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Canine visceral leishmaniasis: Incidence and risk factors for infection in a cohort study in Brazil

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ABSTRACT

Zoonotic visceral leishmaniasis in Brazil is caused by *Leishmania infantum* parasites and is transmitted by sand flies of the Phlebotominae family. Dogs are the main urban reservoirs and represent the major source of contagion for the vectors. Studies have shown that most infected dogs are polymerase chain reaction-positive months before seroconversion. Herein, we describe a cohort study designed to identify the incidence of and risk factors for *L. infantum* infection as detected by polymerase chain reaction-restriction fragment length polymorphism. To determine the risk factors for infection, we conducted a baseline canine survey ($n = 1443$) from which dogs were selected for the cohort study ($n = 282$) involving three evaluations over the course of a 26-month follow-up period. Serology, molecular tests, and a structured questionnaire were used. The risk factors for infection were identified by means of the Cox regression model. The overall infection incidence was 5.8 per 100 dog-months (95% confidence interval 5.1–6.5). Increased risk of infection was associated with the presence of previous cases of canine visceral leishmaniasis in the domiciles (hazard ratio [HR] 1.4; 95% confidence interval [CI] 1.1–1.8) and unplastered house walls (HR 3.6; 95% CI 1.6–8.1). These risk factors suggest that insecticide spraying in cracks and crevices in unplastered walls can reduce biting rates within and around homes. Furthermore, our results demonstrate that the Visceral Leishmaniasis Control and Surveillance Program should adopt environmental management measures in homes with previous cases of canine visceral leishmaniasis, because these homes are more likely to maintain the transmission cycle.

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1. Introduction

Visceral leishmaniasis is caused by *Leishmania donovani* or *L. infantum*, protozoan parasites that are transmitted to human and animal hosts by the bite of phlebotomine sand flies (Chappuis et al., 2007). Depending on whether or not a reservoir host is present, there are two basic types of epidemiological cycles: zoonotic, generally caused by *L. infantum*, and anthroponotic, generally caused by *L. donovani* (Palatnik-de-Sousa et al., 2001). Dogs are the main urban reservoirs of *L. infantum* and represent the major source of contagion for the vectors by virtue of the high prevalence of infection and intense cutaneous parasitism (Molina et al., 1994; Giunchetti et al., 2006).

Canine visceral leishmaniasis (CVL) is present in approximately 50 countries, mainly in South America and the Mediterranean region (Solano-Gallego et al., 2009; Dantas-Torres et al., 2012). Epidemiological studies in endemic areas have demonstrated that the prevalence of infection as determined by molecular techniques (50–80%) is higher than the seroprevalence (10–30%) (Solano-Gallego et al., 2009; Wang et al., 2011). A cohort study conducted by Oliva et al. (2006) showed that most of the study animals were polymerase chain reaction (PCR)-positive months before seroconversion. The CVL infection rate depends on several factors including the length of the transmission season, the vector density, the susceptibility of the dog population, dog behavior, the degree of exposure to vectors, and dog owners' attitudes toward prevention (Baneth et al., 2008; Dantas-Torres et al., 2012). Preventing the expansion and urbanization of zoonotic visceral leishmaniasis (ZVL) requires that the risk factors associated with human and canine infection be identified. Some cross-sectional serological surveys have suggested that canine susceptibility to infection is associated with dog size, fur length, age, and housing conditions (Franca-Silva et al., 2003; Almeida et al., 2009; Galvez et al., 2010; Cortes et al., 2012). During a previously reported cross-sectional study carried out in an urban area of Brazil (Belo Horizonte, southeastern Brazil), we evaluated the prevalence of and risk factors associated with *L. infantum* infection in dogs, as identified by means of a molecular test (polymerase chain reaction-restriction fragment length polymorphism, PCR-RFLP) (Coura-Vital et al., 2011b). Some studies have evaluated the risk factors associated with *L. infantum* infection in dogs, however few have used a cohort studies (Belo et al., 2013), which is the most appropriate observational design to establish causal inference. Recently in cohort study we demonstrated that dogs with PCR positive for *L. infantum* showed approximately twice the risk of seroconversion as those that were PCR negative (Coura-Vital et al., 2013). Herein, we report the results of a cohort study designed to identify the incidence of and risk factors for *L. infantum* infection as identified by PCR-RFLP; we evaluated the domiciliary and peridomiciliary environments, the socioeconomic status of the owners, the type of care provided for the animals, and specific animal behaviors.

