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Research paper

Parasite load in the blood and skin of dogs naturally infected by *Leishmania infantum* is correlated with their capacity to infect sand fly vectors

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ABSTRACT

The sand fly *Lutzomyia longipalpis* is primarily responsible for the transmission of visceral leishmaniasis (VL) in the New World, and dogs are considered to be the main urban reservoir of this disease. In order to improve the efficacy of control measures, it is essential to assess the transmission capacity of *Leishmania infantum* to the sand fly vector by naturally infected dogs. The present study investigated the existence of correlations between canine clinical presentation and the intensity of parasite load in the blood, skin and spleen of naturally infected dogs. In addition, we also attempted to establish correlations between the intensity of parasite load in canine tissue and the parasite load detected in sandflies five days after feeding on naturally infected dogs. A total of 23 dogs were examined and classified according to clinical manifestation of canine VL. Blood samples, splenic aspirate and skin biopsies were collected and parasite DNA was quantified by qPCR. Canine capacity to infect *Lu. longipalpis* with parasites was evaluated by xenodiagnosis and parasite loads were measured five days after feeding. No significant differences were observed with respect to canine clinical manifestation and the parasite loads detected in the blood, skin and spleen samples obtained from naturally infected dogs. Regardless of clinical canine visceral leishmaniasis (CVL) presentation and the degree of parasite burden, almost half of the dogs successfully infected sandflies with parasites, albeit to a low number of sandflies with correspondingly low parasite loads. Parasite loads in both canine blood and skin were shown to be positively correlated with the canine infectiousness to the sand fly vector, and positive correlations were also observed with respect to these tissues and the sand fly infection rate, as well as the parasite load detected in sandflies following xenodiagnosis. In conclusion, this indicates that parasite loads in both blood and skin can function as potentially reliable markers of canine capacity to infect sand fly vector.

1. Introduction

Visceral leishmaniasis (VL) is a severe systemic disease that affects humans, dogs and other mammalian vertebrates (WHO, 2010). In the New World, VL generally results from infection by the protozoan parasite *Leishmania infantum* (syn. *L. chagasi*), which is transmitted to mammalian hosts mainly by the *Lu. longipalpis* sand fly vector (WHO, 2010). Abundant evidence indicate that domestic dogs are the main reservoir of VL in urban areas, which is supported by reports of canine epidemics preceding outbreaks in humans in endemic regions (Alvar et al., 2004; Bevilacqua et al., 2001).

Due to variability in the immune response to infection, dogs with visceral leishmaniasis (CVL) can present as either asymptomatic or with a progressively symptomatic form of the disease (Ciaramella et al., 1997; Rallis et al., 2005). In general, the capa-

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bility to transmit parasites to sandflies has been associated with disease severity. Several studies have reported that most symptomatic dogs are capable of transmitting parasites to sandflies in endemic areas, and that these animals are more infectious than asymptomatic dogs, confirming their importance in the transmission cycle of VL (Courtenay et al., 2002; da Costa-Val et al., 2007; Guarga et al., 2000). By contrast, other studies have demonstrated that a given dog's capacity to transmit parasites to sand fly vectors is independent of its clinical CVL presentation (asymptomatic, oligosymptomatic or polysymptomatic) (Guarga et al., 2000; Molina et al., 1994). Unfortunately, none of these studies has attempted to elucidate parameters for distinguishing dogs that are capable of transmitting parasites to sand fly vectors from those that are incapable of transmission. Since VL control measures in Brazil are based on serological diagnosis and the euthanasia of seropositive dogs (Ministério da Saúde et al., 2006), to increase the efficacy of such measures, it is essential to ascertain infectiousness of naturally infected dogs.

Parasite transmission occurs by the contact of sand fly proboscides with the skin and blood of an infected host vertebrate during blood-feeding (Bates, 2007). We hypothesized that a given infected dog would infect sandflies with *L. infantum* in accordance with the intensity of its tissue (skin or blood) parasite burden. To test this hypothesis, we quantified parasite load in the skin and blood of naturally infected dogs presenting a range of clinical manifestations and correlated the intensity of parasite load in these tissues with a given dog's capacity to infect sandflies with parasites.

2. Materials and methods

2.1. Ethical considerations

All experiments involving canine specimens were performed in compliance with Brazilian federal law for animal experimentation (Law 11794), in conformity with the Oswaldo Cruz Foundation (FIOCRUZ) animal experimentation guidelines, and according to the instructions outlined in the Brazilian Ministry of Health's manual for the surveillance and control of VL. The present study was approved by the Institutional Review Board (CEUA protocol no. 015/2009) of the Gonçalo Moniz Research Center in Bahia, Brazil (CPqGM–FIOCRUZ/BA). Dog owners who agreed to participate in the study signed a Free, Prior and Informed Consent (FPIC) form.

2.2. Canine specimens

A total of 23 dogs were recruited directly from their owners during the period of April 2011 and September 2012, some of whom reside in endemic regions within the State of Bahia, Brazil: (i) 15 animals were pets from the municipality of Dias D'Ávila, (ii) 6 from the municipality of Camaçari and (iii) 2 dogs domiciled in Salvador.

