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ORIGINAL PAPER

Parasitism and inflammation in ear skin and in genital tissues of symptomatic and asymptomatic male dogs with visceral leishmaniasis

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Abstract Canine visceral leishmaniasis (CVL) is transmitted through vector, although venereal transmission has been suggested. This study aimed to compare the parasitic loads and inflammatory processes in genital tissues with ear skin from seropositive male dogs. Forty-five seropositive dogs were separated into groups containing symptomatic (n = 23) and asymptomatic (n = 22) animals. The control group (n = 2) healthy animals with seronegative and negative results in direct parasitological test. Samples of ear tip skin, prepuce, glans penis, testis, epididymis, and prostate were collected for evaluation of parasitic load and inflammatory infiltrate. Although ear tip skin was the most intensely parasitized, prepuce and epididymis revealed no difference in parasitism when compared with ear tip skin ($P > 0.05$). Parasitic loads in testis and prostate were lower than other tissues ($P < 0.05$). Parasitism in glans penis was high, similar to prepuce and epididymis, but lower than ear tip skin. High parasitism was more frequent in symptomatic dogs than asymptomatic animals. Severe inflammatory processes were more frequent within the symptomatic animals compared with asymptomatic and more predominant in prepuce and epididymis. Ear tip skin and genital tissues presented signs of chronic inflammation. There were weak and moderate positive correlations between parasitic loads and inflammatory processes. Our results demonstrate that, likewise with the ear tip skin, the genital of seropositive dogs can carry a large number of *Leishmania infantum* amastigotes and this process are more intense in symptomatic animals. These data have important implications for understanding the possibility of venereal transmission of CVL.

Keywords *Leishmania infantum* · Venereal transmission · Amastigotes · Parasitic load · Inflammatory infiltrate

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Introduction

Visceral leishmaniasis (VL) constitutes an important public health problem in several regions of the world (World Health Organization 2014). The principal mode of transmission of *Leishmania infantum*, the main causative agent of VL in Brazil, is via the bites of infected female sandflies (Spiegel et al. 2016). Furthermore, domestic dogs are considered the main reservoirs of the parasite in the urban environment (Silva et al. 2006).

Although VL is transmitted mainly by insect vectors, examples of vector-independent transmission of the disease have been described. Thus, Pangrazio et al. (2009) discovered DNA of *L. infantum* in placentas and in fetuses derived from seropositive bitches, while Diniz et al. (2005) found *L. infantum* DNA in semen samples from dogs with clinical symptoms of canine visceral leishmaniasis (CVL). Moreover, Silva et al. (2009) reported the transmission of VL through copulation between healthy bitches and seropositive dogs whose semen contained *L. infantum* DNA.

Although a number of studies have focused on the parasitic load and the inflammatory profile of tissues, particularly skin and lymph nodes, derived from dogs naturally infected with *L. infantum* (Solano-Gallego et al. 2004; Tafuri et al. 2004; Reis et al. 2006a, b Lima et al. 2007; Teixeira Neto et al. 2010), investigations involving genital tissues are scarce (Diniz et al. 2005; Benites et al. 2011, Quintal et al. 2016). On this basis, the present study aimed to evaluate the parasitic

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loads and inflammatory processes in genital tissues from seropositive (symptomatic and asymptomatic) male dogs. To the best of our knowledge, our study is the first to compare parasitic loads and inflammatory profiles of samples from the genital region and ear tip skin obtained from *Leishmania*-infected male dogs.

Materials and methods

Ethical issues

Details of the study were submitted to and approved by the Ethics Committee for Research with Animals of the Universidade Federal de São João Del Rei under protocol number 08/2013. All procedures involving experimental animals were conducted according to the guidelines of the Colégio Brasileiro de Experimentação Animal (COBEA).

Animals

The study population was selected from animals held at the zoonosis control centers located in Belo Horizonte and in Divinópolis, MG, Brazil, and comprised forty-five unneutered male dogs of mixed breed. Each dog had been diagnosed VL-positive by enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence test (IIFT; titer >1:40), and the rapid dual path platform (DPP®) test (Bio-Manguinhos, Fiocruz, Rio de Janeiro, RJ, Brazil). Experimental animals were separated into two groups including (i) symptomatic dogs (SD group; $n = 23$) presenting at least three clinical signs of VL (lesions at the tips of the ears, mucosal pallor, onychogryphosis, hepatomegaly, splenomegaly, and exfoliative dermatitis) and (ii) asymptomatic dogs (AD group; $n = 21$) presenting no clinical signs of the disease. Healthy controls (CD group; $n = 2$) comprised animals that were seronegative in the ELISA, IIFT, and DPP tests and negative in the direct parasitological test.

