

Evaluation of the vectorial capacity of *Rhipicephalus sanguineus* (Acari: Ixodidae) in the transmission of canine visceral leishmaniasis

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Abstract The vectorial capacity of *Rhipicephalus sanguineus* in the transmission of canine visceral leishmaniasis has been evaluated through a laboratory-controlled experiment. One healthy *Leishmania*-free dog and two dogs naturally infected with *Leishmania* were infested with *R. sanguineus* in various stages of development. Engorged larvae, unfed nymphs, engorged nymphs, unfed adults, engorged female adults and fed male adults were collected from the experimental animals and examined for *Leishmania* infection by optical microscopy, polymerase chain reaction (PCR) and parasite culture. *Leishmania* forms were not detected in any of the 433 smears prepared from engorged colonies nor in any of the 118 smears prepared from unfed colonies. However, one flagellate structure was identified in one of the smears. All pools of *R. sanguineus* that had fed on the infected dogs tested PCR-positive for *Leishmania* DNA, with the single exception of

the pool of engorged larvae. In contrast, all pools of ticks that had fed on the *Leishmania*-free dog were PCR-negative. *Leishmania* growth was not observed in any of the tick colonies following incubation on culture medium. Considering that no *Leishmania* forms were identified in any of the meticulously analysed smears derived from engorged colonies of *R. sanguineus*, it appears somewhat unlikely that the maintenance and multiplication of *Leishmania* occurs within the tick.

Introduction

Female sand flies of the species *Lutzomyia longipalpis* (Diptera: Psychodidae) are responsible for the natural transmission of *Leishmania chagasi* in the Americas, although it has been proposed that other vectors might also be involved in the epidemiology of canine visceral leishmaniasis (CVL). Such a hypothesis is based on the finding that the rate of infection of *L. longipalpis* by *L. chagasi* is very low ($\leq 0.5\%$; Montoya-Lerma et al. 2003), while the prevalence of CVL in endemic regions of Brazil is very high (França-Silva et al. 2005; Dantas-Torres et al. 2006; Malaquias et al. 2007; Mestre and Fontes 2007; Michalsky et al. 2007; Nunes et al. 2008).

The brown dog tick *Rhipicephalus sanguineus* (Latreille 1806) is the most prevalent parasite amongst urban dogs (Soulsby 1966; Balashov 1972; Linardi and Nagem 1973; Labruna and Pereira 2001; Soares et al. 2006) and is known to be involved in the transmission of various pathogens including *Ehrlichia canis* and *Babesia canis* (Smith et al. 1976; Gothe et al. 1989). Despite the lack of information concerning the interaction between *Leishmania*, host and vector or the capacity of ticks in transmitting *Leishmania*

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either mechanically or biologically, some researchers have hypothesised that *R. sanguineus* is involved in the epidemiology of CVL. In this context, there is evidence that some *Leishmania*-like flagellates are able to survive and develop within the digestive tube of Ixodidae ticks (Sherlock 1964). Moreover, since the process of intracellular digestion in Ixodidae parasites is slow, it is possible that *Leishmania* protozoa would not immediately be exposed to the proteolytic enzymes present in the gut and, hence, could remain viable for a prolonged period (Obenchain and Galun 1982).

Blanc and Caminopetros (1930) demonstrated that experimentally infected *R. sanguineus* is able to carry *Leishmania* during transstadial transmission and that the inoculation of tick macerates can produce infection in the European ground squirrel *Citellus citellus*. In a more recent study conducted in Brazil, Coutinho et al. (2005) collected ticks from dogs that were seropositive for *Leishmania* and reported a rate of natural infection of 15.4% as established by polymerase chain reaction (PCR). Additionally, the experimental inoculation of hamsters (*Mesocricetus auratus*) with macerates prepared from *Leishmania*-positive ticks revealed an infectivity rate of approximately 58.8% as determined by examination of liver and spleen smears.

The somewhat limited success in the control of CVL that has been achieved so far indicates that our knowledge concerning the epidemiology of CVL is still insufficient and that the involvement of other vectors in the transmission of *Leishmania* to canine reservoirs is very plausible. With the aim of evaluating the vectorial capacity of *R. sanguineus* and its possible participation in CVL transmission, a laboratory-controlled experiment involving the exposure of ticks at several stages of development to dogs naturally infected with *Leishmania* has been conducted. Engorged larvae, unfed nymphs, engorged nymphs, unfed adults, engorged female adults and fed male adults were collected from the experimental animals and examined for *Leishmania* infection by optical microscopy, PCR and parasite culture.