2. Methods

2.1. Baseline survey (cross-sectional study)

The rationale for and organization of the study and the data collection methods have been described elsewhere (Coura-Vital et al., 2011b). Briefly, a cross-sectional study was carried out in the northwest sanitary district (36,874 km^2) of Belo Horizonte in 2008. According to a census conducted by the Brazilian Institute of Geography and Statistics, the human population of this area was 331,362 in 2000. The canine population comprised 20,883 animals in 2008, according to the Zoonosis Control Management. At the time of this study, the seroprevalence in Belo Horizonte (7.6%) was similar to that in the northwest sanitary district (7.8%) (PBH, 2007). With an expected CVL positivity in the study area between 5% and 10%, a confidence interval (CI) of 95%, and an estimated precision of 1.5%, the required sample size for the study was approximately 1500 animals. A total of 918 households were visited, and 1443 dogs were included in the baseline survey (Coura-Vital et al., 2011b).

2.2. Follow-up cohort study

We then conducted a follow-up cohort study consisting of three evaluations. Evaluation I was conducted 10 months after the baseline survey (April 2009), and a total of 282 seronegative/PCR-negative dogs were enrolled. This number of dogs was similar to the number of PCR-positive dogs in the cross-sectional study. Households with seronegative/PCR-negative dogs were selected by proximity to the seronegative/PCR-positive dogs. The dogs were selected from 222 owners, all of whom were interviewed; all dogs were clinically examined, and blood was collected by venipuncture. Evaluation II was conducted 16 months after the baseline survey (October 2009), and 225 dogs were tested. Evaluation III was carried out 26 months after the baseline survey (August 2010), and 178 dogs were tested. All the dogs included in evaluations II and III were subjected to the same procedures used in evaluation I.

To evaluate loss trends during the cohort study, we compared the sex, size, and fur length of the included dogs with the same variables for the unincluded dogs in each evaluation.

2.3. Data and sample collection

A trained research team interviewed the owners of the study animals using a previously tested structured questionnaire that sought information regarding (i) the owner's knowledge about the human disease (i.e., form of transmission and clinical signs of human visceral leishmaniasis); (ii) the owner's knowledge about the vector (characteristics and presence in the domicile and peridomicile); (iii) the owner's knowledge about the reservoir host (epidemiological importance of the host, clinical signs of CVL, and dog care); (iv) the owner's socioeconomic characteristics (per capita/family income and schooling); (v) the characteristics of the domicile, annexes, and surroundings (i.e., structure of roof, floor, and walls; number of rooms, including

bedrooms; number of residents; and presence of trees [particularly banana trees], rubble, manure, exposed garbage, dry leaves, and a vegetable garden); (vi) the method of garbage disposal (collected, burned, or buried); and (vii) the presence of other domestic animals (birds, cats, and cattle). The following information was collected for each dog: age, sex, size, fur length, breed, behavior (habits related to the place where the dog slept and spent most of its time, e.g., street, residence, or backyard), dog care, clinical examinations, past history of vaccination, and serological exams previous to leishmaniasis.

A sample of peripheral blood (5 mL) was collected by puncture of the brachiocephalic vein, and an aliquot was transferred to a glass vial containing sufficient anticoagulant (ethylenediaminetetraacetic acid) to achieve a final concentration of 1 mg/mL. The blood sample was centrifuged (1500–1800 × *g*; 20 min), and the buffy coat fraction containing the leukocytes was removed, resuspended in an equal volume of 10 mM Tris–HCl buffer (supplemented with 1 mM ethylenediaminetetraacetic acid), and stored at −70 °C until PCR-RFLP. The remaining portion of the blood sample was transferred to two separate filter papers for subsequent enzyme-linked immunosorbent assay (ELISA) analysis.

