



Participation of *Rhipicephalus sanguineus* (Acari: Ixodidae) in the epidemiology of canine visceral leishmaniasis

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Accepted 4 November 2004

Abstract

The vectorial competence of the tick *Rhipicephalus sanguineus* is discussed in relation to the epidemiology of canine visceral leishmaniasis, taking into account its strict association with dogs and the low indices of natural infection presented by its known vector, the phlebotomine sand fly *Lutzomyia longipalpis*. In order to evaluate natural infection by *Leishmania chagasi* and the infectivity of these parasites in the tick, 39 specimens (6 females, 11 males and 22 nymphs) of *R. sanguineus* were removed from 21 dogs showing diverse symptoms of zoonotic visceral leishmaniasis (ZVL). Six ticks (15.4%) gave positive results for the genus *Leishmania* using the PCR technique. To determine the infectivity of the parasites, 36 hamsters were inoculated orally and peritoneally with macerates of ticks removed from nine dogs symptomatic for visceral leishmaniasis. After 6 months the hamsters were sacrificed and necropsied. Serum was removed for IFAT, as well as spleen and liver fragments to make imprint smears and for PCR. Eight (88.9%) of these dogs presented ticks that were infective for 14 hamsters (41.2%), 12 (85.7%) of them infected peritoneally and two (14.3%) orally. PCR revealed 27 smears (40.9%) to be positive, 20 (62.5%) of them infected peritoneally and seven (20.6%) orally. IFAT showed 14 positive animals (41.2%). Based on these findings, we suggest that the vectorial capacity of *R. sanguineus* for *L. chagasi* should be evaluated further, opening new perspectives in the epidemiology of ZVL.

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Keywords: *Rhipicephalus sanguineus*; *Leishmania chagasi*; Ticks; Acari: Ixodidae; Canine visceral leishmaniasis; Vectorial capacity

1. Introduction

Zoonotic visceral leishmaniasis (ZVL) is caused by parasites of the genus *Leishmania*, digenetic protozoa of the order Kinetoplastida, family Trypanosomatidae.

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[✉] In memoriam.

The etiological agent of the disease, *Leishmania chagasi* (= *L. infantum*), has a wide distribution in the New World, including Brazil. The amastigote forms live and multiply by binary division within cells of the monocytic phagocytatory system of their mammalian hosts (Desjeux, 1991). The extracellular promastigote forms of the protozoan occur in the guts of phlebotomine sand flies (Diptera: Psychodidae), the main vector being *Lutzomyia longipalpis*. The life cycles of all trypanosomatids involve insects as invertebrate hosts, principally members of the Orders Hemiptera, Diptera and Siphonaptera (Wallace, 1966; Hoare, 1972; Williams, 1976; Lainson, 1983; Lainson and Shaw, 1987; Beard et al., 1989; Kettle, 1995).

Although natural transmission of *L. chagasi* occurs principally by the bite of infected *L. longipalpis* other mechanisms may be involved, since in some South American countries these insects usually show low indices of natural infection: 0.28% in Venezuela (Felicangeli et al., 1999), 0.29–0.9% in Colombia (Corredor et al., 1989; Ferro et al., 1995). In Brazil, except the 7.1% natural infection rate reported by Lainson et al. (1985) for Santarém, State of Pará, other rates ranged from 0.2 to 0.5% (Sherlock, 1999; Ryan et al., 1984). In the absence of sand flies transmission could possibly be guaranteed by other ectoparasites of dogs, particularly the flea *Ctenocephalides felis felis* and the tick *Rhipicephalus sanguineus* given the frequency and intensity with which both occur on dogs (Linardi and Nagem, 1973).

Researchers such as Deane (1958) have drawn attention to the need to investigate possible transmission of *Leishmania* by the bite of infected animals, ingestion of contaminated viscera or during copulation. Sherlock (1964) suggested that such mechanisms would be insufficient to guarantee transmission, since the protozoan requires an invertebrate host in its cycle. However, in the absence of sand flies, *Leishmania* transmission might involve other arthropods.

Ticks are potential vectors of several vertebrate pathogens, are widely distributed and abundant, possess high reproductive rates and longevity and are able to maintain high population densities. Furthermore, they are not effectively controlled by predation, parasitism or host immune mechanisms (Friedhoff, 1990).