Materials and methods

Study animals

One healthy *Leishmania*-free dog (control animal) and two dogs that had been naturally infected with *Leishmania* (experimental animals) were used in the experiment. All three dogs were maintained in separate standardised cages at the kennel of the Parasitology Department of the Universidade Federal de Minas Gerais. Infection in the experimental dogs was previously confirmed by clinical examination (symptoms compatible with CVL), enzyme-linked immunosorbent

assay (ELISA), indirect immunofluorescent antibody test (IFAT), rapid immunochromatographic test (TRALd/rK39; InBios International, Seattle, WA, USA) and xenodiagnosis with *L. longipalpis* as described by Michalsky et al. (2007). Absence of infection in the control dog was demonstrated by negative ELISA and IFAT assays carried out twice at 30-day intervals.

Establishment and maintenance of *Leishmania*-free and *Leishmania*-infected *R. sanguineus* colonies

Engorged *R. sanguineus* females were collected from adult seronegative (as determined by ELISA and IFAT) dogs and immediately cleaned, washed with distilled water and blot-dried over filter paper. Thirty selected engorged females were placed separately in 24-well culture plates and maintained in an incubator at $24\pm 1^\circ\text{C}$ at a relative humidity $\geq 80\%$. Eggs were collected at 5-day intervals during the oviposition period and transferred to disposable plastic syringes (50 mg/syringe). The harvested eggs were kept in the syringe (closed at one end by the plunger and at the other by a cotton wool plug) and incubated under the conditions described above. Hatched larvae (approximately 1,250 larvae/syringe) were maintained in an incubator for 15–20 days until required for infestation experiments.

Each of the study dogs was infested at the dorsal region of the head with approximately 2,500 larvae by fixing two infestation chambers (1,250 larvae/chamber) prepared according to Pinter et al. (2002) with slight modification for dogs. In order to prevent removal of the chambers, the head and ears of the dog were screened off using an Elizabethan dog collar (Fig. 1). Every 2 days, the infestation chambers were opened in order to collect the



Fig. 1 Experimental dog being prepared for experiments. The infestation chambers were fixed on the top of the head, which was screened off using an Elizabethan collar

naturally released engorged larvae, which were then placed into labelled syringes, closed and maintained in an incubator under the conditions described above. Following ecdysis, nymphs were kept in an incubator for 15–20 days until required for further infestation experiments. Each study dog was infested with approximately 400 unfed nymphs using two infestation chambers (200 nymphs/chamber). Every 3 days, the infestation chambers were opened in order to collect the naturally released engorged nymphs, which were then placed into labelled syringes, closed and maintained in an incubator under the conditions described above. After ecdysis, adults were kept in an incubator for 15–20 days, following which they were separated according to sex and immediately employed in further infestation experiments. Each study dog was infested with 300 unfed adults using two infestation chambers (75 females and 75 males per chamber). Every 4 days, the infestation chambers were opened for the collection of naturally released engorged female adults. The males were removed from the infestation chambers after the engorged females had been collected.

Investigation of infection of *R. sanguineus* larvae, nymphs and adults with *Leishmania*

R. sanguineus at various stages of development (i.e. engorged larvae, unfed nymphs, engorged nymphs, unfed adults, engorged female adults and fed male adults) were tested for *Leishmania* infection using parasitological (microscopic examination of smears), molecular (polymerase chain reaction—PCR) and biological (parasite culture) methods. Prior to analysis, ticks were incubated at $24\pm 1^\circ\text{C}$ at a relative humidity $\geq 80\%$ for 4–7 days for those in the engorged phases or for 15 days for those in the unfed phases.

Parasitological examination was performed on engorged larvae, nymphs and adult ticks collected from infected dogs. The number of individuals that needed to be examined was calculated on the basis of an expected prevalence of 15% (Coutinho et al. 2005), within a confidence interval of 95% and an error margin of 5%. Thus, 196 engorged larvae and 196 engorged nymphs were analysed. For adult ticks, ten engorged females and 31 fed males were analysed. Larvae and nymphs were squashed between two glass slides, whereas adults were dissected and smears of the intestine were prepared. Slides were stained with Panotic stain (Laborclin, Pinhais, PR, Brazil) and examined under the optical microscope at $\times 1,000$ magnification. A similar methodology was employed for the unfed colonies of *R. sanguineus*, except that the number of unfed nymphs and adults was 59 individuals each (95% probability of detecting at least one positive individual and confidence limit of $\alpha=0.05$).