Dogs were included in the study if they tested positive for CVL by aspirate spleen culture or qPCR of blood, skin or spleen tissue. All dogs were clinically examined by a veterinarian and subsequently classified in accordance with the intensity of the most common clinical signs of CVL, including weight loss, alopecia, cutaneous lesions, conjunctivitis, onychogryphosis and lymphadenopathy. Thereafter, each detected clinical sign was categorized in grades of 0, 1, 2 or 3 in accordance with its intensity. Clinical scores were then calculated by summing the grades assigned for each clinical sign present in infected dogs. Animals were classified as without clinical manifestation (subclinical infection, clinical score 0–3) or with clinical disease (symptomatic infection, clinical score 3 +). The Municipal Center for Zoonotic Disease Control received notification regarding all positive dogs.

2.3. Sample collection

To perform parasitological diagnosis and determine parasite loads, blood collection, spleen aspiration and skin biopsies were performed. Samples of peripheral blood were collected by venipuncture (minimum of 5 mL) then aliquoted and stored at −80 °C. The dogs were anesthetized with acepromazine (0.1 mg/kg iv, Vetnil, Brazil) and splenic aspirate samples were obtained using a puncture technique previously described by Barrouin-Melo et al. (2006), which was modified to incorporate guidance by a portable ultrasound device (Solca Mda et al., 2014). Lidocaine solution (0.1%) was used as local anesthesia to perform cutaneous punch biopsies. Skin samples with a diameter of 4 mm were collected from the internal surface of a single lesion-free ear.

2.4. Parasitological testing and serological evaluation

Splenic aspirate samples were collected as explained above and culturing was performed as previously described (Barrouin-Melo et al., 2006; Solca Mda et al., 2014). Briefly, splenic aspirates were cultured for a period of four weeks in Novy–MacNeal–Nicolle (NNN) biphasic medium, supplemented with 20% FBS (Fetal Bovine Serum, Gibco BRL, New York, USA) and 50 μg/mL of gentamicin to avoid contamination (Sigma Chemical Co., St. Louis, MO) at 23 °C (Barrouin-Melo et al., 2006). Microscopy for parasite detection was performed at weekly intervals for no less than four weeks. Each splenic culture was prepared in duplicate and double-checked to avoid misidentification.

The serological evaluation was realized usind the immunochromatographic rapid test DPP® LVC (Bio-Manguinhos, Brazil). The diagnostic test procedures with DPP® LVC was realized in accordance with manufacturer recommendations.

2.5. Xenodiagnosis

All 23 animals were submitted to xenodiagnosis no later than 10 days following CVL diagnosis and tissue sample collection. Adult *Lu. longipalpis* were obtained from closed colonies at the Laboratory of Veterinary Infectious Diseases at the Federal University of Bahia and the other at the Gonçalo Moniz Research Center (FIOCRUZ-BA). Both sand fly colonies were originally formed by sandflies captured in the endemic municipality of Ipecaetá, located in northeastern Bahia. Insects from 8 to 12 generations were used in xenodiagnosis experimentation. To carry out this procedure, sandflies, including both 30–40 females and 10–15 males, were starved for 24 h, then placed in transparent PVC cylinders covered with fine mesh over one end. Each dog had a cylinder affixed to one of its ears without lesions or scabs, so as to maintain contact for 40 min, thus allowing the insects to feed on the dogs' blood. Xenodiagnosis was only considered successful when an estimated 70% or more of the sandflies were observed taking a blood meal. In only one xenodiagnosis procedure, the estimated percentage was lower than 70%, thus the procedure was considered invalid, and was repeated the next day. All sandflies were subsequently kept for a period of five days and allowed to feed on a saturated glucose solution. After five days, all surviving sandflies were frozen at −80 °C until DNA extraction. qPCR was then performed to determine the presence of parasite DNA and quantify parasite load. The capacity of naturally infected dogs to infect sand fly vectors with *L. infantum* was assessed by considering multiple factors: (1) canine infectiousness to sandflies, estimated as the percentage of dogs that successfully infected sandflies with *L. infantum* during xenodiagnosis; (2) sand fly infection rate, estimated as the number of sandflies that were successfully infected during xenodiagnosis; and (3) the average parasite load detected in successfully infected sandflies following xenodiagnosis.

2.6. DNA extraction from canine tissue and sandflies

DNA extraction from splenic aspirates, skin biopsies (4 mm), and whole blood (200 μL) samples was performed using the DNeasy Blood & Tissue kit in accordance with the manufacturer's instructions (Qiagen, Hilden, Germany). The females *of Lu. longipalpis* used in xenodiagnosis were individually transferred to 1.5 mL microcentrifuge tubes and DNA extraction was performed as previously described (Michalsky et al., 2007). DNA concentration and quality were then assessed and each sample was aliquoted and stored at −20 °C.