The preferred hosts of *R. sanguineus* are wild and domestic canids and this tick species presents a wide distribution throughout Brazil (Linardi and Nagem,

1973). These ticks may harbor fungi, pyroplasm, *Hepatozoon*, *Trypanosoma*, nematodes, bacteria, rickettsiae, *Anaplasma*, *Babesia* and viruses (Jenkins, 1964; Walker, 1994).

Working in France, Blanc and Caminopetros (1930) demonstrated the capacity of *R. sanguineus* to be infected and sustain *Leishmania* experimentally, as well as its ability to transmit the infection by means of the inoculation of triturates in rodent *Citellus citellus*. Giraud et al. (1954) studied the epidemiology of human and canine ZVL in the Mediterranean region and suggested that different groups of arthropods could be responsible for the two known manifestations of the disease. Richardson and Kendall (1963) reinforced the hypothesis of Blanc and Caminopetros in suggesting that *R. sanguineus* could be involved in the transmission of *L. chagasi*. In Brazil, Sherlock (1964) found forms morphologically similar to *Leptomonas*, indicating that flagellates of a genus closely related to *Leishmania* could not only be maintained but also develop in the digestive tube of ticks.

Other factors such as the high prevalences of canine ZVL and low rates of human infection (Alencar et al., 1962; Costa et al., 2001; Profeta et al., 2003), as well as the difficulty of finding sand fly breeding sites in cities, lead to the conclusion that there may be one mechanism of *L. chagasi* transmission for dogs and another for humans. Since no effective treatment exists for canine ZVL there is a constant danger of *L. chagasi* transmission from dogs to humans in urban foci of the disease in Brazil, whatever the vector or mode of transmission involved. This motivated us to investigate the possible participation of ixodid ticks in canine ZVL.

2. Materials and methods

The study was carried out in two stages: (i) ticks were removed from dogs naturally infected with *L. chagasi* to determine the parasite infection rate in *R. sanguineus* and (ii) the infectivity of *L. chagasi* in *R. sanguineus* was evaluated.

2.1. Natural infection of *R. sanguineus* by *L. chagasi*

Dogs found to be positive for *L. chagasi* based on a serological diagnostic method (Indirect Fluorescent

Antibody Test, or IFAT) were brought to the CCZ laboratories, as part of the standard ZVL control policy in Brazil. After immobilization and clinical ectoscopic examination, each dog was sedated for the removal of all ticks on the entire body, using forceps and/or by picking off with the fingers. Parts of the body known to be preferred infestation sites of the ticks were especially well examined, including the ears, interdigital spaces and around the orbits.

After being removed from dogs, ticks were stored in glass tubes with rubber stoppers for 3 days. Individually, they were identified and dissected on a slide in a drop of 0.9% saline solution under a stereomicroscope. After removing the gnathosoma, the guts of ticks were exposed by an incision on the lateral margins of the idiosoma. Part of this material from the digestive system was homogenized in approximately 20 μ l of saline with the tick exoskeleton, transferred to a microtube and mixed with an equal volume of Tris EDTA buffer. Polymerase chain reaction (PCR) was used to detect DNA characteristic for the genus *Leishmania* and distinguish it from other trypanosomatids possibly infecting the ticks or dogs. Slides were also made from these homogenized contents. After fixing in methanol and staining with Giemsa, they were then examined under the oil immersion lens for the detection of *Leishmania*.

DNA was extracted from the material removed from ticks after thawing and homogenization of the samples (80 μ l) with the aid of a disposable plastic pestle. Subsequently 12 μ l of proteinase K (final concentration 100 mg/ml) and 10 μ l of MQ water were added to the samples to reach a final volume of 100 μ l. This was incubated for 3 h at 56 °C and homogenized every 30 min to digest all the material. After inactivation of the proteinase K by boiling for 15 min, the sample was centrifuged for 5 min at 14,000 rpm and the supernatant transferred to another tube, which was labeled and subsequently frozen. The PCRs were performed using primers derived from the small circle kDNA sequence, expressed in Degraeve et al. (1994) and Passos et al. (1996).