2.4. ELISA

Each of the eluates from the blood dried on the filter papers was tested by means of two ELISA protocols. The first protocol used a Canine Leishmaniasis EIE Kit (Bio-Manguinhos/Fiocruz, Rio de Janeiro, Brazil), which employs a soluble antigen from promastigote forms of *L. major*-like parasites (ELISA-*L. major*-like). This serology was performed in the Laboratory of Zoonosis of the Belo Horizonte Health Department according to the manufacturer's instructions. The second ELISA protocol was performed in parallel with soluble *L. infantum* (MHOM/BR/1070/BH46) antigen and was carried out in the Laboratory of Immunopathology of the Federal University of Ouro Preto as described by Coura-Vital et al. (2011b).

2.5. PCR-RFLP

DNA was extracted from the buffy coat fractions by means of a Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. The primers used to amplify the conserved region of the *Leishmania* kDNA minicircle were as follows: forward, 5′-GGG (G/T)AG GGG CGT TCT (G/C)CG AA-3′; reverse, 5′-(G/C)(G/C)(G/C) (A/T)CT AT(A/T) TTA CAC CAA CCC C-3′ (Passos et al., 1999). A single PCR product of 120 bp was generated (Degrave et al., 1994). PCR was performed as described by Coura-Vital et al. (2011b).

PCR amplicons (5 μL) were digested for 3 h at 37 °C in 1 U of *Hae*III (Invitrogen, Carlsbad, CA, USA) in 1× buffer (10 mM Tris–HCl, 10 mM $MgCl_2$ [pH 7.5]) and enough Milli-Q water to bring the final volume to 15.0 μL/well (MicroAmp Fast Optical 96-Well Reaction Plate, Applied Biosystems, Foster City, CA, USA) (Volpini et al., 2004). Restriction fragments, together with a 25-bp DNA ladder (Invitrogen, São Paulo, Brazil), underwent electrophoresis in 10% polyacrylamide gels at 40 mA in 89 mM Tris base (pH 8.0), 89 mM boric acid, and 2 mM ethylenediaminetetraacetic acid. Bands were detected by silver staining, and the patterns were compared with those obtained with DNA from *L.* (*L.*) *amazonensis* (strain MHOM/BR/1973/M2269), *L.* (*Viannia*) *braziliensis* (strain MHOM/BR/1975/M2903), and *L.* (*L.*) *infantum* (strain MHOM/BR/1972/BH46) from the DNA reference library at the Laboratory of Immunopathology of the Federal University of Ouro Preto.

2.6. Statistical analysis

Databases were generated with EpiData software (version 3.2, EpiData Association, Odense, Denmark) by double entry of the results and were subsequently corrected, compared, and analyzed with Stata software (version 11.0, StataCorp, College Station, TX, USA). The incidence of *L. infantum* infection as indicated by PCR-RFLP was estimated per 100 dog-months. The Cox proportional hazard model was used to evaluate the risk factors associated with infection by *L. infantum*. Variables that were statistically significant but exhibited colinearity were excluded from the multivariate analysis, and categorical variables were transformed into dummy variables. The univariate analysis was performed by means of Kaplan–Meir survival analysis following by a log-rank test to examine the associations between each variable and time to infection. A multivariable adjusted model was fitted with the variables that were statistically significant at $p < 0.25$ in univariate analyses. A step-by-step backward selection procedure was used to select the variables and to produce the final multivariate regression model. Only adjusted variables showing a significant association ($p < 0.05$) with the occurrence of infection by *L. infantum* remained in the final model. The strength of association was determined by a hazard ratio and a 95% CI. The Schoenfeld test was performed to test the proportional hazards assumption.

2.7. Ethics

The study was approved by the Ethics Committee in Animal Experimentation of the Federal University of Ouro Preto (protocol no. 083/2007), of the Federal University of Minas Gerais (protocol no. 020/2007), and of the Belo Horizonte City Council (protocol no. 001/2008). All procedures in this study were conducted according to the guidelines set out by the Brazilian Animal Experimental College (federal law number 11794). Owners of dogs participating in the project were informed of the research objectives and were required to sign an informed consent form before sample and data collection.