Sampling

Experimental animals were submitted to euthanasia immediately prior to sampling by intravenous injection of thiopental (20 mg/kg) followed by potassium chloride (100 mg/kg). Bone marrow samples were collected in order to identify the species of *Leishmania* sp. using polymerase chain reaction (PCR) methodology, while tissue samples from ear tip skin, prepuce, glans penis, testis, epididymis, and prostate were collected for evaluation of parasitic load by immunohistochemistry (IHC) and of inflammatory infiltrate by hematoxylin and eosin (HE) staining. Ear tip skin was selected as the reference for comparative evaluations because this tissue normally presents a high parasitic load in seropositive dogs.

PCR analyses

Bone marrow samples were stored in microcentrifuge tubes in a freezer at −20 °C until required for analysis. DNA extraction was performed using QIAamp® DNA Blood Mini Kits (Qiagen, Santa Clarita, CA, USA) following the instructions of the manufacturer. The target for PCR amplification was the gene encoding the 70-kD heat shock protein (Hsp70) in *Leishmania* sp. since this family of genes exhibits highly conserved sequences (Folgueira et al. 2007). PCR reactions were carried out with 5 μL of DNA template and primers designed to amplify a 1300-bp fragment of Hsp70 as follows: 5′GACGGTGCCTGCCTACTTCAA3′ (Hsp70-forward) and 5′CCGCCCATGCTCTGGTACATC3′ (Hsp70-reverse). The amplicons were separated by electrophoresis on 2 % agarose gels and subsequently stained with ethidium bromide. PCR-positive DNA samples were submitted to restriction fragment length polymorphism (RFLP) using the restriction endonuclease *Hae*III (Garcia et al. 2004). RFLP restriction fragments were separated by electrophoresis on 2 % agarose gels and the patterns compared with those obtained by digestion of the PCR products from control strains of *Leishmania amazonensis* (IFLA/BR/67/PH8), *Leishmania braziliensis* (MHOM/BR/75/M2903), and *L. infantum* (MHOM/BR/74/PP75).

IHC analyses

The IHC protocol described by Tafuri et al. (2004) was employed in order to obtain immunostained *Leishmania* sp. amastigotes. Slides were examined under a Zeiss (Jena, Germany) Axio light microscope coupled with an AxioCam ERc 5s camera, and image analysis was performed with the aid of AxioVision Rel. 4.8 imaging software, with ×400 magnification. Images of 10 fields from each slide were analyzed, and parasitic load was determined according to a modified version of the method described by Giunchetti et al. (2008). Considering that the IHC and Leishman Donovan Unit (LDU) techniques exhibit a strong positive correlation and that each immunostained amastigote occupies an area in the image, the parasitic load of each tissue was estimated from the mean percentage of immunostained area (% area) in each picture, according to the follow definitions: 0 (absent), 0.01–0.05 (low parasitism; 1 to 4 stained amastigotes/field), 0.06–0.39 (moderate parasitism; 5 to 25 stained amastigotes/field), and >0.4 (high parasitism; >25 stained amastigotes/field) (Giunchetti et al. 2008).

Hematoxylin and eosin staining

In order to characterize the inflammatory infiltrate, tissue samples were prepared and mounted onto slides, stained with HE, and examined under a light microscope at magnifications of

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×200 and ×400. Semiquantitative evaluation of the samples was performed by estimating the area occupied by the inflammatory infiltrate expressed according to the scores 0 (absent), 1 (light—infiltrate area less than 1/3/field), 2 (moderate—infiltrate area between 1/3 and 2/3/field), and 3 (severe—infiltrate area more than 2/3/field).