2.7. Leishmania *kinetoplast DNA (kDNA) Quantification*

To determine parasite load in canine samples and sandflies, *Leishmania* DNA was quantified by qPCR assays employing an amplification procedure previously described (Francino et al., 2006) and modified by Solcà and collaborators (Solca Mda et al., 2014). The taqMan-MGB probe and PCR primers were designed to target conserved DNA regions of the kinetoplast minicircle DNA from *L. infantum* to obtain a 120-bp amplicon. The qPCR amplification protocol (Solca Mda et al., 2014) employed the following primers: forward primer 5′-AACTTTTCTGGTCCTCCGGGTAG-3′ (Leish-1) and reverse primer 5′-ACCCCCAGTTTCCCGCC-3′ (Leish-2), both at a final concentration of 900 nM. A fluorogenic probe 5′-AAAAATGGGTGCAGAAAT-3′ was used for detection, synthesized using a FAM reporter molecule attached to the 5′ end, as well as a MGB-NFQ quencher linked to the 3′-end (Perkin-Elmer Applied Biosystems) at a final concentration of 200 nM. Parasite load was determined by comparing cycle threshold (CT) values derived from a standard curve, which was obtained from DNA amplification using 10-fold serial dilutions of *Leishmania* DNA performed in triplicate, ranging from 10^5 to 10^{-1} parasites. In order to minimize interplate variation, the averaged CT values for each sample were normalized based on a common fluorescence detection baseline value. A CT value was recorded at the point at which its fluorescence signal crossed the established detection baseline. In order to overcome the limitations caused by melanin present in skin samples submitted to PCR, all steps leading up to DNA amplification were performed in the presence of bovine serum albumin (2.5 μg/each reaction) (Sigma Chemical) to prevent the inhibition of PCR (Giambernardi et al., 1998).

Two separate receiver operating characteristic (ROC) curves were plotted to obtain the optimal CT cut-off point for considering either a canine or sand fly sample as positive. First, to determine the CT cut-off point for *Leishmania* DNA in canine samples, the amplification results from 20 *Leishmania*-negative samples and 20 *Leishmania*-positive samples were plotted on a ROC curve. Next, for *Leishmania* DNA detection in sand fly samples, a ROC curve was constructed using the amplification results from 40 sandflies used in the xenodiagnosis of two confirmed negative animals (non-infected dogs from a non-endemic area with negative results in serology by DPP® LVC, screening test recommended by Brazilian Health Ministry, and negative results in PCR and culture of spleen aspirate) and 12 male sandflies reared in a laboratory spiked with *Leishmania* DNA (positive controls). Finally, a range of CT cut-off points across each ROC curve were analyzed for sensitivity and specificity to determine its optimal CT cut-off value. Canine tissue and sand fly samples were considered positive when CT values were less than or equal to its corresponding ROC curve CT cut-off value.

To evaluate dog DNA integrity, amplification of the 18S rRNA gene (Perkin-Elmer Applied Biosystems) was used as an internal reference of genomic canine DNA. Only canine DNA samples that successfully amplified the 18S rRNA gene were included. Predeveloped TaqMan assay reagents were used in accordance with manufacturer's recommendations. The slope of the standard curve for the 18S rRNA gene corresponded to −3.406 (SD ± 0.415) and an average slope was determined from CT values obtained from five independent experiments with a corresponding correlation coefficient (r^2) of 0.993 (SD ± 0.002). The results of parasite loads in infected dogs are expressed as the number of parasites per 10 mg of DNA of canine host tissue.

2.8. Statistical analysis

Differences in parasite loads among dogs with different clinical manifestations, as well as parasite loads in infected sandflies used for xenodiagnosis, were evaluated using the Mann-Whitney test. Fisher's exact test was used to compare infectiousness of dogs with presenting different clinical manifestation. Spearman's rank correlation coefficient was used to conduct correlation analysis. All p values < 0.05 were considered to be statistically significant.

3. Results

3.1. Study group

Of the 23 dogs included in this study, 13 were classified as dogs with subclinical infection and 10 as with symptomatic infection (Table 1). Cultures of splenic aspirate were positive in nine animals (39%), 6 with subclinical infection (46%) and 3 with symptomatic infection (30%) while serology by DPP was positive in eight (35%), 3 with subclinical infection (23%) and 5 with symptomatic infection (50%). All dogs tested positive by qPCR with respect to at least one of the tissue types evaluated, including 18 positive skin samples (78.2%), 10 with subclinical infection, 77%; 8 with symptomatic infection, 80%, 18 splenic aspirate samples (78.2%), 11 with subclinical infection (75%) and 7 with symptomatic infection (70%), and eight blood samples (35%), 4 with subclinical infection (31%) and 4 with symptomatic infection (40%).

3.2. Parasite load in tissue samples from infected dogs with diverse clinical manifestations of CVL

The median values found for parasite load in each evaluated canine tissue type were: 316 parasites in skin and 1335 in splenic aspirate (Table 1). Since in 15 out of 23 dogs, parasite DNA was not detected in blood samples, the median parasite load resulted in 0. Considered only samples from dogs that qPCR tested positive (n = 8), the median parasite load resulted in 309. No significant differences were found when comparing detected parasite load values within a given tissue type with respect to clinical manifestations of CVL (dogs with subclinical or symptomatic infection) (Fig. 1).