3. Results

3.1. Characteristics of the dogs and housing conditions

The gender, size, and fur length of the dogs in the cohort study (50.0% female, 50.0% medium sized, and 57.1% short fur) were similar to those in the baseline survey. The mean age was 54.3 months (standard deviation [SD] 41.7), the median (interquartile range [IQR]) was 48 months (IQR 24;

Table 1
Characteristics of the dogs in the cohort study ($n = 282$), Brazil 2010.

Variable	No.	%
Gender		
Female	141	50.0
Male	141	50.0
Size		
Small	81	28.7
Medium	141	50.0
Big	60	21.3
Fur length		
Long	161	57.1
Short	121	42.9
Age		
≤24 months	97	34.4
>24 and ≤84 months	123	43.6
>84 months	62	22.0
Origin of the animal		
District of residence	158	56.0
Other district	124	44.0
Dog stayed predominantly in the backyard		
No	35	12.4
Yes	247	87.6
Sleeping place		
Inside the house	46	16.3
In the backyard	236	83.7
Had access to the street		
No	253	89.7
Yes	29	10.3
Veterinary checkups		
Yes	141	50.0
No	141	50.0

72). One hundred twenty-three dogs (43.6%) were between 24 and 84 months. Most of the animals lived and slept in the backyard (87.6 and 83.7%, respectively) rather than inside the residence. Only 29 (10.3%) of the dogs had easy access to the street. Half of the dogs were regularly checked by a veterinarian (Table 1).

A total of 222 households were selected. They had a mean of 1.3 (SD 0.8) dogs per household (1–7 dogs/house) and median of 1 (IQR 1; 1). Most of the dwellings (142; 63.9%) were detached houses, 216 (97.3%) had plastered walls, 183 (82.4%) had a garden, and 100% were served by a sewage main. Garbage was collected at least 3 times per week from 202 (91.8%) residences. The mean numbers of rooms and bedrooms per house were 7.2 (SD 2.8) and 2.6 (SD 0.9), respectively. Each dwelling had an average of 4.1 (SD 1.8) residents (data not shown).

3.2. Losses to follow-up

Prior to evaluations II and III, 57 and 47 dogs were lost to follow-up respectively. The reasons for losses to follow-up were euthanasia (seroconversion), death, change of address, household closed, refusal, and dog escape. The closed houses were visited 3 times before the dogs were considered lost to follow-up. Comparisons of the characteristics of the evaluated dogs and those lost to follow-up in each evaluation phase did not reveal any differences.

3.3. Incidence and risk factors for L. infantum infection

Among 282 seronegative and PCR-RFLP-negative dogs, 234 showed failure events as indicated by PCR-RFLP during follow-up (26 months), and the overall incidence for 4061 dog-months was 5.8 per 100 dog-months (95% CI 5.1–6.5). The first evaluation showed 109 events (PCR-RFLP), and an incidence of 3.1 per 100 dog-months (95% CI 2.5–3.7). Evaluations II and II showed 112 and 13 events, with incidences of 25.7 (95% CI 21.3–30.9) and 19.4 per 100 dog-months (95% CI 11.1–33.1), respectively (Table 2). The results of the preliminary selection of the variables from the univariate analysis ($p < 0.25$) as well as the hazard ratios (HR), number of failure events, and incidences per 100 dog-months are shown in Table 3. The variables selected to build the final model ($p < 0.15$) were dog fur length (long/short), previous case of CVL in the household (yes/no), unplastered house walls (yes/no), regular garbage collection (<3× per week/≥3× per week), access to the street (yes/no), and previous serological examination for CVL (yes/no). Infection with *L. infantum* (PCR-RFLP) was associated with a previous case of CVL in the household (HR 1.4; 95% CI 1.1–1.8) and unplastered house walls (HR 3.6; 95% CI 1.6–8.1) (Table 4).

Table 2
Failure events (PCR-RFLP), dog-months of follow-up, and incidence rates with 95% CIs, Brazil 2010.