Statistical analyses

Analyses were performed with the aid of GraphPad Prism™ version 5.0 (GraphPad Software, San Diego, CA, USA), Epi Info™ version 7.1.3 (Center of Disease Control and Prevention, Atlanta, GA, USA), and R version 3.1.0 (R Development Core Team, Wirtschaftsuniversität, Vienna, Austria) software with the level of significance set at 5 % for all tests. The distribution of the data was nonparametric according to *Q–Q* plots and Shapiro test; therefore, potential associations between inflammatory infiltrate and parasitic load (expressed as mean percentage of immunostained area detected by IHC) in different tissues were investigated using Spearman's rank correlation coefficient (*r*) with the classification adopted by Dancey and Reidy (2006). The Kruskal-Wallis test was employed to detect differences in parasitism between the tissues, while the Dunn test was performed to identify in which tissues these differences were significant. Differences in parasitic load between the SD and AD groups were established using the Wilcoxon signed rank test.

Results

Identification of *Leishmania* species

PCR amplification of the 1300-bp fragment of the gene encoding Hsp70 in *Leishmania* species was observed in 30 of the 44 bone marrow samples analyzed. PCR-RFLP analyses with the restriction enzyme *Hae* III revealed the presence of DNA from *L. infantum* in 19 of the PCR-positive samples. In the other 11 samples, the amount of amplified product digested by *Hae* III did not allow the conclusive evaluation of the *Leishmania* species involved in the infection. In none of the samples was observed the digestion pattern similar to *L. amazonensis* or *L. braziliensis*. These results, along with the symptomatology of dogs, are described in Table 1.

Parasitic load in sample tissues

Leishmania sp. amastigotes were found in tissue samples obtained from ear tip skin, glans penis, prepuce, testis, epididymis, and prostate of seropositive dogs (Fig. 1). The parasitic loads in tissues derived from the SD group were significantly higher ($P < 0.05$) than those from the AD group, with the single exception of prostate samples for which the *P* value (0.056) was close to significance (Fig. 2).

Leishmania sp. amastigotes concentrated around blood vessels and hair follicles in ear tip skin and prepuce samples presenting low or moderate parasitism, whereas in samples with high parasitism, the amastigotes were scattered throughout the tissue. In glans penis tissue, amastigotes occurred adjacent to the epithelium even in samples with high parasitism. Samples from testis of members of both SD and AD groups presented low parasitism, and *Leishmania* sp. amastigotes could be observed only in the areas between the testis. Epididymis samples displayed high parasitism in the intertubular space of the duct. No *Leishmania* sp. amastigotes were observed in the lumen of the seminiferous tubules of the testis, but amastigotes were observed in SD epididymis imprint (Fig. 3).

Significant differences ($P < 0.001$) were observed between parasitic loads determined in samples of ear tip skin and genital tissues from seropositive dogs, with ear tip skin showing the most intense parasitism (Fig. 4). Pairwise comparisons revealed that parasitic loads in the prepuce and epididymis were similar ($P > 0.05$) to that of the ear tip skin, while those of the testis and prostate samples were significantly lower in comparison with other tissues ($P < 0.05$). Parasitic loads in samples of glans penis were not significantly different from those of prepuce and epididymis samples but significantly lower than that of ear tip skin.

Inflammatory status of sample tissues

Frequency and intensity of inflammation in the various tissues derived from members of the SD, AD, and CD groups (Fig. 5) indicated that severe inflammatory processes were more frequent within the SD than the AD group and more predominant in prepuce, epididymis, and glans penis than in the other tissues.

Ear tip skin and genital tissue samples presented signs of chronic inflammation characterized by the presence of mononuclear cells together with large quantities of lymphocytes and plasmocytes. Inflammatory infiltrate of ear tip skin samples exhibiting light to moderate inflammation was more frequent around the blood vessels and skin annexes such as hair follicles and glands, whereas the distribution of infiltrate was rather diffuse in samples presenting severe inflammation. The distribution of inflammation in prepuce samples was similar to that established in ear tip skin, while in glans penis samples, the inflammatory infiltrate was observed in areas adjacent to the epithelium. In samples of testis and epididymis tissue, the inflammatory process was evident in the intertubular spaces and was associated with thickening of the ducts. In prostate samples, the inflammatory process could be observed in the interstitial septa of the prostatic tissue.