PCR was conducted according to Michalsky et al. (2007) using generic primers (5' GGGGAGGGGGCTTCTGCGAA 3'; 5' CCGCCCCTATTTTACACCAACCCC 3'; 5' GGCCCACTATATTACACCAACCCC 3') in order to amplify the conserved region within the minicircles of *Leishmania* kDNA (Degraeve et al. 1994). DNA was extracted from 14 different tick pools (each consisting of ten individuals) characterised as follows: pool 1—engorged larvae from experimental dogs; pool 2—engorged larvae from the control dog; pool 3—positive control comprising macerate of engorged larvae from the control dog mixed with 10 μL of stationary-phase promastigotes of *L. chagasi* (strain MHOM/BR/74/PP75) previously cultured in liver infusion tryptose (LIT) medium; pool 4—unfed nymphs from experimental dogs; pool 5—unfed nymphs from the control dog; pool 6—positive control comprising macerate of unfed nymphs from the control dog mixed with *Leishmania* promastigotes (as in pool 3); pool 7—engorged nymphs from experimental dogs; pool 8—engorged nymphs from the control dog; pool 9—positive control comprising macerate of engorged nymphs from the control dog mixed with *Leishmania* promastigotes (as in pool 3); pools 10 to 14—unfed adults from experimental dogs.

In order to isolate *Leishmania* from the various tick colonies, parasite cultures were established in Novy, McNeal and Nicolle (NNN)/LIT medium from engorged larvae, engorged nymphs, engorged females and fed males using 10–20 individuals for each isolation. Inocula of the various colonies were prepared by immersing each colony pool in 70% commercial alcohol, washing in 1% phosphate-buffered saline and macerating in LIT medium. In the case of engorged females, the intestines were dissected and macerated in LIT medium. All cultures were incubated at 25°C for 50 days with periodic subculture at 7-day intervals.

Results

Leishmania forms were not detected in any of the 433 smears prepared from colonies of engorged ticks or in any of the 118 smears from unfed colonies. However, one flagellate structure was identified in one of the smears prepared from engorged larvae fed on one of the experimental dogs (Fig. 2).

PCR assays revealed that pools 4, 7 and 10–14 of *R. sanguineus* that had fed on the experimental dogs were positive for *Leishmania* DNA, while pool 1, comprising engorged larvae from experimental dogs, presented a negative PCR test. All of the pools fed on the control dog (i.e. pools 2, 5 and 8) were negative. It is important to note that the control dog remained seronegative for *Leishmania* and without CVL symptoms until the end of the experi-

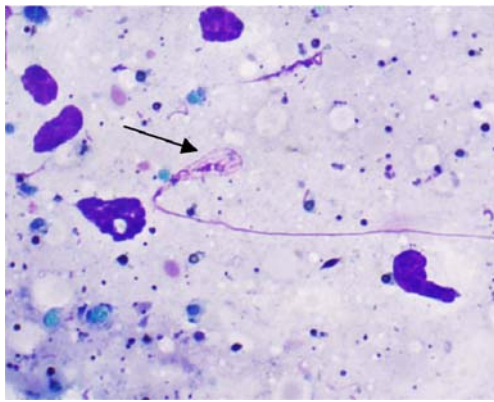


Fig. 2 Flagellate structure (arrowed) found in a smear prepared from *R. sanguineus* engorged larvae fed on *Leishmania*-infected dogs ($\times 1,000$ magnification; 1.25 objective)

ment. As expected, the PCR of the positive controls (pools 3, 6 and 9) were positive for the presence of *Leishmania* DNA (Figs. 3 and 4). *Leishmania* growth was not observed in any of the tick colonies cultured in NNN/LIT medium.