With respect to correlation testing considering parasite burden among the three tissue types evaluated, positive correlations were observed between parasite load in the blood and spleen (Spearman's; r = 0.57, P < 0.001), blood and skin (Spearman's; r = 0.64, p < 0.0001), and spleen and skin (Spearman's; r = 0.57, p < 0.001).

3.3. Capacity of naturally infected dogs to infect sand fly vectors with parasites

An assessment of the capacity of naturally infected dogs to infect sand fly vectors with parasites revealed that among the 653 sandflies used in xenodiagnosis, only 74 (11.4%) tested positive for *Leishmania* DNA by qPCR (Table 1). Canine infectiousness to sandflies, calculated as the percentage of dogs that successfully transmitted parasites to sandflies during xenodiagnosis, was similar in dogs with subclinical infection (54%; 07/13) and symptomatic infection (40%; 4/10) (p = 0.39). Further analysis of these dogs' capacity to infect sandflies showed that 44% of the infected animals

Table 1
Evaluation of naturally infected dogs with different clinical manifestations according to positivity in diagnostic tests for Leishmania infection and the capacity to infect sand fly vector.
Clinical classification/Dog no.

Clinical classification/Dog no.	Clinical Score	Diagnostic Test Type					Canine capacity of infect sandflies		
		DPP	Aspirate Spleen Culture	qPCR (*Leishmania* kDNA)			Infectiousness	Infection rate	Parasite load detected
				Skin	Spleen	Blood			
Subclinical infection									
01	0	–	+	89	274	1335	1	01/22 (4.5%)	3
02	0	–	–	0	0	113	0	0/31 (0%)	0
03	0	–	+	1238	0	660	0	0/42 (0%)	0
04	0	–	–	0	0	447	1	02/32 (6.25%)	3
05	2	+	+	18,020,075	6315	339,826	1	01/36 (2.7%)	45
06	1	–	–	0	0	18437	0	00/39 (0%)	0
07	2	–	–	24	0	0	0	0/14 (0%)	0
08	1	–	–	66	118	0	1	01/23 (4.3%)	4
09	1	–	–	645	0	628	1	1/41 (2.4%)	3
10	3	–	+	1553	0	966	1	25/31 (80.6%)	689
11	1	+	–	1014	0	26,274	0	0/25 (0%)	0
12	2	+	+	97	0	602	0	0/28 (0%)	0
13	2	–	+	59,187,691	509	17,105	1	01/28 (3.5%)	3
Symptomatic infection									
14	5	–	–	316	0	0	0	00/32 (0%)	0
15	7	–	–	67	0	0	0	0/23 (0%)	0
16	6	–	–	0	0	9382	0	00/25 (0%)	0
17	8	+	–	42,632	0	5640	0	0/25 (0%)	0
18	10	–	–	147	0	0	1	01/24 (4.1%)	8
19	12	+	–	737,615	58	17,602	0	0/13 (0%)	0
20	10	+	–	0	0	2198	0	0/23 (0%)	0
21	17	+	+	3,451,889,073	151194	17,863,788	1	28/36 (77.7%)	29774
22	8	–	+	30,625,119	344	19,058,029	1	11/29 (38%)	197
23	7	+	+	22,904,007	179	3440,041	1	02/28 (7.1%)	970
Total positivity rate		08/23 (35%)	09/23 (39.1%)	18/23 (78.2%)	08/23 (35%)	18/23 (78.2%)	11/23 (47.8%)	74/653 (11.3%)	1338