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Table 1 Results of canine bone marrow PCR test

	PCR(+) *Leishmania* spp.	% (PCR(+)/SD)	PCR(+) *L. infantum*	% (*L. infantum*(+)/ PCR(+)	PCR(−)	Total
SD	18	78.3	12	66.7	5	23
AD	12	57.1	7	58.3	9	21
Total	30	68.2	19	63.3	14	44

SD symptomatic dogs, *AD* asymptomatic dogs, (+) positive results, (−) negative results

Correlation between parasitic load and inflammatory response

In the ear tip skin samples, the correlation between parasitic loads (mean percentage of immunostained areas) and inflammatory processes was moderate ($r = 0.41$) and statistically significant ($P < 0.05$), but in the genital tissues, the correlations did not showed statistical significance ($P > 0.05$) (Fig. 6).

Discussion

Canine VL is a chronic infection that can affect the genital tissues of seropositive male dogs. In the present study, in an unprecedented way, we have demonstrated that prepuce and epididymis were heavily parasitized by *L. infantum*, in the same way that ear skin.

The parasitic load in genital tissues of symptomatic dogs (SD group) was significantly higher than that of animals

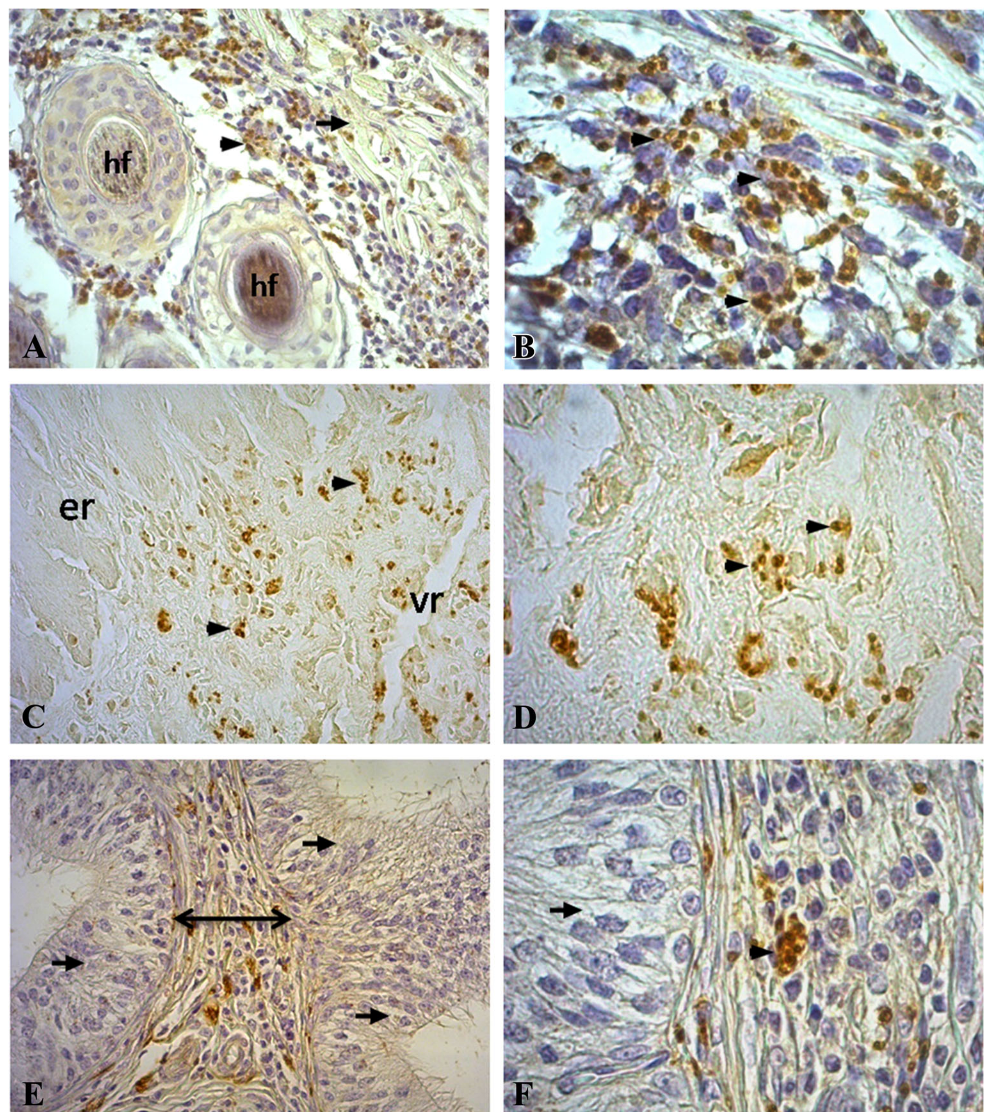

Fig. 1 Tissues evaluated with IHC technique. **a** Intensely parasitized ear tip (×400). Notice the large amount of immunostained (*black arrowhead*) around the hair follicles (*hf*), compared to areas with large amounts of connective tissue (*black arrow*). **b** Ear tip (×1000). Presence of amastigote nests in the tissue (*black arrowhead*). **c** Glans with intense parasitism (×400). A number of immunostained (*black arrowhead*) in the vascular region (*vr*) and a small amount in the epithelial region (*er*). **d** Glans (×1000). Amastigote forms in the tissue (*black arrowhead*). **e** Epididymis. Moderate parasitism (×400). Immunoblasts were found in the intertubular region (*two-headed black arrow*) but were not observed in the seminiferous tubules (*black arrow*). **f** Epididymis (×1000). Amastigotes form (*black arrowhead*). It was not observed in the markings on the wall of the seminiferous tubules (*black arrow*)