The experimental protocol used was that of Marques et al. (2001). Gels were fixed for 10 min (15 ml ethanol PA solution + 750 μ l acetic acid + 150 ml MQ water); then stained with 0.2% silver (Santos et al., 1973).

2.2. Infectivity of *Leishmania* found in *R. sanguineus*

To determine the infectivity of parasites encountered in the ticks, 36 hamsters were sorted into groups of two in 18 cages from May to August 2001.

Several dogs positive by IFAT for *L. chagasi* were made available for euthanasia at the laboratories of the Centro de Controle de Zoonoses (CCZ) in Belo Horizonte and Sabará (Minas Gerais). These animals were immobilized according to the methodology already described and determine the quantity of ectoparasites, only those bearing more than 10 ticks being included in the study. Blood samples were taken from the dogs to confirm serological diagnoses based on IFAT and TRALd (Camargo and Robonato, 1969; Burns et al., 1993).

The ectoparasites were identified, counted and macerated in a tissue grinder. The macerate was diluted in 2 ml of 0.9% saline solution to which was added potassic penicillin G (5,000,000 u) and streptomycin.

Each tick homogenate was removed with a syringe and injected into four hamsters, two peritoneally and two orally, the latter after removal of the needle. A total of nine dogs positive for ticks were used in the experiment.

After approximately 6 months all the hamsters were necropsied (December 2001–January 2002). Portions of their viscerae (spleen and liver) were used to prepare slide smears and to provide material for PCR. Blood samples were also collected for serological diagnostic (IFAT). Signs of possible visceral abnormalities were noted.

The chi-square test corrected by Yates was used to evaluate the degree of association between infection and factors considered.

3. Results

3.1. Natural infection of *R. sanguineus* by *L. chagasi*

A total of 39 specimens (6 females, 11 males and 22 nymphs) of *R. sanguineus* were removed from 21 dogs showing diverse symptoms of ZVL (Mancianti et al., 1988). Six of these ticks were positive for the genus *Leishmania*, giving an infection rate of 15.4% by PCR.

Table 1
Determination of infectivity of *R. sanguineus* by *Leishmania* sp using PCR

<i>R. sanguineus</i>	Hamsters		
	Analyzed	Infected	
		Number	%
Males	11	–	–
Females	6	2	33.3
Nymphs	22	4	18.2
Total	39	6	15.4

However, none of them presented trypanosomatids in slide smears.

Two of the positive ticks were females (33.3%) and four nymphs (18.1%). With respect to the total number of examined and infected 5.1% were females and 10.2% nymphs (Table 1).

The positive ticks were collected on different dogs, two of the infected nymphs being found on the same animal. Thus, six (28.6%) of the dogs were infective for their ticks.

3.2. Infectivity of *Leishmania* found in *R. sanguineus*

Two hamsters (one inoculated peritoneally and the other orally with material from the same dog) died before the end of the experiment. Thus, 34 hamsters in all were necropsied.

Of the nine dogs, eight (88.9%) presented ticks that were infective for 14 hamsters (41.2%); 12 of these were infected peritoneally (85.7%) and two (14.3%) orally. A total of 68 slide smears were prepared, 34 each from spleen and liver. The number and percentage of positive hamsters after inoculation are shown in Table 2.

Intraperitoneal inoculations produced the most significant positive results: 12 (70.6%) slides of spleen and eight (47.0%) in the liver, totaling 12 (35.2%) hamsters infected. Eight of these harbored infections both in the liver and the spleen, while in the other four parasites were only seen in the latter (12%). None of the hamsters inoculated peritoneally presented infections in the liver alone. With regard to oral inoculations only one slide from spleen and one other from liver (from two different animals) gave positive results.

Diagnoses based on molecular biological (Table 3) and serological techniques were also used to show

Table 2
Results of smears from liver and spleen of hamsters inoculated peritoneally and orally by macerates of ticks removed from dogs positive for *Leishmania chagasi*, Belo Horizonte, Minas Gerais, 2001

Smears	Examined number	Positive	
		Number	%
Hamsters with inoculated peritoneally			
Spleen	17	12	70.6 ^b
Liver	17	8	47.0 ^a
Total	34	20	58.8 ^c
Hamsters with inoculated orally			
Spleen	17	1	5.9 ^b
Liver	17	1	5.9 ^a
Total	34	2	5.9 ^c
Total	68	22	32.4

^a χ^2 (Yates) = 5.44; $P < 0.01$.

