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ORIGINAL ARTICLE

Serological Evidence of *Leishmania donovani* Infection in Apparently Healthy Dogs using Direct Agglutination Test (DAT) and *rk39* Dipstick Tests in Kafta Humera, north-west Ethiopia

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Summary

Leishmania (Kinetoplastida: Trypanosomatidae) are protozoan parasites of significant medical and veterinary importance. Over the last decade, visceral leishmaniasis (VL) has emerged as a major opportunistic infection associated with HIV/AIDS in North Western Ethiopia. This paper reports on serological evidence of possible *Leishmania donovani* (*L. donovani*) infection in dogs using two serological tests: direct agglutination test (DAT) and Kalazar detect rapid test (KDRT). Two hundred and seventeen asymptomatic local breed dogs were examined for *L. donovani* antibodies. Performance of the DAT and KDRT was assessed in 162 matching samples of blood collected on filter paper and serum, respectively. Using DAT and KDRT testing in parallel, the overall seroprevalence of *L. donovani* infection was 27.7% and 14.8%, respectively. The degree of agreement was found to be fair (68.8%, $k = 0.234$). Univariable logistic regression analysis of some risk factors for *L. donovani* infection in dogs using DAT indicates that place of residence, sex, age, dog keeping purpose and dog housing condition were not significantly associated with seropositivity. The high proportion of positive dogs suggests the exposure of these animals to *L. donovani* infection and needs further investigation. Isolation and typing of the parasite aiming at confirming the role of these animals in maintenance and transmission of kala-azar is advocated.

Introduction

Leishmaniases are protozoan diseases caused by members of the genus *Leishmania*, parasites infecting numerous mammalian species, including humans, and transmitted by the bite of phlebotomine sand flies (Gramiccia and Gradoni, 2005).

Leishmaniases are endemic in Ethiopia and caused by the species *L. major, L. aethiopica* and *L. donovani* sensu *lato* (Hailu et al., 1995). *Leishmania major* is considered to be the causative agent of Cutaneous Leishmaniasis (CL) in the lowlands of southwestern Ethiopia. *Leishmania aethiopica* causes diffuse CL in the highlands and is widely distributed at altitudes between 1700 and 2700 m (Ashford et al., 1973; Hailu et al., 1995). The disease is zoonotic, with rock hyraxes being the main reservoir host (Ashford et al., 1973). Transmission is via two species of phlebotomines: *Phlebotomus longipes* and *P. pedifer* (Lemma et al., 1969). As in other East African countries, Somalia, Sudan, Kenya and Uganda, *L. donovani* causes visceral leishmaniasis (VL) or kala-azar (Marlet et al., 2003; Basiye et al., 2010). The principal vector is *P. orientalis* (Gebre-Michael et al., 2010).

In the Kafta Humera district of north-west Ethiopia, VL is particularly associated with migration of non-immune labourers from the surrounding highland regions to the extensive agricultural development schemes in the lowlands (Lyons et al., 2003; Gebre-Michael et al., 2010). The disease has also emerged as a major opportunistic infection associated with HIV (Lyons et al., 2003; Ritmeijer et al., 2006). Transmission is thought to be anthroponotic. Epidemiological studies of VL have incriminated several animal species as reservoirs for *L. donovani*. Studies in neighbouring countries, Sudan and Kenya, have detected *L. donovani* in domestic animals (Mukhtar et al., 2000; Dereure et al., 2003), but whether these play a role in Ethiopia is unknown.

Serology, the detection of specific antibodies, is probably the most widely used method to assess *Leishmania* prevalence in canines. Antibody detection tests such as the direct agglutination test (DAT) (el Harith et al., 1988) and the *rk39* immunochromatographic strip test (Chappuis et al., 2005; Ritmeijer et al., 2006; ter Horst et al., 2009; Sharma et al., 2009) are easy to perform and provide valuable information in a relatively short time (França-Silva et al., 2003) compared to microscopic examination of tissue smear or cultivation of bone marrow aspirates that suffer from low sensitivity. The result report is a serological survey of *L. donovani* infection in apparently healthy dogs with the main objectives of exploring the existence of *L. donovani* sero-positive dogs and some associated risk factors for *L. donovan*i seropositivity. Direct agglutination test and Kalazar detect rapid test (KDRT) were chosen for our survey, as these tests have proven to be very suitable for the serodiagnosis of VL (Oskam et al., 1996; Mettler et al., 2005; Sharma et al., 2009).

