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Short communication

Ectoparasites and anti-*Leishmania* antibodies: Association in an observational case–control study of dogs from a Brazilian endemic area

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ABSTRACT

It has been proposed that the transmission of canine visceral leishmaniasis might involve the participation of mechanical vectors, including ticks of the family Ixodidae, in particular the brown dog tick *Rhipicephalus sanguineus*, and the cat flea *Ctenocephalides felis felis*. Here, the association between the infestation by *R. sanguineus* and *C. felis felis* and the occurrence of anti-*Leishmania* antibodies was evaluated in an observational case–control study of dogs living in a Brazilian endemic area for canine visceral leishmaniasis. Blood samples were taken once every three months for one year from 96 initially seronegative domestic dogs, and submitted to indirect immunofluorescence antibody assay. All dogs were evaluated for the presence of ticks and fleas, and the results were expressed qualitatively as infested or non-infested, irrespective of the intensity of infestation. At the end of follow-up, twenty dogs had turned seropositive, while 68 remained seronegative and 8 were excluded because of incomplete data. All the dogs were asymptomatic. The odds of infection was significantly greater (OR = 3.54, CI95% = 1.10–12.53) for dogs infested by *C. felis felis* compared to their non-infested counterparts. In contrast, the odds of infection showed no significance difference between non-infested and *R. sanguineus*-infested groups of dogs (OR = 0.31, CI95% = 0.03–1.52). This study provides further evidence for the potential role of *C. felis felis* in mechanically transmitting *Leishmania* among the canine population.

1. Introduction

Zoonotic visceral leishmaniasis is a severe vector-borne parasitic disease caused by *Leishmania infantum* (syn. *L. chagasi*), a protozoan widely distributed in the South American continent, with 90% of cases occurring in Brazil (Bern et al., 2008). Dogs are reported as the main reservoir of visceral leishmaniasis, with its occurrence preceding human cases of infection (Desjeux, 2003). In South America, the natural transmission of *L. infantum* occurs by the bite of the phlebotomine sand fly *Lutzomyia longipalpis* (Diptera: Psychodidae), although it has been proposed that mechanical vectors, such the brown dog tick *Rhipicephalus sanguineus* and the cat flea *Ctenocephalides felis felis*, might also be involved in the epidemiology of canine visceral leishmaniasis (CVL) (Coutinho et al., 2005; Coutinho and Linardi, 2007; Ferreira et al., 2009; Paz et al., 2010a,b). This hypothesis is based on the following observations: (i) the rate of infection of *L. longipalpis* by *L. infantum* is generally low (0.28% in Venezuela; 0.29–0.90% in Colombia; and generally ≤0.5% in

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Brazil) (Montoya-Lerma et al., 2003); (ii) the prevalence of CVL in endemic regions is very high (Malaquias et al., 2007; Michalsky et al., 2007; Nunes et al., 2008); (iii) the occurrence of natural infection by *Leishmania*-like flagellates, or of the DNA of *Leishmania* sp. in *C. felis felis* and *R. sanguineus* (Machattie and Chadwick, 1930; Sherlock, 1964; Coutinho et al., 2005; Coutinho and Linardi, 2007; Silva et al., 2007; Ferreira et al., 2009); (iv) the persistence of *L. infantum* kinetoplast DNA (kDNA) in unfed nymphs and adults of *R. sanguineus* that, before molting, had previously fed on infected dogs (Paz et al., 2010a,b); (v) in a case–control study, a strong association between the prevalence of infestation by *C. felis felis* and *R. sanguineus* and the presence of anti-*Leishmania* antibodies in dogs (Paz et al., 2010a,b). The aim of the present study was to evaluate the association between the infestation by *C. felis felis* and *R. sanguineus*, and the appearance of anti-*Leishmania* antibodies in an observational case–control study of dogs living in a Brazilian endemic area for CVL.

2. Materials and methods

The study was submitted to, and approved by, the Ethics Committee for Research with Animals of the Oswaldo Cruz Foundation (CEUA/Fiocruz) under protocol number LW-76/12. All procedures involving experimental animals were conducted according to the guidelines of the Brazilian College for Experiments with Animals (Colégio Brasileiro de Experimentação Animal/COBEA).