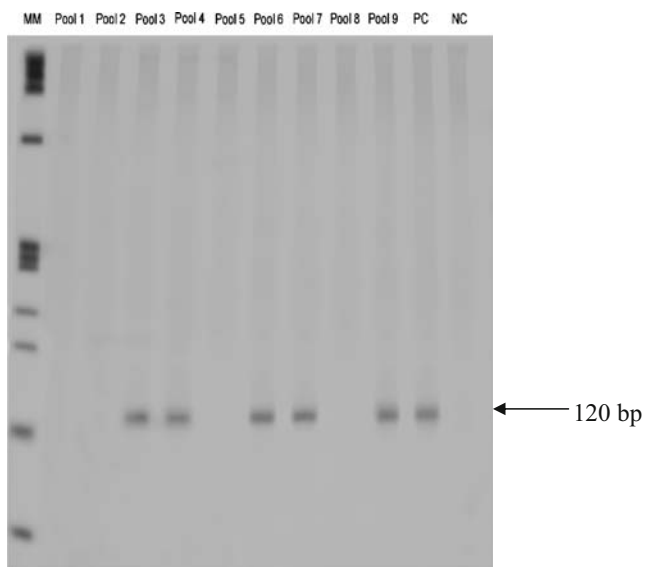


Fig. 3 Polyacrylamide gel electrophoresis (6%; silver stain) of PCR amplicons obtained using *Leishmania* generic primers. DNA was extracted from nine different tick pools (each consisting of ten individuals) characterised as follows: *pool 1* engorged larvae from experimental dogs; *pool 2* engorged larvae from the control dog; *pool 3* positive control comprising macerate of engorged larvae from the control dog mixed with 10 μ L of stationary-phase promastigotes of *L. chagasi* (strain MHOM/BR/74/PP75) previously cultured in liver infusion tryptose medium; *pool 4* unfed nymphs from experimental dogs; *pool 5* unfed nymphs from the control dog; *pool 6* positive control comprising macerate of unfed nymphs from the control dog mixed with *Leishmania* promastigotes (as in pool 3); *pool 7* engorged nymphs from experimental dogs; *pool 8* engorged nymphs from the control dog; *pool 9* positive control comprising macerate of engorged nymphs from the control dog mixed with *Leishmania* promastigotes (as in pool 3). *MM* molecular marker, *PC* positive control (*L. braziliensis*/M2903), *NC* negative control (no DNA)

Discussion

The importance of *R. sanguineus* as a vector for pathogenic organisms such as *B. canis* and *E. canis* is unquestionable (Smith et al. 1976; Gothe et al. 1989). With regard to *Leishmania*, the natural infection of *R. sanguineus* is favoured by many factors including the high prevalence of both ectoparasite and protozoan, in urban dogs within CVL-endemic areas, the prolonged duration of the blood feeding process, the prolonged contact between ticks and dogs, the slow digestion of ticks and the swapping of hosts during the life cycle of the tick. However, in order to confirm the postulated role of *R. sanguineus* as a vectorial agent in CVL, it is necessary to establish the capacity of the tick in supporting the growth and multiplication of *Leishmania*, together with the subsequent transstadial transmission of the protozoan.

The results obtained in the present study offer only limited support for a vectorial role of *R. sanguineus* but are insufficient to confirm or reject the hypothesis outright. *Leishmania* DNA was evidently present in the various developmental stages of *R. sanguineus*, including in unfed (post-ecdysis) nymphs and adults, suggesting the possibility of maintenance and transstadial transmission of *Leishmania* by the tick. However, PCR is a very sensitive technique, and small amounts of *Leishmania* DNA (≤ 1 ng) can be detected in samples (Prichard 1997). The results obtained with PCR can be misleading because DNA from just a few protozoa or from a single organism can give a positive