Follow-up	PCR-RFLP		
	No. of failure events	Dog-months	Incidence rate (95% CI)[a]
Evaluation I[b]	109	3557	3.1 (2.5–3.7)
Evaluation II[c]	112	436	25.7 (21.3–30.9)
Evaluation III[d]	13	67	19.4 (11.1–33.1)
Total	234	4061	5.8 (5.1–6.5)

[a] Per 100 dog-months.
[b] 10 months after the baseline survey.
[c] 16 months after the baseline survey.
[d] 26 months after the baseline survey.

4. Discussion

The present investigation showed that the condition of the domicile and the presence of a previous case of CVL in the household were associated with early *L. infantum* infection in dogs. Thus, it was possible to determine the role of household structures as a predictor of canine infection occurrence in urban areas. These results are relevant because they allow a better understanding of how household structure may affect the urbanization of ZVL and thus what control measures might be adopted to more effectively control the spread of the disease.

Studies have shown that molecular methods not only lead to more accurate *Leishmania* detection (Solano-Gallego et al., 2009) but also reveal the presence of protozoan DNA very early, before seroconversion and clinical manifestations (Quinnell et al., 2001; Oliva et al., 2006; Coura-Vital et al., 2011a). In experimentally infected dogs, seroconversion can take from 1 to 6 months (Moreno and Alvar, 2002), whereas in naturally infected animals, the median time to seroconversion is 10.5 months (range, 4–22 months) (Oliva et al., 2006). These facts emphasize the importance of early detection of infection by molecular methods. For CVL diagnosis, PCR can be performed on samples from a broad range of tissues with various degrees of sensitivity (Manna et al., 2004; Di Muccio et al., 2012).

Table 3
Univariate analysis using Kaplan–Meier survival analysis and the Cox regression model, to determine risk factors for *Leishmania infantum* infection, Brazil 2010.

Variable	No. of failure events	No. of measured	Time at risk	Incid./100 dog-month	HR	95% CI	*p*
Vector/reservoir							
Seen the vector							
No	220	267	3859	5.7	1		
Yes	14	15	210	6.7	1.47	0.85–2.53	0.17
Previous case of CVL in the household							
No	162	198	2918	5.5	1		
Yes	72	84	1151	6.3	1.35	1.02–1.78	0.04
Housing conditions							
Unplastered house walls							
No	228	276	4000	5.7	1		
Yes	6	6	70	8.6	3.37	1.49–7.63	0.00
Regular garbage collection							
≥3×/week	216	257	3682	5.9	1		
<3×/week	15	22	343	4.4	0.63	0.37–1.05	0.08
Trees in backyard							
No	122	152	2236	5.4	1		
Yes	112	130	1832	6.1	1.17	0.91–1.52	0.22
Characteristics of dogs							
Fur length							
Long	95	121	1747	5.4	1		
Short	139	161	2322	6.0	1.21	0.93–1.57	0.16
Dog stayed predominantly in the backyard							
No	27	35	543	5.0	1		
Yes	207	247	3526	5.9	1.40	0.93–2.08	0.10
Had access to the street							
No	207	253	3681	5.6	1		
Yes	27	29	387	7.0	1.38	0.92–2.05	0.12
Previous serological examination for CVL							
Yes	179	217	3161	5.7	1		
No	48	55	755	6.4	1.38	1.00–1.91	0.05

HR, hazard ratio; CI, confidence interval; CVL, canine visceral leishmaniasis.

Although the peripheral blood is not the most sensitive sample, it is the most useful for mass surveys because sample collection is less invasive and the use of peripheral blood allows serology to be performed concurrently (Lachaud et al., 2002a,b), allowing the detection of most of the infected dogs.

The incidence of *L. infantum* infection as detected by PCR-RFLP was high; at the end of 26 months of follow-up, almost all the dogs had been infected. These results indicate that Belo Horizonte is an area of active CVL transmission. The incidence of CVL is an important epidemiologic parameter to consider in prioritizing target control areas. Detection of DNA by PCR effectively assists in CVL diagnosis, mostly for dogs with uncertain serology status or for dogs that have not seroconverted (Martinez et al., 2011).