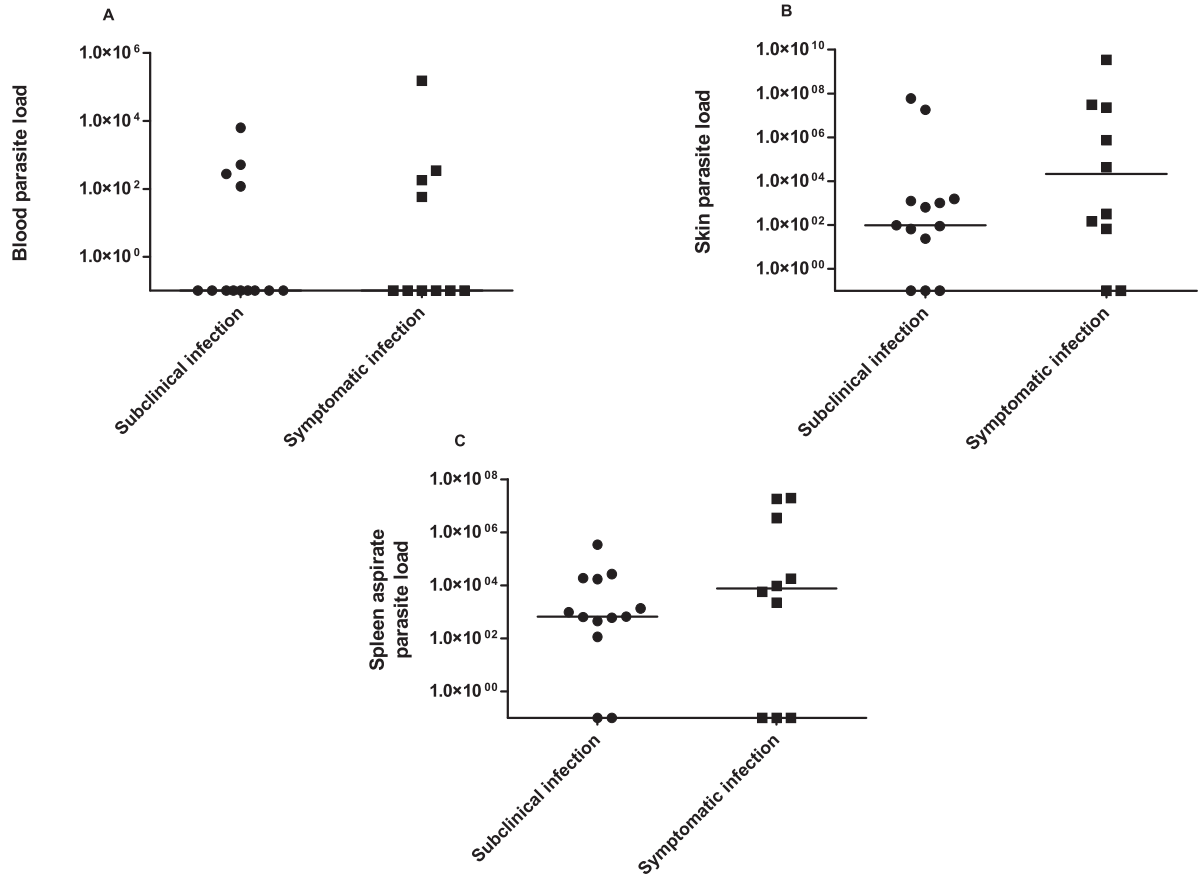

Fig. 1. Blood, skin and spleen samples parasite load of infected dogs according to clinical classification. Blood (A), skin (B), and spleen aspirate samples (C) were obtained from dogs classified with a different form of clinical manifestations of CVL: subclinical infection or symptomatic infection. Parasite load was detected using qPCR in all skin, blood, and splenic aspirate samples. Each plot represents the detected parasite load in an individual dog.

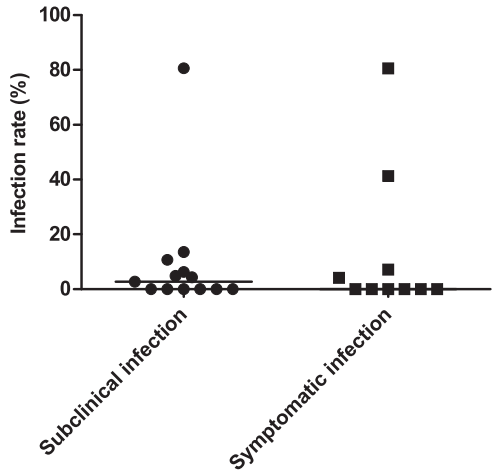

Fig. 2. Sand fly infection rate of naturally infected dogs according to clinical classification. Sand fly infection rates, calculated as the number of successfully infected sandflies following xenodiagnostic procedures, distributed according to clinical manifestation.

were capable of successfully infect sand fly vector with parasites, although 80% of these dogs infected to a relatively small number of sandflies (n = 1 to 2). Sand fly infection rate, which measures the number of sandflies that were positively infected following xenodiagnosis, remained similar between dogs with subclinical infection (9%) and with symptomatic infection (13%) dogs (Fig. 2).

Parasite load quantification performed in each sand fly submitted to xenodiagnosis revealed that parasite load ranged from 1 to 5971 parasites in the 74 infected sandflies, with a median value of 28 parasites, the 25% percentile was 4 and 75% percentile was 112.5 parasites. The median parasite loads among sandflies that became successfully infected was 10, after feeding on dogs with subclinical infection, and 84, after feeding on dogs with symptomatic infection. No differences were found when comparing the average parasite loads in infected sandflies, irrespective of the clinical classification of the animals on which the vectors fed (Fig. 3A). Fig. 3B illustrates individual sand fly parasite loads five days after xenodiagnosis. Most sandflies showed low parasite loads and, notably, a single highly symptomatic dog (score = 17, dog no 21) was found to be responsible for the highest parasite loads detected in sandflies (total: 29,774 parasites counted in 28 infected sandflies) (Fig. 3B).