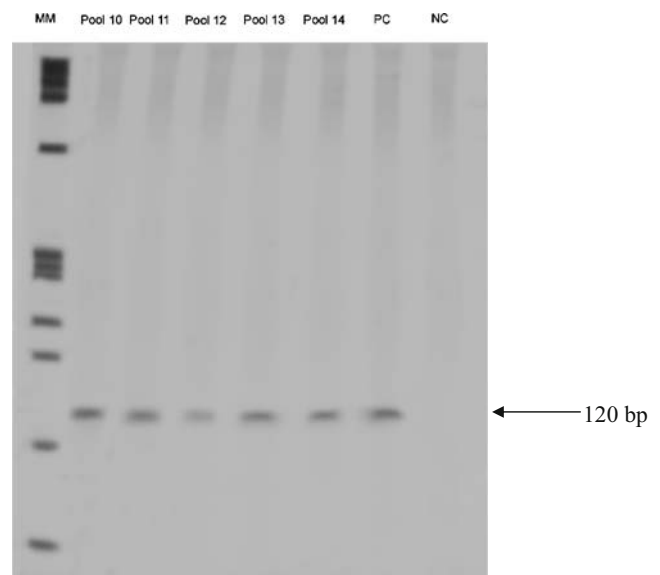


Fig. 4 Polyacrylamide gel electrophoresis (6%; silver stain) of PCR amplicons obtained using *Leishmania* generic primers. DNA was extracted from five different colonies (pools 10–14) of unfed adults collected from experimental dogs, each consisting of ten individuals. *MM* molecular marker, *PC* positive control (*L. braziliensis*/M2903), *NC* negative control (no DNA)

result, and the technique can also detect fragments of the target DNA. Hence, a positive PCR does not necessarily characterise a valid interactive infection of this protozoa with *R. sanguineus*.

According to the study conducted by Coutinho et al. (2005), 15.6% of the *R. sanguineus* forms collected from naturally infected dogs were infected with *Leishmania*, and the viability of such forms were confirmed by inoculating macerates into *C. citellus*. However, the methodology used by these authors is questionable since the macerates of *R. sanguineus* were inoculated immediately after collection of the ticks. It is possible, therefore, that ticks still contained undigested blood monocytes from the vertebrate host in which infecting *Leishmania* amastigotes were present. In the study described here, tick colonies were incubated under controlled conditions for between 4 and 15 days following removal from the experimental animals in order to assess the survival of *Leishmania* in the gut of *R. sanguineus*.

The study of the digestion process in Ixodidae species is rendered somewhat difficult because of the slow and prolonged feeding process that occurs continuously whilst the tick is in contact with the host. Indeed, ticks feed during all developmental stages from engorgement until the drop-off point. Another problem is that protease activity in the gut depends on a number of factors including the development stage of the tick, the feeding period and the sex of the parasite (Obenchain and Galun 1982). There is, however, no evidence that the *Leishmania* forms ingested with blood are destroyed in the intestine by the direct action of digestive enzymes (Balashov 1972).

The flagellate structure found in one of the smears prepared from engorged *R. sanguineus* larvae suggests the presence of organisms belonging to the family Trypanosomatidae since similar flagellates have been previously detected, but not characterised, in Ixodidae ticks (Sherlock 1964). Moreover, the occurrence of *Crithidia hyalommae* flagellates within eggs and haemolymph of *Hyalomma* and *Boophilus calcaratus* ticks has been reported (Balashov 1972), and *Trypanosoma theileri* epimastigotes have been found in haemolymph from Ixodidae larvae (Ribeiro et al. 1988).

Considering that no *Leishmania* forms were identified in any of the numerous (433 in total) meticulously analysed smears derived from engorged colonies of *R. sanguineus*, it appears somewhat unlikely that the maintenance and multiplication of *Leishmania* occurs within *R. sanguineus*. Hence, the hypothesis concerning the possible vectorial capacity of *R. sanguineus* remains in doubt, and the interspecific relationship between *Leishmania* and *R. sanguineus* demands further investigation. In particular, information is essential concerning which of the developmental stages of *R. sanguineus* might be susceptible to *Leishmania* infection, whether *Leishmania* can survive and

multiply within the gut of the tick during digestion and subsequently develop into an infecting form for the vertebrate host and the possibility of transstadial transmission of *Leishmania* by *R. sanguineus*.

The two infected dogs, which had their infection checked through serology and xenodiagnosis with *L. longipalpis*, were sufficient to provide the number of *R. sanguineus* stages previously determined by the statistical method employed. The PCR results showed that different stages of *R. sanguineus* ingested *Leishmania*, thus demonstrating that the number of dogs was enough to reach our objective. Further investigations should be designed so as to eliminate any possible effects of the characteristics of the individual vertebrate host on the interaction between *R. sanguineus* and the infected animals.

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Ethical standards The study was submitted to and approved by the Comissão de Ética no Uso de Animais (CEUA/FIOCRUZ) under protocol no. L-044/08. All procedures involving experimental animals were conducted according to the guidelines of Colégio Brasileiro de Experimentação Animal (COBEA).

Conflict of interest None.

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