The observational case–control study was conducted during one year (August 2011 to August 2012) in an endemic area for CVL located to the northeast of the city of Belo Horizonte, state of Minas Gerais, Brazil. In order to identify domestic dogs for enrollment into the observational study that were initially seronegative for anti-*Leishmania* antibodies, blood samples were collected from a total of 338 dogs, corresponding to almost every resident dog present in the study area (total population 340; unpublished data from the Secretaria Municipal de Saúde de Belo Horizonte). An initial screening for the presence of anti-*Leishmania* antibodies in canine sera was performed using enzyme-linked immunosorbent assays (ELISA-Bio-Manguinhos® kit). Samples with absorbance values equal to, or higher than, 3 times the standard deviation of the cut-off value were considered positive. These samples were then submitted to indirect immunofluorescence antibody assays (IFAT-Bio-Manguinhos® kit), and those with fluorescence at a 1:40 dilution were confirmed to be positive. Both assays were performed according to the manufacturer's instructions and the recommendations of the Brazilian Ministry of Health (Ministério da Saúde do Brasil, 2006). The specificity of ELISA and IFAT assays for CVL is between 86–100% and 100%, respectively. The sensitivity varies in the range of 71–98% for ELISA and 72–100% for IFAT, when the parasitological method is taken as reference (Romero and Boelaert, 2010). In the initial enrollment screening, a total of 283 (83.7%) dogs were seronegative for both ELISA and IFAT. Among these seronegative dogs, 96 dogs were randomly selected for the follow-up study during the next 13 months. One month after the initial screening, serological testing of the 96 selected dogs was performed by IFAT every three months for 1 year: days 0, 90, 180, 270 and 360 after the start of the observational study. Hypothetically, this would result in 40 seropositive dogs after the fifth serological test (day 360), if we consider a *Leishmania* spp. incidence of 0.10. This would allow us to detect a minimum difference of 16% between the proportions of infestation by ticks and fleas in the two populations using a 95% confidence interval for this difference. The prevalence of the infestation by fleas and ticks in seropositive and seronegative populations were considered to be 0.385 and 0.290, respectively (Paz et al., 2010a,b), and the initial population of dogs in the area 340.

All the dogs enrolled in the study were inspected every 30 days, starting from day 0, for ectoparasites until seroconversion, after which the dogs were no longer examined for ticks and fleas. For example, in the first serological test of the observational study (day 0), one month after the initial screening, 12 dogs tested seropositive. Thus, the inspection for ectoparasites was performed only on day 0 for this group of dogs. In the second serological test (day 90), when six dogs tested seropositive, the inspection for ectoparasites was performed on days 0, 30, 60 and 90. In the third serological test (day 180), two dogs tested seropositive, the inspection for ectoparasites was performed every 30 days until day 180, etc.

Inspection was performed as following: muzzle and cheek, ears, neck, back, flanks, under the chest and within the paws, especially in the spaces between the toes. During the inspection procedure, 50 samples of fleas and ticks were randomly collected throughout the body of the infested animals, and transferred to tubes containing 70% ethanol for subsequent identification. The ticks and fleas collected during examination of the study animals were unambiguously identified as *R. sanguineus* and *C. felis felis* (Aragão and Fonseca, 1961; Linardi and Guimarães, 2000). Dogs were considered infested if they had at least one stage of the ectoparasites during the examination.

The results of the examinations for ectoparasites were expressed qualitatively as either infested or non-infested, independent of the intensity of infestation. Among the 76 seronegative dogs, 8 were not evaluated in some of the inspections, and hence were removed from the group of seronegative dogs giving a final number of 68 seronegative dogs included in the analysis. In the analysis comparing the infestation with ectoparasites, we included the data from all of the examinations of seropositive dogs, but only the data from the examinations conducted between days 120 and 180 inclusive for the seronegative dogs. Comparisons of the proportions of seronegative and seropositive dogs for each variable investigated were performed using Pearson's χ^2 or Fisher's exact tests, depending on the sample sizes of each group. Associations between the presence of anti-*Leishmania* antibodies and infestation by ticks and fleas were evaluated by means of odds ratios (OR) and their respective 95% confidence intervals (CI95%). Owners of the selected households were interviewed using a semi-structured questionnaire covering questions on the sex of the dogs, whether the dog is castrated or not, their type of fur, animal insecticide use, environment insecticide use and walked by the owner on the street.