CVL is currently spreading to non-endemic areas in Europe (Dereure et al., 2009) and recently emerged in North America (Petersen, 2009). Furthermore, an increase in the prevalence of ZVL has been observed in urban areas, which may be attributed to high population density, increased migration, environmental changes, inadequate living conditions, and the presence of vector and reservoir in the domestic environment (Desjeux, 2004; Oliveira et al., 2008). Because dogs are the main reservoirs for *L. infantum* in humans, we investigated the risk factors associated with *L. infantum* infection in dogs. Identification of factors associated with CVL might be useful for identifying areas of higher ZVL risk, because infection in dogs generally precedes the occurrence of human cases (Nunes et al., 2010).

We observed that canine infection by *L. infantum* was associated with the presence of unplastered house walls. Sand flies are crepuscular and nocturnal, and during the day, they rest in comparatively cool, humid areas, including houses, latrines, basements, and wall fissures (Killick-Kendrick, 1999). Sand flies can hide in cracks in unplastered walls, which facilitates contact between the vector and dogs. The occurrence of *Lutzomyia longipalpis* in cracks and crevices of house walls has been observed in endemic areas of northeastern Brazil (Deane and Deane, 1962). In a case-control study conducted in Nepal, cracks in house walls

Table 4
Risk factors for *Leishmania infantum* infection in dogs, Brazil 2010.

Variable	Crude hazard ratio (95% CI)	Adjusted hazard ratio (95% CI)
Previous case of CVL in the household Yes versus no	1.3 (1.0–1.8)	1.4 (1.1–1.8)
Unplastered house walls No versus yes	3.4 (1.5–7.6)	3.6 (1.6–8.1)

CI, confidence interval.

were found to be associated with elevated human visceral leishmaniasis risk (Bern et al., 2000). A case-control study by Costa et al. (2005) in Teresina, Brazil, revealed no association between risk of infection by *L. infantum* and unplastered walls, ceiling, floor, and water supply; however, the lack of sewerage or regular collection of garbage was associated with increased ZVL incidence. These findings demonstrate that basic public services are important in controlling the spread of the disease (Costa et al., 2005). In the current study, almost all households evaluated had sewerage and garbage collection, making it impossible to detect the influence of these factors on canine infection.

Housing conditions generally reflect socioeconomic status. The cross-sectional study on which this cohort study was based demonstrated that one factor associated with *L. infantum* infection (as indicated by PCR-RFLP) was the socioeconomic status of the owner (Coura-Vital et al., 2011b). Indeed, Oliveira et al. (2006) demonstrated an association between ZVL and family income in the Belo Horizonte metropolitan area. Our data are also consistent with literature confirming that ZVL is more incident in areas of precarious socioeconomic status (Werneck et al., 2007).

In Brazil, the main strategies used by the Visceral Leishmaniasis Control and Surveillance Program have been vector control and culling of infected dogs. However, these measures have been found to be insufficient to contain disease dissemination (Harhay et al., 2011). In this study, we observed that dogs residing in households with previous case of CVL showed a higher risk of infection than dogs residing in households with no previous cases. Similar results were observed in a study conducted in Teresina, Brazil, where in households with a history of dog removal by the program, the odds of having at least one infected dog were 5 times the odds in dwellings with no history of dog removal (da Silva et al., 2012). This result may have been due to an environment favorable to the development of the vector in the domiciles, causing the occurrence of new cases. According to da Silva et al. (2012), simply removing a seropositive dog from a household, without taking environmental management measures, does not prevent future infections in other animals in that household.

One limitation of cohort studies is loss to follow-up; however, in the current study, the dogs lost to follow-up did not differ substantially from those that remained, as indicated by comparison of several characteristics. Therefore, the effect of loss to follow-up was minimized. The present study was designed not to evaluate a representative sample of Belo Horizonte but to assess the incidence of and identify risk factors for *L. infantum* infection. However, the northwest sanitary district is representative of the city in terms of buildings, commerce, residences, and green areas.

5. Conclusion

We identified domicile characteristics that were associated with an increased risk of canine infection by *L. infantum*. The identification of these risk factors indicates the necessity for insecticide spraying in cracks and crevices in the walls of houses to reduce biting rates within and around houses. Furthermore, our results demonstrate that the Visceral Leishmaniasis Control and Surveillance Program should adopt environmental management measures in homes with previous cases of CVL because these households are more likely to maintain the transmission cycle.

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