Materials and Methods

Study area

Kafta Humera district (Western Tigray, north-west Ethiopia) is a remote, tropical region where extensive agriculture is performed manually by large numbers of migrant labourers from the surrounding highlands. Its geographical location is 13° 42′–14° 28′ North latitude and 36° 23′–37′31′ East longitude (Fig. 1). Its elevation ranges from 560 to 2800 m above sea level. The study area is bordered to the North by Eritrea, to the South by Gonder, to the East by Tahtai Adiabo and to the West by Sudan. The study

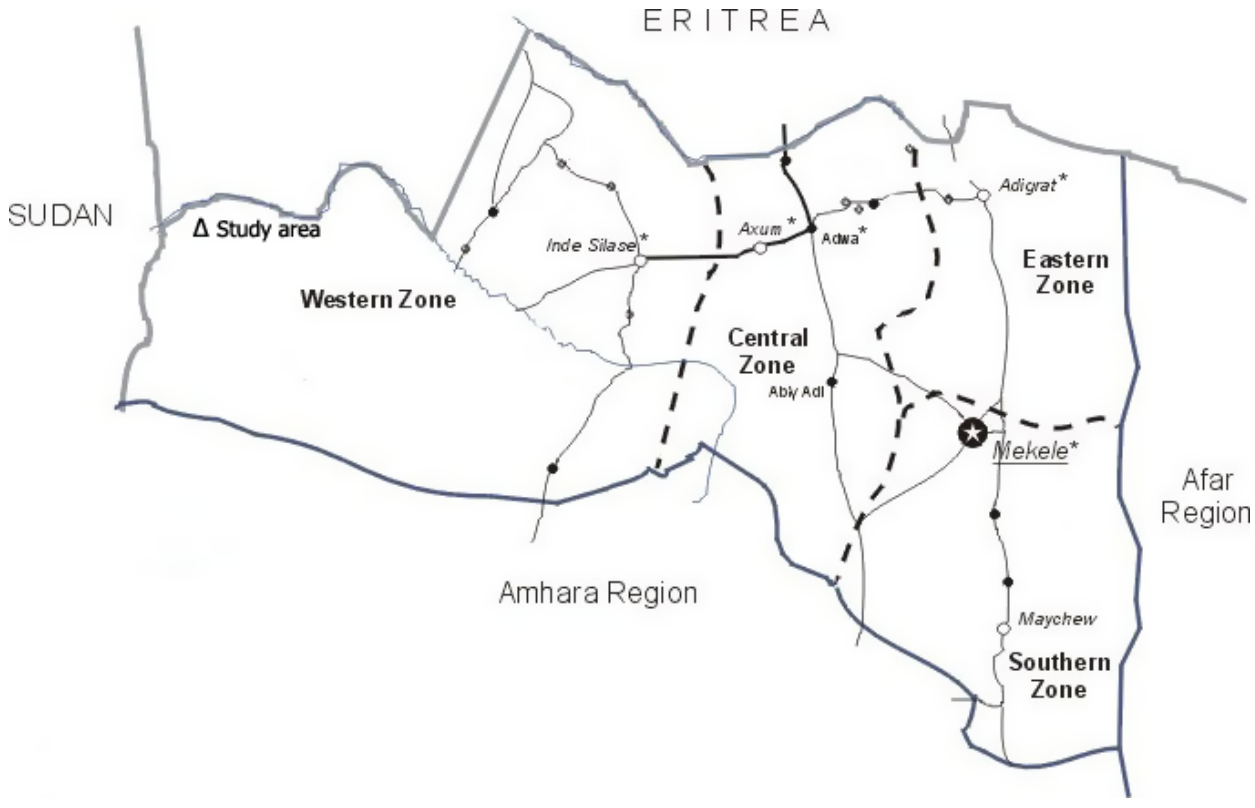

Fig. 1. Administrative provinces of Tigay Regional State, Northern Ethiopia, showing the study area Kafta Humera.

area's position near the Eritrean and Sudanese borders means that it is a transit point for cross-border trade and traffic. As in any district of the region, dogs are historically intimate friends to daily labourers and to the local population, and almost every residence owns a dog for various purposes including guarding and herding. In addition, there are numerous unclaimed dogs that roam on the streets.