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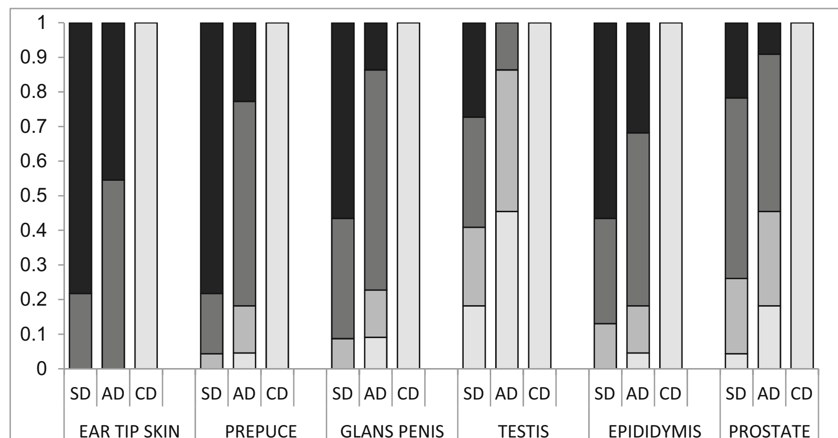

Fig. 2 Distribution of symptomatic (*SD*), asymptomatic (*AD*), and control (*CD*) groups with respect to the parasitic load of ear tip skin and genital tissues. Parasitic loads are distributed according to intensity defined as high (■), moderate (■), low (■), and absent (□)

presenting no signs of the disease (AD group). Although there are few specific references to genital male tissues (Diniz et al. 2005; Manna et al. 2012), we could observe that these results were analogous to those reported for other types of tissues (Solano-Gallego et al. 2004; Giunchetti et al. 2006, 2008; Reis et al. 2006a, 2006b; Xavier et al. 2006). Such findings demonstrate the clinical progression of CVL, since asymptomatic animals tend to exhibit mild degrees of parasitism in comparison with their symptomatic counterparts.

In the present study, no significant difference in the intensity of inflammation in ear tip skin and prepuce samples could be detected in SD or AD animals, although the frequency of severe inflammation was higher in symptomatic dogs. Giunchetti et al. (2006) reported that the frequencies of inflammation and severe inflammation were higher in ear skin samples from symptomatic dogs. A similar inflammatory pattern was reported by Lima et al. (2007).

Samples from glans penis tissue exhibited lower parasitic loads than those of ear tip skin, although higher levels of parasitism and of inflammatory processes were more frequent in the SD group than in the AD group. This result differs from that of Diniz et al. (2005) who noted the equivalence between symptomatic and asymptomatic animals regarding the mean percentage of immunostained area in glans tissue. Nonetheless, our results showed that parasitism in glans tissue was high and comparable with that of epididymis.