Parasite load values in the dogs' blood and skin were shown to be positively correlated with canine infectiousness to sandflies (blood $r = 0.65$, $p < 0.001$; skin $r = 0.42$, $p = 0.05$) (Fig. 4A and B), yet no correlations were found with respect to spleen tissue ($r = 0.27$, $p = 0.21$). Interestingly, positive correlations were also found between parasite load in blood ($r = 0.59$ $P < 0.01$) or skin ($r = 0.43$, $P \leq 0.05$) and sand fly infection rate (Fig. 4C and D). Finally, a positive correlation was additionally found between parasite load in canine blood ($r = 0.66$, $P < 0.001$) and skin ($r = 0.54$, $P < 0.01$) and parasite burden in sandflies following xenodiagnosis (Fig. 5A and B). No correlations were found when comparing the parasite loads detected in splenic aspirate samples (Fig. 5C) to the parasite burden found in vectors five days after feeding.

4. Discussion

The present study represents an initial attempt to establish correlations between the intensity of parasite load in canine tissue and the parasite load detected in sandflies five days after feeding on naturally infected dogs with a range of clinical presentations. Almost half of the dogs were found to be capable of infect sand fly vectors with parasites, regardless of their clinical classification and corresponding clinical score. This finding supports the notion that a particular canine clinical manifestation of VL is not indicative of a dog's capacity to infect sand fly vectors with parasites. Remarkably, among these infective dogs, most (82%) infected to a relatively limited number of sandflies (between 1 and 2), which corresponds to approximately 10% of the total number of sandflies used in xenodiagnosis procedures. These results are consistent with those obtained by Courtenay et al. (2002), who similarly showed that most of the infective dogs studied infected no more than 10% of the sandflies employed. Taken together, these findings would seem to suggest that, in a given endemic area, one would expect a low number of infective dogs to successfully infect a proportionately low number of sandflies with parasites.

The present study utilized qPCR to detect parasite DNA in vectors five days following xenodiagnosis procedures, with low parasite burden found in the vast majority of sandflies. This may be explained by the choice to assess parasite load in the vectors at only five days post-xenodiagnosis. Undoubtedly, by quantifying the number of infective metacyclic forms of promastigotes under direct observation at seven days or later following blood-feeding, sensitivity would be enhanced. Additionally, this important information would provide a notion of how many infective parasites could be successfully transmitted to another mammalian host in a subsequent sand fly blood feeding. Another possibility to improve sensitivity with respect to this procedure is performing xenodiagnosis in animal lesion sites, as previously described by Aslan et al. (2016) in an experimental model of CVL. Future studies should strive to not only comprehensively address the proper site on the animal's skin to perform xenodiagnosis in naturally infected dogs, but also quantify parasite burden, in the sandflies that were successfully blood-fed, at time points seven days or later following blood-feeding.

The low infection rates and low parasite loads detected in sandflies in the present study support the notion that most vectors do not significantly impact the urban VL transmission cycle. Interestingly, we found a very high parasite load in the blood and skin of one of the dogs we evaluated, which was able to infect a high number of sandflies (78%) resulting in high parasite loads (5.9 to 10×10^3 parasites) at five days after feeding. We recognize that since our dog population represents a convenience sampling, the present findings are not indicative of the actual transmission cycle seen in a given dog population within an endemic area. Nonetheless, we speculate that a low number of highly infected dogs would likely have a more significant impact on parasite transmission to sandflies, while higher numbers of less-infected dogs should present less risk of transmissibility in the urban VL transmission cycle in endemic areas.

In the present report we also found that neither canine infectiousness nor sand fly infection rates have been found associated to CVL clinical manifestations similar to other authors (Guarga et al., 2000; Laurenti et al., 2013; Magalhaes-Junior et al., 2016; Molina et al., 1994). Different from us, other authors have demonstrated that both the capacity of dogs infect sandflies and sand fly infection rates are positively correlated with the presence of clinical signs in infected dogs (da Costa-Val et al., 2007; Vercosa et al., 2008). These discrepancies in the literature may be due to the lack of standardization regarding xenodiagnoses.

Xenodiagnosis is considered the only way to accurately identify which dogs are infective to sand fly vectors (Guarga et al., 2000; Michalsky et al., 2007; Molina et al., 1994), yet, due to its inherent limitations, this technique is unable to improve the effectiveness of programs designed to control leishmaniasis in endemic areas. Thus, the identification of tissues in which parasite load is positively correlated with canine infectiousness is paramount. Deane and Deane (1954) showed that the skin of asymptomatic dogs in an endemic area can harbor *Leishmania* parasites, indicating this tissue type could be a potential marker of infectiousness. Interest-