Study design

A cross-sectional survey was carried out in March and April 2010 to determine the existence of *L. donovani* sero-positives in the population of dogs in three human VL endemic towns, Humera, Maykadra and Bereket. Initially, the census of all domestic dogs was reviewed from the district agriculture and rural development bureau. In April 2010, dog owners in the three towns were informed through the district veterinary services to bring their dogs for blood sampling and deworming. These animals served as the study population and their descriptive information were documented in a short standard questionnaire during sera collection. The information collected included gender, age, purpose of dog keeping, presence/absence of shelter, human visceral leishmaniasis history in the household, owner's perception regarding its zoonotic importance, symptoms of leishmaniasis in dogs, treatment and vaccination history, if any. The study group comprised of a total of 217 domestic dogs. As part of selection criteria, only dogs of 6 months of age and above that had lived in towns for at least 6 months during the year prior to the study were eligible for sampling. A summary of the dogs sampled per town and numbers evaluated using each type of serological test is indicated in Table 1.

Table 1. Summary of dogs sampled and tests used to detect anti-leishmanial antibodies in dogs of three towns of Western Tigray, North Western Ethiopia

			Number tested by	
Town	Dogs sampled	Total dog population[a]	Direct agglutination test	Kalazar detect rapid test
Humera	44	680	12	37
Maykadra	96	489	95	77
Bereket	77	210	77	75
Total	217	1379	184	189

[a]Total dog populations of the study areas are only approximate and do not include the hundreds of dogs in various agricultural camps nor the many stray dogs.

Direct agglutination test (DAT)

Capillary blood samples were collected on filter paper (Whatman no.3) for DAT. Filter paper labelled with unique identifiers, consisting of identification number, sex, site and date of sampling, was transported to Kahsay Abera Hospital in Humera town. The stained freeze-dried DAT antigen employed is derived from *L. donovani* promastigotes (Lot number 8789; KIT Biomedical Research, Amsterdam, The Netherlands). Antigen concentration for the DAT was 5×10^7 promastigotes/ml. The protocol employed was that described previously (Meredith et al., 1995; Oskam et al., 1996; Hailu et al., 2002; Schallig et al., 2002). Briefly, 5.5-mm-diameter filter paper was punched out from the middle of the blood-spotted filter paper and eluted overnight at 4°C in 0.9% saline containing 1.56% mercaptoethanol (Lot: A016455501; Acros organics, Geel, Belgium) to produce the equivalent of a 1 : 100 serum dilution. Twofold dilution series were made from 1 : 100 to 1 : 102 400 in a V-shaped micro titre plate (Fig. 2) and incubated for 1 h at 37°C. Fifty microlitres of reconstituted DAT antigen was subsequently added to each well containing 50 μl of diluted serum. Negative control (antigen only) and a known positive control serum of *L. donovani* were included. Quantitative results obtained with DAT are expressed as an antibody titre, i.e. the reciprocal of the highest dilution at which agglutination is still visible after 18 h incubation at room temperature (Schallig et al., 2002). Three individuals read the tests independently. A sample was considered positive if it had a titre of 1 : 3200 and above (Mukhtar et al., 2000; Hassan et al., 2009).

Kalazar detect rapid test (KDRT)

The Kalazar detect rapid test™ (P/N 900003.9; InBios International, Seattle, WA, USA) is an immunochromatographic qualitative antibody assay used both in human and canine against *L. donovani* complex *rk*39 antigen (Benson et al., 1996; Mettler et al., 2005; Sharma et al., 2009). The test strip membrane is coated in the middle with a band of *rk*39 antigen and on the top with immobilized anti-protein-A antibody to detect IgG. A protein A-gold conjugate is used as the immunochromatographic detection reagent. Approximately 3–5 ml of peripheral blood was collected from the cephaneous/cephalic vein of each dog using disposable syringe or plain vacutainer, and subsequently serum was obtained by either passively decanting or using centrifuges at 350 ***g*** for 5 min. The protocol was performed according to the procedures given by the manufacturer. One drop of serum (approximately 20 μl) was placed on the absorbent pad at the bottom of the strip, three drops of the test buffer were added to the pad, and the mixture was