^b χ^2 (Yates) = 12.45; $P < 0.01$.

^c χ^2 (Yates) = 19.40; $P < 0.01$.

infection in hamsters after inoculated of tick macerates (Table 4).

IFAT results for the dogs varied from 1:40 to 1:40960. The only dog whose ticks did not infect the hamsters presented a titre of 1:40. The total number of ticks macerated was 233, varying from 10 to 48 per inoculum in each experiment (Table 5).

Table 3
Results of PCR analyses of liver and spleen fragments of hamsters inoculated peritoneally and orally with macerates of ticks removed from ZVL-positive dogs during 2001 in Belo Horizonte, Minas Gerais

Smears	Examined numbers	Positive	
		Numbers	%
Hamsters with inoculated peritoneally			
Spleen	16	11	68.7
Liver	16	9	56.2
Total	32	20	62.5 ^a
Hamsters with inoculated orally			
Spleen	17	2	11.8
Liver	17	5	29.4
Total	34	7	20.6 ^a
Total	66	27	40.9

^a χ^2 (Yates) = 12.07; $P < 0.01$.

Table 4
Serum IFAT titres in hamsters inoculated peritoneally or orally with macerates of *R. sanguineus*, removed from ZVL-positive dogs during 2001 in Belo Horizonte, Minas Gerais

IFAT			
Inoculation route	Examined number	Positive	
		Number	%
Peritoneally	17	11	64.7 ^a
Orally	17	3	17.6
Total	34	14	41.2

^a χ^2 (Yates) = 5.94; $P < 0.05$.

Based on the combined results of slide smears, PCR and IFAT, 14/34 animals were found to be positive (41.2%) (Table 5).

4. Discussion

Although no experiment had been made to evidence the persistence of leishmanial infections into the ticks, these results confirm and expand on the results of experiments carried out by Blanc and Caminopetros (1930). During the present study, *R. sanguineus* was shown to be susceptible to infection with *L. chagasi* and to be able to carry out it to the experimental host.

PCR is a highly sensitive instrument, allowing diagnosis of leishmaniasis by the detection of parasite DNA. Very light infections of *Leishmania* have been identified in sand flies using hybridization with kDNA

probes (Rogers et al., 1988; Rodgers et al., 1990; Rodriguez et al., 1999). When PCR was used to detect *Leishmania* in *R. sanguineus* during the present study an infection rate of 15.4% was obtained for the ticks examined (Table 1). Examination of slide smears was a less sensitive technique, the relatively large volume of blood ingested by the ticks making detection of the parasite difficult.

Significant differences were encountered when infections of the liver and spleen by different routes of inoculation were compared ($\chi^2 = 5.44$ and 12.45, respectively at $P < 0.01$). Intraperitoneal inoculation was more significant both for inoculation in the spleen and liver, as demonstrated in smears ($\chi^2 = 19.40$; $P < 0.01$), PCR ($\chi^2 = 12.07$; $P < 0.01$) and IFAT ($\chi^2 = 5.94$; $P < 0.05$) (Tables 2–4).

Peritoneal inoculation produced an infection rate ranging from 58.8 to 64.7% in function of different diagnoses, confirming the presence of *L. chagasi* and guaranteeing its infectivity at the moment of inoculation.

The number of ticks macerated and inoculated did not follow a progression, i.e., the greatest number of ticks inoculated did not produce the highest rate of infection for the hamsters (Table 5). However, the macerates containing between 10 and 20 ticks caused 50% of infection in hamsters; those prepared with 21–31 ticks infected 60% of the animals and others including more than 32 ectoparasites provided 75% of infection when inoculating (Table 5).

