present study aimed to evaluate under controlled conditions, the acquisition of *H. canis* by *A. ovale* after feeding on infected dogs, and the subsequent induction of infection in uninfected dogs that ingested the experimentally infected ticks.

2. Materials and methods

2.1. Acquisition of H. canis by ticks

Two male dogs from the rural area of Botucatu, state of São Paulo, were shown to be naturally infected by *H. canis*, as demonstrated by blood smears and polymerase chain reaction (PCR), as described below. Both infested dogs had low parasitemia, with only two or three gamonts observed on the entire blood smear. PCR products generated from the blood of these two puppies were DNA sequenced (as described below) and shown to be 100% identical to the corresponding sequence of a Brazilian isolate of *H. canis* (GenBank accession number FJ743476).

Engorged females of *A. ovale* collected from naturally infested dogs in Monte Negro, state of Rondônia, northern Brazil, were used to start a laboratory colony. Larvae and nymphs of first generation were fed on tick naïve rabbits as previously described for other tick species (Pinter et al., 2002). For the first experiment, 300 unfed nymphs of *A. ovale* were placed on an infected dog, but no nymph was able to complete engorgement. Thereafter, a second experiment with unfed adult ticks was conducted.

One of the infected dogs was infested with 100 *A. ovale* adult ticks and the other with 50 adults. Tick infestations were conducted inside cotton sleeves glued to the shaved back of dogs, as previously described (Pinter et al., 2002). Adult females were allowed to feed through engorgement completion, whereas males were allowed to feed until engorgement completion of the last female, when they were removed from dogs and examined, as described below. Infected dogs were fed with commercial pellets and water *ad libitum*, and monitored daily with blood smears to confirm *Hepatozoon* infection throughout the period of tick feeding. After blood meal, adult ticks were recovered and analyzed for the presence of oocysts, or used for the study of vectorial competence.

For analysis of the presence of oocysts, hemolymph smears were prepared from each adult tick in physiological saline solution at 0.85%. For this purpose, tick capitulum was pierced with a hypodermic needle and its idiosoma was slightly compressed to force the exit of hemolymph (Forlano et al., 2005). Hemolymph smears were examined immediately in a light microscope with an image analyzer (Leica). Oocysts were identified and characterized morphologically and morphometrically using an ocular micrometer.

2.2. Vectorial competence of A. ovale

Three uninfected puppies from a same litter, ≈2 months old, were used for the study of vectorial competence. These dogs had never been in contact with ticks. Before the experiment, the animals were dewormed with febantel and pyrantel (Puppy, Bayer), immunized against major canine infectious diseases (V-8 vaccine, Biovet) and monitored during 30 days for presence of *Hepatozoon* infection through the techniques of capillary blood smears and by PCR, which resulted in negative results. Dogs used anti-tick collar (Kiltix, Bayer) and were housed in a kennel that was cleaned daily with a 2.5% hypochlorite solution. On day 0, each puppy was fed with *A. ovale* adult ticks (males and females) derived from the previous experiment on acquisition of *H. canis* by ticks. Dogs were fed with a mixture of intact and macerated ticks mixed with fresh minced beef. After this procedure, the dogs were monitored weekly by capillary blood smears to verify the presence of *H. canis* gametocytes.

2.3. Molecular analysis

Genomic DNA of canine blood samples was extracted using the GFX™ Blood Genomic DNA Purification kit (GE Healthcare) according to the manufacturer's recommendations. Extracted DNA was kept at −20 °C until used. PCR amplification and DNA sequencing of a 650-bp fragment of the 18S rDNA gene of *Hepatozoon* spp. was attempted using primers HEPF and HEFR according to Rubini et al. (2005). Generated sequences were visually inspected and aligned by using the programs Merger (http://bioweb.pasteur.fr/seqanal/alignment/intro-uk.html) and CLUSTALX Version 1.8 (Thompson et al., 1997). The consensus was submitted to BLAST analysis for comparison with available sequences in GenBank.

3. Results

3.1. Acquisition of H. canis by ticks

In the first experiment, infestation with nymphs failed, since few partially engorged nymphs (37) were recovered and none molted to adults. In the second experiment, two *H. canis*-infected dogs were infested with 150 *A. ovale* adult ticks for approximately 10 days, to allow a possible