The intensity of parasitism and the severity of inflammation in testis samples were lower than those of other tissues, and the differences between the SD and AD groups were not statistically significant. Nevertheless, the frequency of high parasitism and inflammation was higher in the SD group, a finding that is in agreement with the reports of Diniz et al. (2005) and Manna et al. (2012). Even though the number of amastigotes in testis samples was low, such parasitism may have triggered inflammatory processes that precede degenerative alterations. Similar to Diniz et al. (2005), we observed signs of degeneration in the seminiferous tubules of the testis that were associated with the severity of inflammation and with the number of amastigotes in the tissue. In this context, Benites et al. (2011) described alterations in blood leukocytes in the testis of *Leishmania*-infected dogs, with higher numbers in symptomatic animals. Immunostained amastigotes were not observed in the seminiferous tubules of animals involved in the present study, probably because of the Sertoli cells. Such cells isolated the inner side of the seminiferous tubules containing the germ cells from the outer side that contacts with interstitial blood cells including leukocytes (De Cesaris et al. 1992). Thus, the inflammatory process was restricted to the interstitial zones

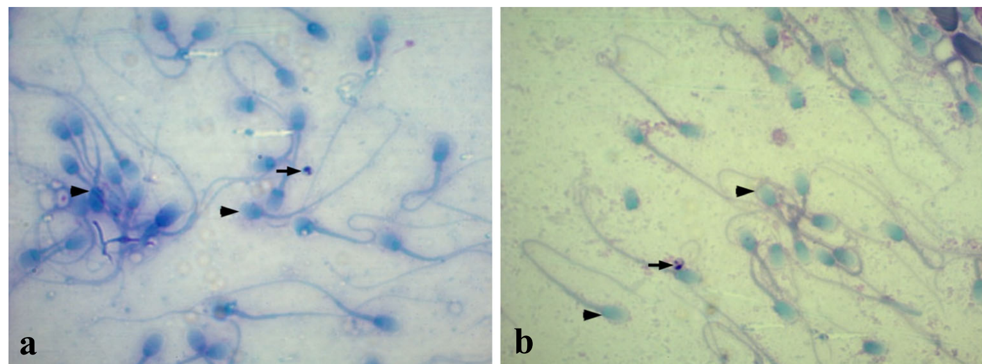

Fig. 3 **a**, **b** Epididymal imprint showing spermatozoa (*black arrowhead*) and amastigotes forms of *Leishmania* sp. (*black arrow*). ×1000

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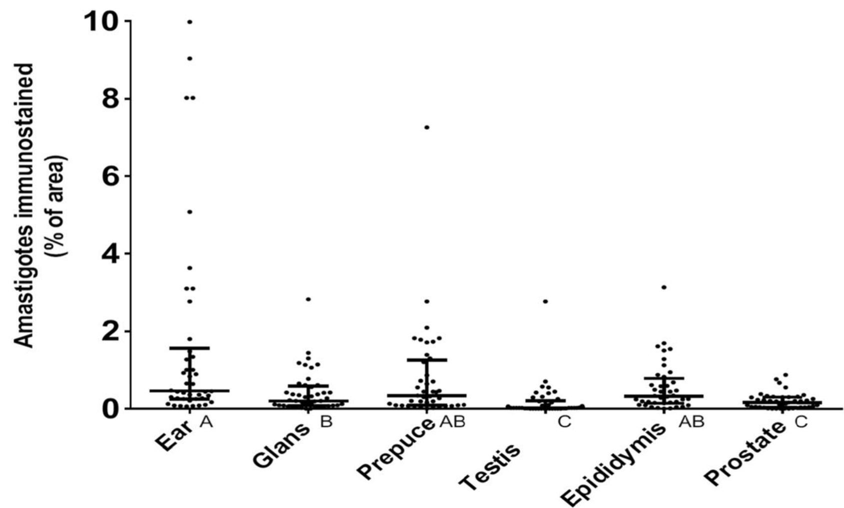

Fig. 4 Determination of parasite load of different tissues using the IHC technique. The same letter indicates similarity of parasitism: A > B > C

towards which the leukocytes migrate, as has been previously reported (Diniz et al. 2005; Benites et al. 2011).