Although the correlation between the titres of IFAT of dogs and the rates of infection for the hamsters was

Table 5
Relationship between IFAT of nine dogs and the number of hamsters positive and smears for *Leishmania chagasi* inoculated with macerates of ticks removed from dogs brought to laboratories of the Centro de Controle de Zoonoses (CCZ) in Belo Horizonte and Sabará, May–August 2001

IFAT of dogs	Ticks number	Smears positive ($N = 68$)	PCR-positive ($N = 66$)	IFAT of hamsters ($N = 34$)	Hamsters-positive ($N = 34$)
1:40	17	–	–	–	–
1:1280	24	1	2	2	2
1:1280	32	1	4	1	2
1:2560	22	5	5	2	3
1:10240	25	2	2	1	1
1:20480	10	2	3	1	2
1:20480	14	4	4	3	4
1:40960	41	2	1	1	2
1:40960	48	5	6	3	4
Total	233	22	27	14	20

not progressive, the highest infectivity was produced using a titre of 1:40960, revealed by 14 positive tests (Table 5). When analyzed by groups at the level of 1:10240, IFATs with titres greater than or equal to of this value were more infective to the hamsters (72.2%) than those with lower titres (43.8%) without significant differences (Table 5).

Because the promastigotes were not found in slide blood smears the development of *Leishmania* in ticks cannot be comprovod. However, when grooming dogs remove ticks and other ectoparasites with their teeth. Also when fondling some dogs lick others increasing the chances of ingesting tick gut contents. Thus, the infection rate of 5.9–29.4% (Tables 2–4) produced by oral infections in the present study indicates that grooming and ingestion of ticks or licking of sores and tick gut contents might represent an important manner of infection by *L. chagasi*. In addition, the habit by male ticks of changing hosts frequently could also favor the spread of ZVL to healthy dogs, in spite of the infections in hamsters have not been produced by macerated male ticks in view of the small sample analyzed in this study (Table 1). In summary, the results of the present study indicate that ticks might be an alternative vector for the transmission of *L. chagasi* between dogs, opening new perspectives in the epidemiology of ZVL. The vectorial capacity of *R. sanguineus* for this parasite thus needs to be evaluated in relation to the other important factors as the persistence, viability and multiplication of the parasites into the gut of the ticks, since: (a) *R. sanguineus* is a three-host tick with a broad distribution in Brazil; (b) experimentally, it has been shown to be capable of transmitting the causative agent of ZVL; (c) the parasite persists in ticks after moulting from larvae to nymphs and from nymphs to adults (Blanc and Caminopetros, 1930); (d) flagellate forms can be found in the gut of this tick (Sherlock, 1964); (e) peritrophic matrix has been demonstrated in the gut of feeding larvae, nymphs and adults of the ticks *Ixodes dammini* (Rudzinska et al., 1982) and *I. ricinus* (Zhu et al., 1991).

Although prior authors have experimentally transmitted *L. chagasi* in animals using ticks, this is the first time that the oral transmission of that parasite by *R. sanguineus* is noticed.

Finally, it is important to point out that experimental transmission to dogs by live ticks would be

conclusive to demonstrate the role of these arthropods in the transmission of visceral leishmaniasis.

Acknowledgements

This research is part of the Ph.D. thesis of M.T.Z.C. in Parasitology/Programa de Pós-graduação em Parasitologia/Instituto de Ciências Biológicas/Universidade Federal de Minas Gerais/UFMG. P.M.L. is a research fellow from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Brasil). This project received partial support from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), CNPq/Brasil and Fundação Nacional de Saúde (FUNASA). We thank the members of Control Center of Zoonosis of Belo Horizonte-MG, Sabará-MG, Montes Claros-MG and Ana Maria Salvador Pereira, Roberto Teodoro Costa, João Carlos França Silva and Elaine Matias do Amaral for technical assistance. Maria Norma Melo and Nelder F. Gontijo for suggestions and comments, as well as Ivan Barbosa Machado Sampaio for help with statistical analysis.