Table 1
Proportion of dogs ($n = 96$) seropositive and seronegative for anti-*Leishmania* antibodies according to the variables listed. The *P*-value shown is for Pearson's Chi-square test, unless otherwise indicated.

Variable	Seropositive, *n* (%)	Seronegative, *n* (%)	*P*-value
Sex			
Male	6 (30.0)	34 (44.7)	0.350
Female	14 (70.0)	42 (55.3)	
Animal insecticide use			
Yes	9 (45.0)	42 (55.3)	0.571
No	11 (55.0)	34 (44.7)	
Environment insecticide use			
Yes	5 (25.0)	24 (31.6)	0.767
No	15 (75.0)	52 (68.4)	
Dogs walked by their owners in street			
Yes	6 (30.0)	18 (23.7)	0.772
No	14 (70.0)	58 (76.3)	
Fur type			
Short-haired breeds	10 (50.0)	33 (43.4)	0.784
Long-haired breeds	10 (50.0)	43 (56.6)	
Castrated[a]			
Yes	4 (21.1)	11 (14.6)	0.495[b]
No	15 (78.9)	64 (85.3)	

[a] No data available for two dogs.
[b] Fisher's exact test.

3. Results

During the initial screening of dogs living in the study area, a total of 33 (9.8%; $n = 338$) of those tested were seropositive for anti-*Leishmania* antibodies as indicated by ELISA and IFAT tests, while a further 22 (6.5%) were seropositive only by ELISA, and 283 (83.7%) were seronegative for both tests. Among the randomly selected seronegative animals ($n = 96$) included in the observational study, 20 were seropositive by the end of follow-up (cumulative incidence of 20.8% in twelve months). After day 180, blood samples were collected from all 68 seronegative animals on days 270 and 360, and all remained seronegative. At the end of follow-up, all the dogs were also asymptomatic. For all the variables studied except the ectoparasite infestation, no significant difference was detected between the groups of seropositive and seronegative dogs (Table 1).

The odds of infection (i.e., conversion from seronegative to seropositive for anti-*Leishmania* antibodies) was significantly greater for dogs infested by *C. felis felis* compared with their non-infested counterparts (OR = 3.54, CI95% = 1.10–12.53). However, the odds of infection showed no significant difference between the non-infested and *R. sanguineus*-infested groups of dogs (OR = 0.31, CI95% = 0.03–1.52) (Table 2).

4. Discussion

Fleas and ticks are the principal ectoparasites of dogs throughout the world, causing annoyance to the animals and acting as vectors of diseases (Rust and Dryden, 1997). In the city of Belo Horizonte *R. sanguineus* and *C. felis felis* are the most prevalent species of ectoparasites and therefore of potentially of greatest epidemiological importance (Linardi and Nagem, 1973; Paz et al., 2008). Recently, these ectoparasites have been mentioned as possible mechanical vectors of *L. infantum*, representing a potential alternative route for the transmission of *L. infantum* between dogs (Coutinho et al., 2005; Coutinho and Linardi, 2007; Ferreira et al., 2009; Paz et al., 2010a,b).