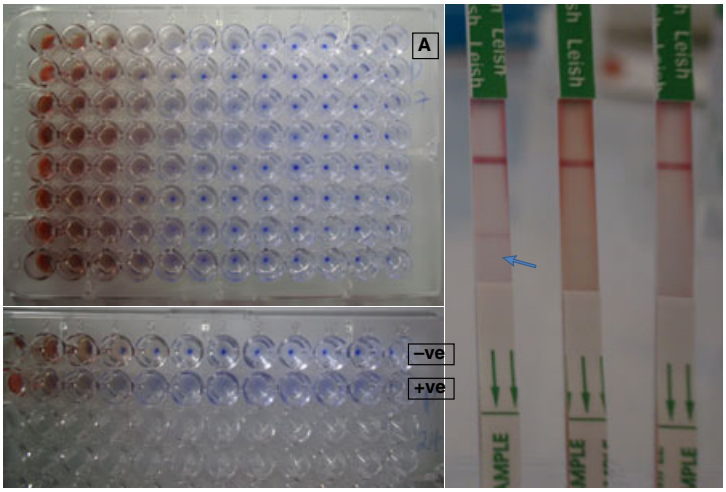

Fig. 2. Direct agglutination test (DAT) and Kalazar detect rapid test (KDRT) testing from sera of dogs. A twofold dilution series of test samples in V-shaped microtitre plates, –ve-negative serum, +ve- positive control serum and arrow (right) KDRT indicating the presence of rk39 IgG for positive test serum.

allowed to migrate up the strip by capillary action. After 10 min, the appearance of a red upper (control) line indicated the presence of IgG and proper test functioning; a red lower line indicated the presence of anti-*rk*39 IgG and a positive test result (Fig. 2).

Statistical analysis

Data obtained from the serological tests and questionnaire survey were stored in a Microsoft Excel spreadsheet (Microsoft Corp. Redmond, WA, USA). These data were analysed by descriptive statistics and univariable regression using the SPSS 15 statistical package (SPSS Inc., Chicago, IL, USA).

We estimated individual dog-level seroprevalence using SPSS with seropositivity (positive/negative) as the outcome of interest and stratifying according to town, gender and age. Questionnaire data that included risk factors associated with dog keeping such as purpose, housing condition, treatment and vaccination history, together with parameters such as incidence of *leishmaniasis* in the household and perception of the family as to its zoonotic importance was analysed in conjunction with the serological results.

A two-sided Fisher's exact test was used to determine a significant correlation between all the risk factors. A univariable logistic regression model was applied to measure the strength of that association. The logistic regression model was fitted with individual dog seropositivity (positive/negative) as the outcome. The model was built using the forward stepwise (conditional)-selection procedure by applying the iterative maximum-likelihood estimation procedure while the statistical-significance contribution of individual predictors to the models was tested using the Wald's test and likelihood-ratio tests. The logistic model was checked for goodness-of-fit using the Hosmer and Lemeshow test. $P < 0.05$ was taken as significant.

The degree of agreement between DAT and KDRT was determined by calculating kappa (k) values and their associated 95% CIs using SPSS 15. This was done on 162 matching blood on filter paper and serum samples. *K* values express the agreement beyond chance, with a *K* value of 0.21–0.60 representing a fair to moderate agreement, a *K* value of 0.6–0.8 representing a substantial agreement and a $K > 0.8$ representing almost perfect agreement (Altman, 1991).

Results

Seroprevalence of *L. donovani* in dogs

A total of 217 canine sera were examined by DAT and/or KDRT from three human VL endemic towns in the Western Zone of Tigray Regional state, North Western Ethiopia. Using DAT and KDRT testing, the overall seroprevalence of *L. donovani* infection was 27.7% (51/184) and 14.8% (28/189), respectively. The results of DAT and KDRT testing of 162 matching sera in the towns are presented in Table 2. Fifty-one dogs were found seropositive with DAT using a cut-off titre of 3200. KDRT found 28 dogs seropositive. Twelve KDRT positive had DAT titre of less than 3200, of which four were close to the cut-off value. Thirty five KDRT negative dogs had a DAT titre of 3200 and above. The observed agreement between DAT and KDRT was 68.5%, with a *k* value of 0.234 representing a fair agreement beyond chance (Table 3).

Univariable logistic regression analyses of some risk factors for *L. donovani* infection in dogs

The analysis of *L. donovani* infection seroprevalence in dogs and some of the risk factor categories, together with their associations, are summarized in Table 4. The first

Table 2. DAT and rk39 results in 162 matching samples of dogs from Kafta Humera, North Western Ethiopia

		Kalazar detect rapid test	
DAT titre[a]	*N*	Positive (*n*)	Negative (*n*)
<400	55	8	47
400	0	0	0
800	13	2	11
1600	43	2	41
3200	7	6	1
6400	30	8	22
12 800	13	2	11
25 600	1	0	1
51 200	0	0	0
Total	162	28	134

[a]Direct agglutination test (DAT) cut-off ≥3200.