References

- Alencar, J.E., Pessoa, E.P., Costa, O.R., 1962. Calazar em Santarém, Estado do Pará. Rev. Brasil. Malariol. D Trop. 14, 371–377.
- Beard, C.B., Butler, J.F., Orshar, E.C., 1989. Growth characterization and host parasite relationship of *Leptomonas pulexsimulantis* n. sp., tripanosomatid flagellate of the flea *Pulex simulans*. J. Parasitol. 75, 658–668.
- Blanc, G., Caminopetros, J., 1930. La transmission du Kala – Azar méditerranéen par une tique: *Rhipicephalus sanguineus*. C. R. Acad. Sci. 191, 1162–1164.
- Burns Jr., J.M., Shreffler, W.G., Benson, D.R., Ghalib, H., 1993. Molecular characterization of a kinesin related antigen of *L. chagasi* that detect specific antibody in African and American visceral leishmaniasis. Proc. Natl. Acad. Sci. U.S.A. 90, 775–779.
- Camargo, E.M., Robonato, C., 1969. Cross reactivity in fluorescence tests for *Trypanosoma* and *Leishmania* antibody. A single inhibition procedure to ensure specific results. Am. J. Trop. Med. Hyg. 18, 500–501.
- Corredor, A., Gallego, J.F., Tesh, R.B., Morales, A., Carrasquilla, C.F., Young, D.G., Kreutzer, R.D., Boshell, J., Palau, M.T., Caceres, E., Pelaez, D., 1989. Epidemiology of visceral leishmaniasis in Colombia. Am. J. Trop. Med. Hyg. 40, 480–486.