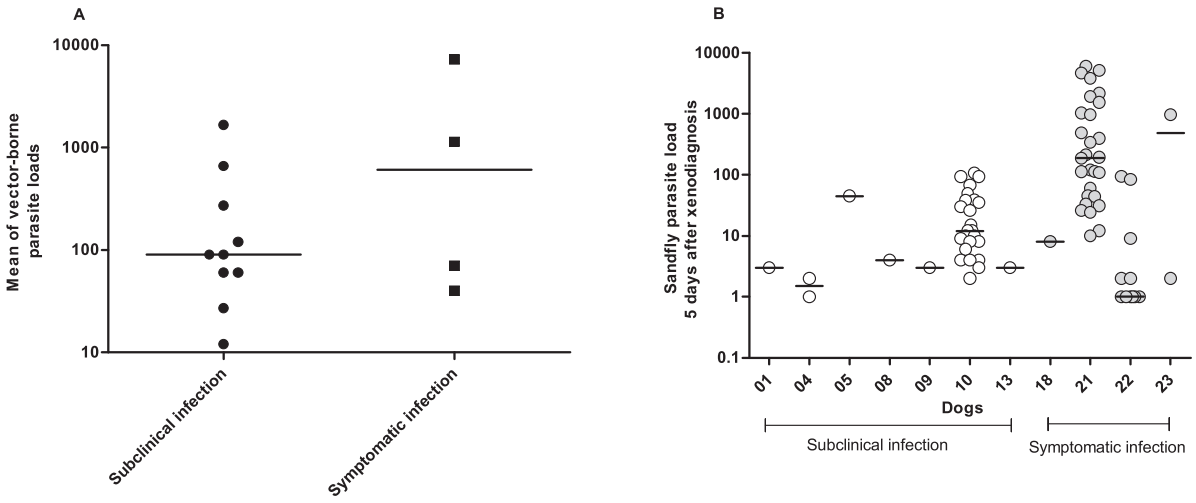

Fig. 3. Parasite load in infected sandflies fed on naturally infected dogs with a range of clinical classifications. (A) Mean parasite loads in sandflies measured 5 days after being fed on naturally infected dogs, which were classified according to clinical VL presentation. (B) Transmission capacity of infective dogs, measured as the parasite load in each sand fly 5 days following xenodiagnosis, according to clinical manifestation of CVL.

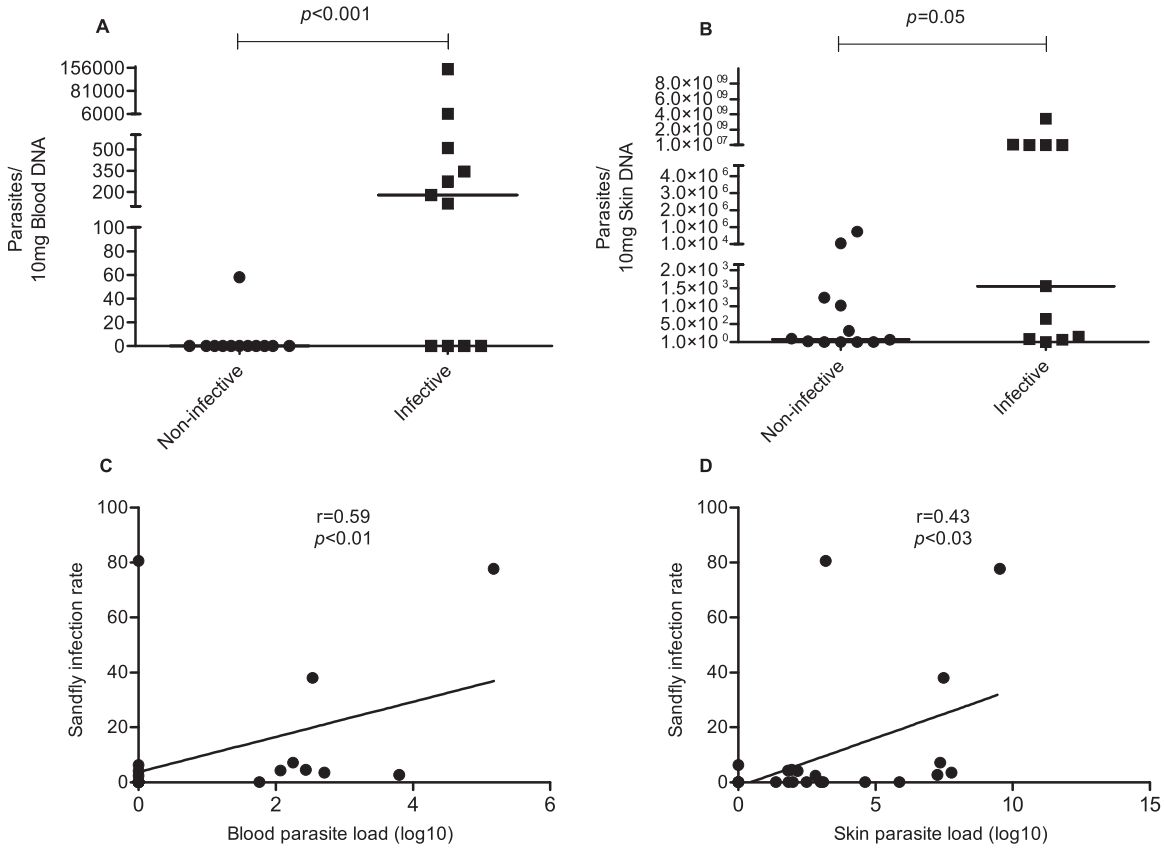

Fig. 4. Parasite load in the blood and skin of naturally infected dogs in association with canine capacity to infect sand fly: distribution of parasite load in blood (A) and skin (B) of non-infective and infective dogs. Correlation between parasite load in blood (C) and skin (D) of dogs with sand fly infection rate following xenodiagnosis. Parasite load was detected by qPCR in blood and skin samples and it is represented as the number of parasites measured in 10 mg of DNA. Each plot represents the detected parasite load in an individual dog. Relevant Spearman correlation coefficients (r) and *p* values are shown.