A previous case–control study found that the probability of seropositivity for *Leishmania* was 53% higher in tick-infested dogs and 300% higher in flea-infested dogs in comparison with non-infested animals (Paz et al., 2010a,b). The present observational study showed a statistical association only between flea infestation and the risk of visceral leishmaniasis in dogs. This result together with the presence of *Leishmania*-like trypanosomatids and *Leishmania* DNA in (or on) fleas (Coutinho and Linardi, 2007; Ferreira et al., 2009) reinforces the possibility of mechanical transmission of *L. infantum* by fleas. Characteristics of the biology and behavior of the flea *C. felis felis*, such as their voracity and feeding frequency (Bibikova, 1977), their remarkable capacity for exchange between hosts (Rust, 1994), together with their feeding behavior, prompts dogs to carry out grooming, facilitating the ingestion of flea gut contents by the dog (Dryden and Gaafar, 1991; Hinkle et al., 1998), which could represent an alternative route for the transmission of *L. infantum* (Coutinho and Linardi, 2007). This study provides further evidence for the potential role of *C. felis felis* in the mechanical transmission of *Leishmania* among the canine population, requiring further research aimed at assessing how the ability of *C. felis felis* to transmit *L. infantum* between different host dogs, and its relationship to the intensity of ectoparasite infestation, might affect their potential participation in the epidemiology of canine visceral leishmaniasis.

Table 2
Association between infestation by *Rhipicephalus sanguineus* and *Ctenocephalides felis felis* and the presence of anti-*Leishmania* antibodies in dogs from an area of Brazil that is endemic for visceral leishmaniasis. The data collected after day 180 were excluded from the analysis.

Ectoparasite infestation	Seropositive dogs, *n* (%)	Seronegative dogs, *n* (%)	Odds ratio	95% confidence interval	*P*-value
C. felis felis					
Yes	14 (70.0)	27 (39.7)	3.54	1.10–12.53	0.0222
No	6 (30.0)	41 (60.3)	1.00		
Total	20	68			
R. sanguineus					
Yes	2 (10.0)	18 (26.5)	0.31	0.03–1.52	0.1435
No	18 (90.0)	50 (73.5)	1.00		
Total	20	68			

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References

Aragão, H.B., Fonseca, F., 1961. Notas de Ixodologia VIII. Lista e chave para os representantes da fauna ixodológica brasileira. Mem. Inst. Oswaldo Cruz 59, 115–129.

Bern, C., Maguire, J.H., Alvar, J., 2008. Complexities of assessing the disease burden attributable to leishmaniasis. Plos. Negl. Trop. Dis. 2, e313.

Bibikova, V.A., 1977. Contemporary views on the interrelationships between fleas and pathogens of human and animal diseases. Annu. Rev. Entomol. 22, 23–32.

Coutinho, M.T., Linardi, P.M., 2007. Can fleas from dogs infected with canine visceral leishmaniasis transfer the infection to other mammals? Vet. Parasitol. 147, 320–325.

Coutinho, M.T.Z., Bueno, L.L., Sterzik, A., Fujiwara, R.T., Botelho, J.R., De Maria, M., Genaro, O., Linardi, P.M., 2005. Participation of *Rhipicephalus sanguineus* (Acari: Ixodidae) in the epidemiology of canine visceral leishmaniasis. Vet. Parasitol. 128, 49–155.

Desjeux, P., 2003. Leishmaniasis. Ann. Trop. Med. Parasitol. 97 (1), 3–15.

Dryden, M.W., Gaafar, S.M., 1991. Blood consumption by the cat flea, *Ctenocephalides felis* (Siphonaptera: Pulicidae). J. Med. Entomol. 28, 394–400.

Ferreira, M.G.P.A., Fattori, K.R., Souza, F., Lima, V.M.F., 2009. Potential role for dog fleas in the cycle of *Leishmania* spp. Vet. Parasitol. 165, 150–154.

Hinkle, N.C., Koehler, P.G., Patterson, R.S., 1998. Host grooming efficiency for regulation of cat flea (Siphonaptera: Pulicidae) populations. J. Med. Entomol. 35, 266–269.

Linardi, P.M., Nagem, R.L., 1973. Pulicídeos e outros ectoparasitos de cães de Belo Horizonte emunicípios vizinhos. Rev. Bras. Biol. 33, 529–538.

Linardi, P.M., Guimarães, L.R., 2000. Sifonápteros do Brasil. Museu de Zoologia USP/FAPESP, São Paulo, pp. 291.

Machattie, C., Chadwick, C.R., 1930. Notes on a trypanosome infection of the dog tick *Rhipicephalus sanguineus* in Iraq. Trans. R. Soc. Trop. Med. Hyg. 23, 417–420.