- Costa, C.H.N., Gomes, A.C.G., Costa, J.M.L., Vieira, J.B.F., Lima, J.W.O., Dietz, R., 2001. Changes in the control program of visceral leishmaniasis in Brazil. *Rev. Soc. Bras. Med. Trop.* 34, 223–228.
- Deane, L.M., 1958. Epidemiologia e profilaxia do calazar americano. *Rev. Brasil. Malariol. D Trop.* 10, 431–450.
- Degrave, W., Fernandes, O., Campbell, D., Bozza, M., Lopes, U., 1994. Use of molecular probes and PCR for detection and typing of *Leishmania*—a mini review. *Mem. Inst. Oswaldo Cruz* 89, 463–469.
- Desjeux, P., 1991. Information on the epidemiology and control of the leishmaniasis by country and territory. Document WHO/LEISH/91.30, pp. 1–47.
- Feliciangeli, M.D., Rodríguez, N., de Guglielmo, Z., Rodríguez, A., 1999. The re-emergence of American visceral leishmaniasis in an old focus in Venezuela. II. Vectors and parasites. *Parasite* 6, 113–120.
- Ferro, C., Morrison, A.C., Torres, M., Prado, R., Wilson, M.L., Tesh, R.B., 1995. Age structure, blood-feeding behavior, and *Leishmania chagasi* infection in *Lutzomyia longipalpis* (Diptera: Psychodidae) at an endemic focus of visceral leishmaniasis in Colombia. *J. Med. Entomol.* 32, 618–625.
- Friedhoff, K.T., 1990. Interaction between parasites and tick vector. *Int. J. Parasitol.* 20, 525–535.
- Giraud, P., Ranque, J., Cabassu, H., 1954. Epidemiologie de la leishmaniose viscérale humaine méditerranéenne, en particulier dans ses rapports avec la leishmaniose canine. *Arch. Fr. Ped.* 11, 337–353.
- Hoare, C.A., 1972. *The Trypanosomes of Mammals*. Blackwell Scientific Publication, Oxford, p. 749.
- Jenkins, D.W., 1964. Pathogens, parasites and predators of medically important arthropods. *Bull. WHO* 30, 79–89.
- Kettle, D.S., 1995. *Medical and Veterinary Entomology*. Cab International, Cambridge, p. 725.
- Lainson, R., 1983. The American leishmaniasis: some observations on their ecology and epidemiology. *Trans. R. Soc. Trop. Med. Hyg.* 77, 569–596.
- Lainson, R., Shaw, J.J., 1987. Evolution, classification and geographical distribution. In: Peters, W., Killick-Kendrick, R. (Eds.), *The Leishmaniasis in Biology and Medicine, Biology and Epidemiology*. Academy Press, New York, pp. 1–120.
- Lainson, R., Shaw, J.J., Ryan, L., Ribeiro, R.S.M., Silveira, F.T., 1985. Leishmaniasis in Brazil: XXI. Visceral leishmaniasis in the Amazon Region and further observations on the role of *Lutzomyia longipalpis* (Lutz and Neiva, 1912) as the vector. *Trans. R. Soc. Trop. Med. Hyg.* 79, 223–226.
- Linardi, P.M., Nagem, R.L., 1973. Pulicídeos e outros ectoparasitos de cães de Belo Horizonte e municípios vizinhos. *Rev. Brasil. Biol.* 33, 529–538.
- Mancianti, F., Gramiccia, M., Grandoni, L., Pieri, S., 1988. Studies on canine leishmaniasis control. I Evolution of infection of different clinical forms of canine leishmaniasis following antimonial treatment. *Trans. R. Soc. Trop. Med. Hyg.* 82, 566–567.
- Marques, M.J., Volpini, A.C., Genaro, O., Mayrink, W., Romanha, A.J., 2001. A simple form of clinical sample preservation and *Leishmania* DNA extraction from human lesion for the diagnosis of American cutaneous leishmaniasis by polymerase chain reaction (PCR). *Am. J. Trop. Med. Hyg.* 65, 902–906.
- Passos, V.M.A., Lasmaz, E.B., Gontijo, C.M.F., Fernandes, O., Degrave, W., 1996. Natural infection of a domestic cat (*Felis domesticus*) with *Leishmania (Viannia)* in the metropolitan region of Belo Horizonte, State of Minas Gerais. *Brazil. Mem. Inst. Oswaldo Cruz* 9, 19–20.
- Profeta, Z., Fiuza, V.O.P., Rabello, A., 2003. Leishmaniose: uma doença em expansão. http://www.cpqrr.fiocruz.br/laboratorio/lab_pclin/1 >03/02/2003.
- Richardson, N.F., Kendall, S.B., 1963. *Veterinary Protozoology*. Oliver and Boyd, Edinburg.
- Rodgers, M.R., Popper, S.J., Wirth, D., 1990. Amplification of kinetoplast DNA as a tool in the detection and diagnosis of *Leishmania*. *Exp. Parasitol.* 71, 267–275.
- Rodriguez, N., Aguilar, C.M., Barrios, M.A., Barker, D.C., 1999. Detection of *Leishmania brasiliensis* in naturally infected individual sandflies by the polymerase chain reaction. *Trans. R. Soc. Trop. Med. Hyg.* 93, 47–49.
- Rogers, W.O., Burnheim, P.F., Wirth, D.F., 1988. Detection of *Leishmania* within sandflies by kinetoplast DNA hybridization. *Am. J. Trop. Med. Hyg.* 39, 434–439.
- Rudzinska, M.A., Spielman, A., Lewengrub, S., Piesman, J., Karakashian, S., 1982. Penetration of the peritrophic membrane of the tick by *Babesia microti*. *Cell Tissue Res.* 221, 471–481.
- Ryan, L., Silveira, F.T., Lainson, R., Shaw, J.J., 1984. Leishmanial infections in *Lu. longipalpis* and *L. antunesi* (Diptera: Psychodidae) on the island of Marajó, Pará State. *Brazil. Trans. R. Soc. Trop. Med. Hyg.* 78, 547–548.
- Santos, F.R., Pena, S.D.J., Eppelen, J.T., 1973. Genetic and population study of a Y-linked tetranucleotide repeat DNA polymorphism with a simple non-isotopic technique. *Hum. Genet.* 90, 655–656.
- Sherlock, I.A., 1964. Nota sobre a transmissão da leishmaniose visceral no Brasil. *Rev. Brasil. Malariol. D Trop.* 16, 19–26.
- Sherlock, I.A., 1999. Ecological interactions of visceral leishmaniasis in the State of Bahia. *Brazil. Mem. Inst. Oswaldo Cruz* 91, 671–683.
- Walker, A., 1994. *The Arthropods of Humans and Domestic Animals. A Guide to Preliminary Identification*. Chapman & Hall, London.
- Wallace, F.G., 1966. The trypanosomatid parasite of insects and arachnids. *Exp. Parasitol.* 18, 124–193.
- Williams, P., 1976. Flagellate infection in cave-dwelling sandflies (Diptera, Psychodidae) in Belize, Central America. *Bull. Ent. Res.* 65, 615–629.
- Zhu, Z., Gern, L., Aeschlimann, A., 1991. The peritrophic membrane of *Ixodes ricinus*. *Parasitol. Res.* 77, 635–641.