The parasitism observed in epididymis samples was intense and, in a unique way, equivalent to that determined in ear tip skin samples. Like for the other tissues, high parasitism was more frequent in members of the SD group than in those of the AD group. The inflammatory process was also elevated in the epididymis. According to Diniz et al. (2005), the presence of *Leishmania* amastigotes in the epididymis could have resulted from a preexistent inflammation in which monocytes recruited macrophages containing *Leishmania*. The intense transmigration of macrophages, neutrophils, and lymphocytes towards the lumen of the epididymis could explain the large number of amastigotes in this duct (Benites et al. 2011), as well as the observation of amastigotes in the tissue imprint. This observation is relevant to the hypothesis venereal transmission of CVL, once the amastigotes from the epididymis could reach the female body together with male dog's semen. It is important to emphasize that amastigotes can remain viable in semen, since they resist to environments with low and neuter pH (Antoine et al. 1990, Cardoso et al. 2010).

The correlation between inflammatory infiltrate and parasitic load was not statistically significant in prostate tissue, confirming the observations reported by Diniz et al. (2005) and Benites et al. (2011). The same pattern was observed for most tissues.

A high parasitic load and the presence of a moderate to severe inflammatory process in reproductive tissues, as observed in the present study, may favor the non-vector-borne transmission of CVL. Macrophages containing *Leishmania* amastigotes, during copulation, can gain entry into the body of healthy females through lesions in genital mucous membranes. This is exemplified by other diseases, for example, the canine transmissible venereal tumor (CTVT) (Flórez et al. 2012). Transmission of CTVT occurs during copulation through the adherence of tumor cells from the affected animal onto the epithelium of the glans penis or vagina in the healthy partner, as long as the mucosal membrane is damaged. In our study, *Leishmania* amastigotes have been found in macrophages, as well as in CTVT cells (Albanese et al. 2002; Catone et al. 2003; Marino et al. 2012). It should be noted, however, that no tumors were observed in the canine population of the present study and, therefore, no diagnostic tests for CTVT were performed.

Although the venereal and vertical transmission hypothesis has been reported here and in a few studies (Silva et al. 2009,

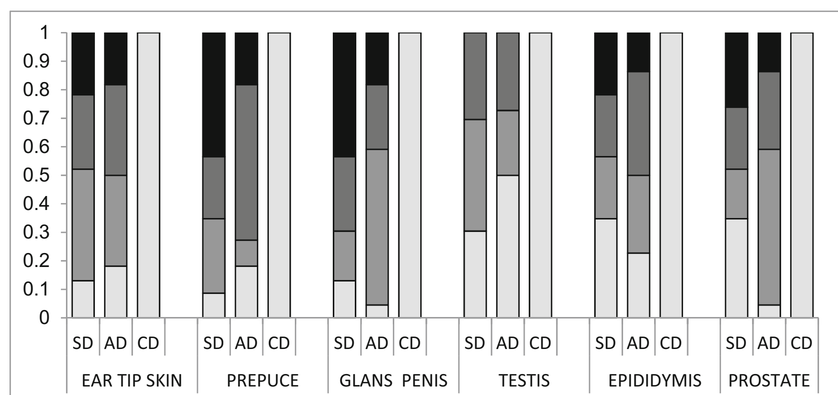

Fig. 5 Frequency and distribution of infiltrate inflammatory in symptomatic (*SD*), asymptomatic (*AD*), and control (*CD*) groups. Inflammatory infiltrates are distributed according to intensity defined as severe (■), moderate (■), light (■), and absent (□)