Table 3. Comparison of Kalazar detect rapid test (KDRT) and direct agglutination test (DAT) in matching sera of dogs from Western Tigray

	DAT		
KDRT	Positive	Negative	Total
Positive	16	12	28
Negative	35	99	134
Total	51	111	162

(k = 0. 234, P = 0.001) showing a fair agreement beyond that because of chance alone

level of each independent variable was used as a reference category in all the statistical analyses. Direct agglutination test Sero-prevalences in Bereket, Humera and Maykadra towns were 23.4%, 50.0% and 36.8%, respectively. The odds of infection in Humera and Maykadra were 3.278 and 1.912, respectively; however, the differences were not statistically significant. Sero-prevalence values among male and female dogs were 33.8% and 25%, respectively. No statistical difference in the level of canine *L. donovani* infection was found for animal sex (P = 0.313, 95% CI [0.67, 3.5]).

The univariable logistic regression model indicated that the highest value of seroprevalence was, in older dogs, 6 years and above (38.7%). However, no differences were observed between the seroprevalence values of the distinct age interval. Similarly, house keeper dogs had a higher infection rate (32.9%) than herder dogs (21.4%), but once again, the difference was not significant. Analysis of housing condition indicated that housed dogs, with at least a shed, had a seroprevalence of 33.3% while those which has no shed or were strays had a prevalence of 29.7%.

Discussion

Dogs are usually in or next to human houses, and thereby can contribute to the domestic transmission cycle of major zoonotic diseases including leishmaniasis. Information on the status of canine VL in Ethiopia is lacking. The definitive reservoir for *L. donovani* in Ethiopia remains unknown. Knowledge of the reservoir hosts is an important pre-requisite for understanding the epidemiology of and designing control programs for VL. The present study is, to our knowledge, the first in Ethiopia to estimate the prevalence of canine VL in apparently healthy dogs in foci where human cases are widespread. The current study area has been known to be endemic for VL since 1970. Since then, the number of human cases in the region has increased with reported death rates as high as 18%, which appears to correspond to an extensive programme of agricultural development with its

Table 4. Summary results of the univariable logistic regression analyses of some risk factors with dependent direct agglutination test seropositivity in dogs in Western Zone of Tigray, North West Ethiopia

						95% CI of OR	
Variables	Category level	*N*	Prevalence (%)	*P* value	Odds Ratio (OR)	Lower	Upper
Town	Bereket	77	23.4	–	–	–	–
	Humera	12	50	0.062	3.278	0.940	11.425
	Maykadra	95	36.8	0.059	1.912	0.976	3.746
Sex	Female	36	25	–	–	–	–
	Male	148	33.8	0.313	1.531	0.669	3.502
Age	<2 years of age	77	35.07	–	–	–	–
	2–6 years of age	76	26.3	0.242	0.661	0.331	1.322
	>6 years of age	31	38.7	0.721	1.17	0.494	2.767
Dog use	Herder	14	21.4	–	–	–	–
	House guard	170	32.9	0.381	1.801	0.483	6.716
Housing condition	No shed/stray	64	29.7	–	–	–	–
	Housed	120	33.3	0.614	1.184	0.614	2.284

annual influx of migrant workers (Berhe et al., 2001; Lyons et al., 2003). Direct agglutination test and KDRT were employed to screen dogs for the presence of *anti-Leishmania* specific antibodies as an indicator of infection. The DAT cut-off titre (1 : 3200) was similar to that of Mukhtar et al. (2000). The former gave a higher estimate of prevalence (27.7% versus 14.8%). The estimated sero-prevalence of leishmania infection in dogs from the present study area was higher than that reported from neighbouring Sudan, which ranged from 5.9% to 8.0% (Dereure et al., 2000, 2003; Mukhtar et al., 2000; Hassan et al., 2009). Direct agglutination test is most frequently used in the detection of anti-leishmania antibodies in dogs because of its increased sensitivity and specificity when compared with other tests (Schallig et al., 2002). In the present study, analysis of the agreement between the two serological tests revealed a fair agreement of 68.8% ($k = 0.234$, $P = 0.001$). In 162 matching sera, 35 KDRT negative had a DAT titre of 3200 and above. Direct agglutination test testing yielded higher detection in apparently healthy dogs. Only 12 DAT negative sera were found to have KDRT positive result that is consistent with previous observations (Diro et al., 2007). It has been stated that anti-leishmania antibody detection by KDRT based on rk39 antigen is an indicator of active disease (Benson et al., 1996; Mettler et al., 2005). The fact that our study population included only apparently healthy dogs and the fact that sera from early or self-healing infected dogs are generally less reactive with rk39 may explain the KDRT's lower detection rate. Infection with other species of leishmania, such as those causing cutaneous forms, may affect the interpretation of *rk*39 test result (Hartzell et al., 2008). The test in some reports has showed cross-reactivity in human patients with CL, with more than 10% of patients with CL having a positive *rk*39 dipstick result (Braz et al., 2002; Hartzell et al., 2008). These reports are, however, not supported in infected dogs. Several studies from India and Sudan have indicated that leishmania species causing the cutaneous form in dogs do not express a *rk*39 repeat-like *L. donovani* complex (Zijlstra et al., 2001, Sharma et al., 2009). Thus, the antibody response to *rk*39 is largely *L. donovani* complex-oriented, and there is virtually no response in an infection with CL (Zijlstra et al., 2001).