ingly, the present report we found positive correlations between parasite loads in both skin and blood with respect to canine infectiousness to the sand fly vector, as well as the sand fly infection rate and parasite load in sand fly vectors following xenodiagnosis. Furthermore, De Amorim et al. (2011) and Courtenay et al. (2002) also demonstrated an association between high parasite load in dog skin and canine infectiousness. These authors considered a high parasite load in dog skin to be a strong predictor of canine infectiousness to sandflies (Courtenay et al., 2014). Moreover, Vercosa et al. (2008) demonstrated a positive association between canine infectiousness to sandflies and an elevated degree of blood parasitism in dogs, which is in agreement with our finding of a positive correlation between parasite load in canine blood and dog capacity to infect sandflies. Taken together, these findings support the notion that

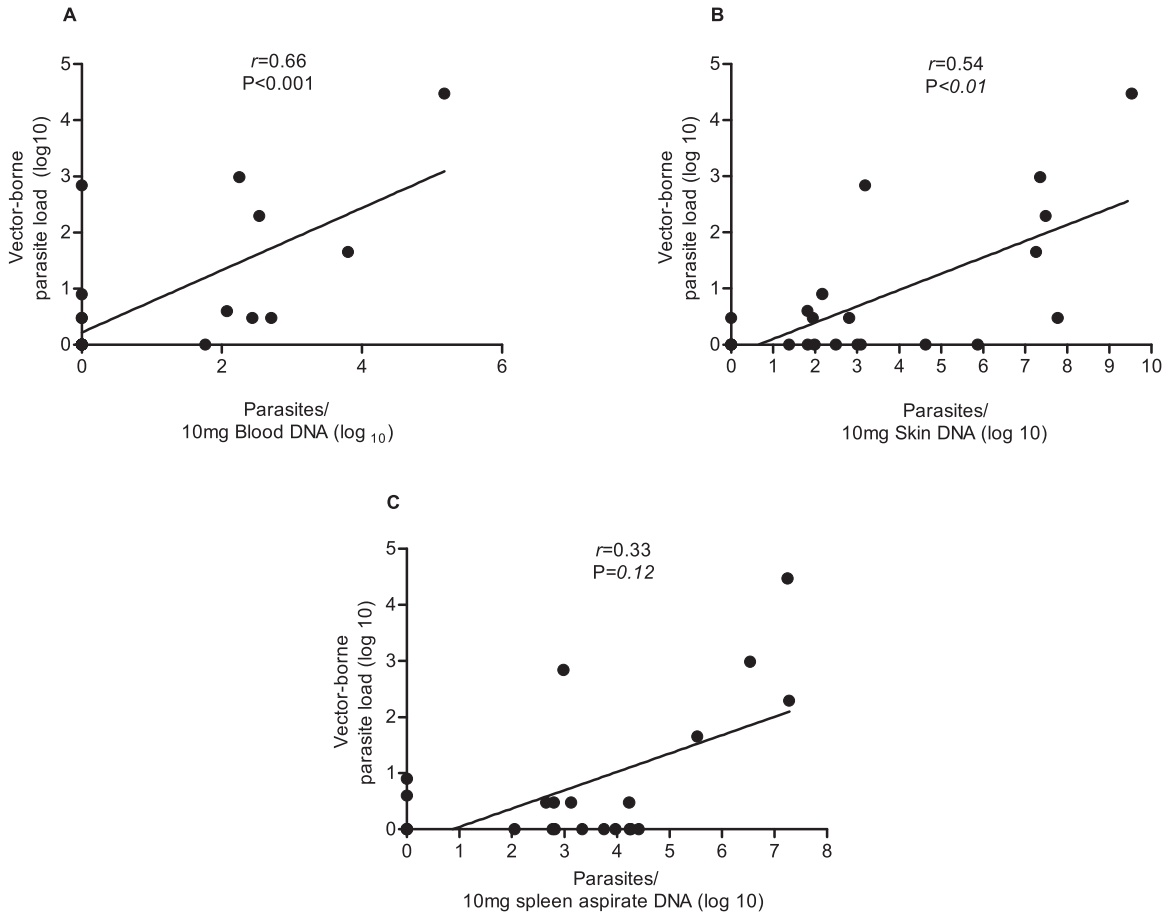

Fig. 5. Evaluation of parasite load in infected sandflies in relation to parasite load in different tissues of naturally infected dogs. Each plot represents the mean parasite load detected in sandflies 5 days following xenodiagnosis in accordance with the parasite load detected in canine tissue type: blood (A), skin (B) and splenic aspirate (C). Relevant Spearman correlation coefficients (r) and *p* values for each tissue type are shown in the respective plots.

parasite load in both canine skin and blood could potentially serve as reliable markers of the canine capacity to infect sand fly vector.

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