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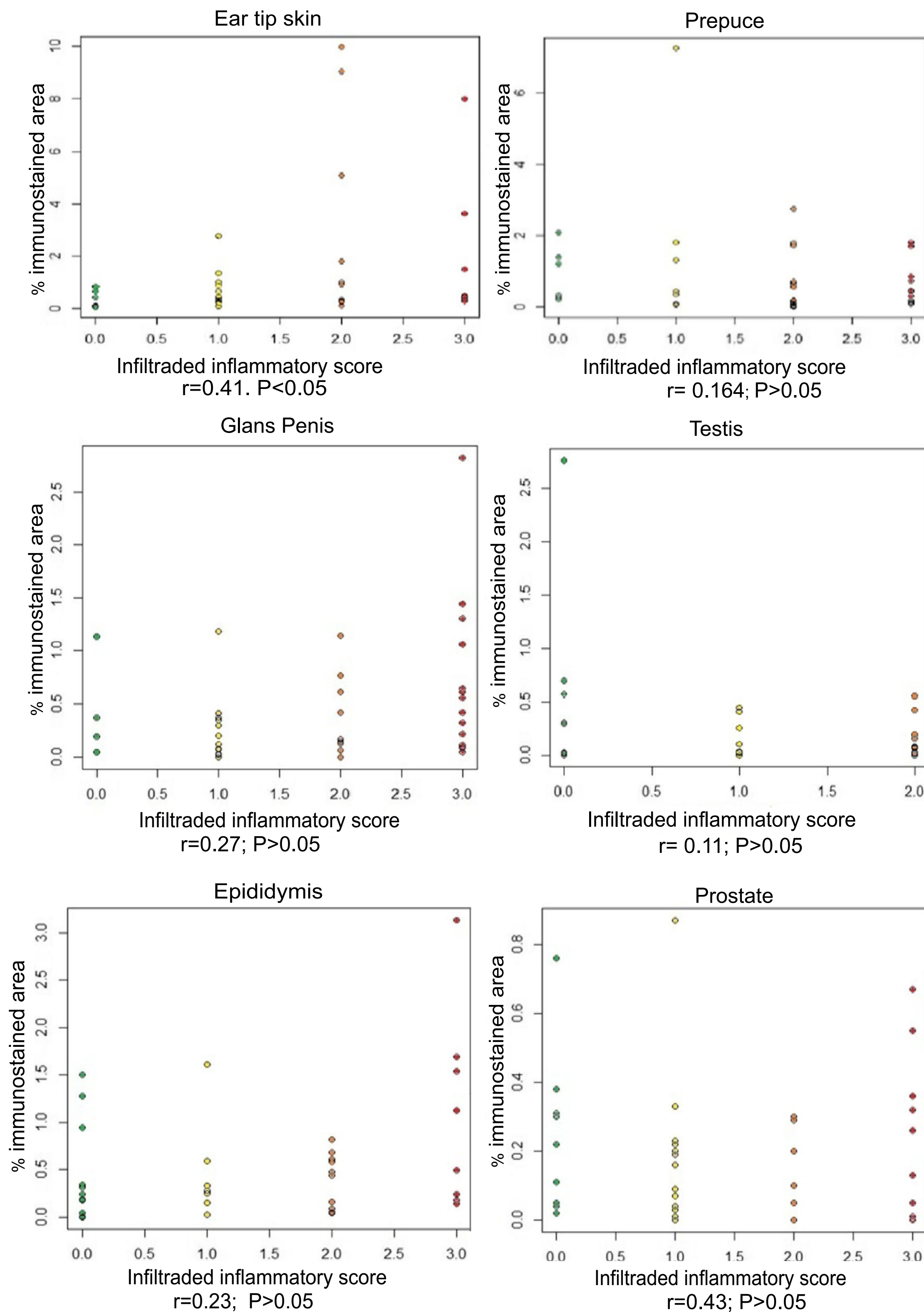

Fig. 6 Correlation between parasitic load (expressed as mean percentage areas of immunostained amastigotes) and inflammatory infiltrate scored as 0 (absent), 1 (light), 2 (moderate), and 3 (severe). The Spearman's rank correlation coefficients varied from moderate (r = 0.30–0.49) to weak (r = 0.10 to 0.29)

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Boggiatto et al. 2011, Turchetti et al. 2014), the relevance of this pathway for the epidemiology of CVL is not well understood. Reports of autochthonous cases in regions with well-established control measures or without the presence of the vector (Duprey et al. 2006; Petersen 2009) reinforce that such topic deserves more attention in the CVL research agenda.

In conclusion, our results demonstrate that the genital tissues of seropositive male dogs, specially that symptomatics, can carry a large number of *Leishmania* amastigotes. Parasitism was particularly high in glans penis, prepuce, and epididymis, with the intensity of parasitism in prepuce and epididymis being similar to that of ear tip skin. Hence, the venereal transmission of CVL could be possible during copulation of a male infected for susceptible females, either through mucosal lesions or via the semen.

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Conflict of interest The authors have declared no conflict of interest pertaining to the study presented herein.

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