Malaquias, L.C., Do Carmo, R.R., Do Anjos Jr., J.B., Giunchetti, R.C., Corrêa-Oliveira, R.C., Reis, A.B., 2007. Serological screening confirms the re-emergence of canine leishmaniosis in urban and rural areas in Governador Valadares, Vale do Rio Doce, Minas Gerais, Brazil. Parasitol. Res. 100, 233–239.

Michalsky, E.M., Rocha, M.F., Da Rocha Lima, A.C., França-Silva, J.C., Pires, M.Q., Oliveira, F.S., Pacheco, R.S., Dos Santos, S.L., Barata, R.A., Romanha, A.J., Fortes-Dias, C.L., Dias, E.S., 2007. Infectivity of seropositive dogs, showing different clinical forms of leishmaniasis, to *Lutzomyia longipalpis* phlebotomine sand flies. Vet. Parasitol. 147, 67–76.

Ministério da Saúde do Brasil, 2006. Manual de Vigilância e Controle da Leishmaniose Visceral. Editora do Ministério da Saúde, Brasília, Available from: http://dtr2001.saude.gov.br/editora/produtos/livros/popup/manual_vigilancia_controle_leishmaniose_visceral.html

Montoya-Lerma, J., Cadena, H., Oviedo, M., Ready, P.D., Barazarte, R., Travi, B.L., Lane, R.P., 2003. Comparative vectorial efficiency of *Lutzomyia evansi* and *L. longipalpis* for transmitting *Leishmania chagasi*. Acta Trop. 85, 19–29.

Nunes, C.M., Lima, V.M., Paula, H.B., Perri, S.H., Andrade, A.M., Dias, F.E., Burattini, M.N., 2008. Dog culling and replacement in an area endemic for visceral leishmaniasis in Brazil. Vet. Parasitol. 6, 19–23.

Paz, G.F., Leite, R.C., Oliveira, P.R., 2008. Control of *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae) in the kennel of the UFMG Veterinary School, Belo Horizonte, Minas Gerais, Brazil. Rev. Bras. Parasitol. Vet. 17, 41–44.

Paz, G.F., Ribeiro, M.F., Michalsky, E.M., da Rocha-Lima, A.C., França-Silva, J.C., Barata, R.A., Fortes-Dias, C.L., Dias, E.S., 2010a. Evaluation of the vectorial capacity of *Rhipicephalus sanguineus* (Acari: Ixodidae) in the transmission of canine visceral leishmaniasis. Parasitol. Res. 106, 523–528.

Paz, G.F., Ribeiro, M.F.B., Magalhães, D.F., Sathler, K.P.B., Morais, M.H., Fiúza, V.O.P., Brandão, S.T., Werneck, G.L., Fortes-Dias, C.L., Dias, E.S., 2010b. Association between the prevalence of infestation by *Rhipicephalus sanguineus* and *Ctenocephalides felis felis* and the presence of anti-*Leishmania* antibodies: a case–control study in dogs from a Brazilian endemic area. Prev. Vet. Med. 97, 131–133.

Romero, G.A., Boelaert, M., 2010. Control of visceral leishmaniasis in Latin America—a systematic review. PLoS Negl. Trop. Dis. 4, e584.

Rust, M.K., 1994. Interhost movement of adult cat fleas (Siphonaptera: Pulicidae). J. Med. Entomol. 31, 486–489.

Rust, M.K., Dryden, M.W., 1997. The biology, ecology, and management of the cat flea. Annu. Rev. Entomol. 42, 451–473.

Sherlock, I.A., 1964. Nota sobre a transmissão da leishmaniose visceral no Brasil. Ver. Bras. Malariol. Doenças Trop. 16, 19–26.

Silva, O.A., Silva, P.B., Silva, O.V., Braga, G.M., Albuquerque Júnior, A., Queiros Neto, V., Rocha, M.E., Silva, E.F., 2007. Canine visceral leishmaniasis in northeast Brazil: epidemiological aspects. Bull. Soc. Pathol. Exot. 100, 49–50.