Studies of the risk factors associated with the geographical location, breed, sex, age, dog keeping purpose and housing condition of dogs are few, and their results are not always in consistent. Our study revealed that seroprevalence was highest in Humera followed by Maykadra and Bereket. Large variation of prevalence to anti-leishmania antibodies can be found between canine populations living in different areas because of ecological conditions that determine the abundance of sand flies, as a focal agent, within an endemic area (Cardoso et al., 2004). These three small towns are a few kilometres apart from each other. The variation among the three was, however, not statistically significant. Most of the study area's ecology seems to be similar to the known VL endemic area of the Sudan where the vector *P. orientalis* is known to thrive best (Gebre-Michael et al., 2010). As a reflection of the high prevalence of infection, all age intervals had seropositive animals. There appears to be an increased prevalence associated with age. Dogs of ≥6 years of age and dogs younger than 2 years of age had higher rates of infection when compared with young adult dogs between two and 6 years of age. This finding is in line with previous reports (Abranches et al., 1991; Cortes et al., 2007; Martín-Sánchez et al., 2009) for *L. infantumi/chagasi* infection. The highest prevalence in older dogs may be related to the increase of time of exposure of dogs to infected sandflies (Zivicnjak et al., 2005, Adel et al., 2010). There was no significant pattern of sex distribution for seropositivity across the study areas.

Guard or semi-stray dogs were more likely to contract the infection than herder dogs (32.9% versus 21.4%). This may be explained by the facts that the domestic sand flies, not the peridomestic, play a major role in the infection. In peridomestic transmission cycle, sand flies acquire the parasite by feeding on the skin of wild canids such as fox and transmit it to dogs. Domestic animals (i.e. cattle, sheep, goat, camel, equids) and dogs and cats live together with humans. Sharing residence with one or more seropositive animal could increase the risk for dogs to become seropositive (Alonso et al., 2010). This hypothesis is supported by a recent study in the region (Gebre-Michael et al., 2010) which showed that bovines account for about 92% blood meal source for *P. orientalis* while only 2% was from human origin. The higher sero-prevalence in house keeper or semi-stray dogs could also be related to their outdoor living habit, which increases time of exposure to infected sand flies, and they may be easier targets for infection (Cortes et al., 2007; Martín-Sánchez et al., 2009; Adel et al., 2010). Better living conditions, such as proper housing, could prevent contact with sand flies and lower the risk of *Leishmania* infection (Abranches et al., 1991; Miranda et al., 2008). Our result indicated prevalences of 29.7% and 33.3% for those dogs with no shed and with sheds, respectively. This finding is contrary to previous reports (Abranches et al., 1991; Miranda et al., 2008). The exact classification of housing condition was difficult to determine as there was not a single pet owner with a regular kennel. This may partly explain the discrepancy with previous reports. The notion of classifying housing condition as shed or no shed can only be taken as a

shelter from rain and sun. It was also common to see many house keeper dogs roaming outside during the night with strays.

In conclusion, our study has revealed the presence of a canine population with seroreactivity against *L. donovani* infection in an area where human VL is endemic. The presence of a high number of apparently healthy dogs with positive agglutination reactions may be related to exposure and development of protective immunity especially in older dogs and a high exposure to Leishmania parasites (Mukhtar et al., 2000; Cardoso et al., 2004), suggesting that such dogs may be harbouring the infection and providing a constant reservoir for the infection. However, validation of such hypotheses could not be supported by the present study and requires additional evidence which could emerge from parasite cultivation and molecular studies.

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