Preventive Veterinary Medicine 132 (2016) 83–87

Contents lists available at ScienceDirect

Preventive Veterinary Medicine

journal homepage: www.elsevier.com/locate/prevetmed

Original article

Correlations between peripheral parasite load and common clinical and laboratory alterations in dogs with visceral leishmaniasis

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ARTICLE INFO

Article history:
Received 20 June 2016
Received in revised form 22 August 2016
Accepted 23 August 2016

Keywords:
Canis lupus familiaris
Leishmania spp
Hematology
Biochemistry
Oxidative stress
Principal components analysis

ABSTRACT

Intensity of peripheral parasite infection has an important role in the transmission of *Leishmania* spp. from one host to another. As parasite load quantification is still an expensive procedure to be used routinely in epidemiological surveillance, the use of surrogate predictors may be an important asset in the identification of dogs with high transmitting ability. The present study examined whether common clinical and laboratory alterations can serve as predictors of peripheral parasitism in dogs naturally infected with *Leishmania* spp. Thirty-seven dogs were examined in order to establish correlations between parasite load (PL) in multiple peripheral tissues and common clinical and laboratory findings in canine visceral leishmaniasis (CVL). Quantitative polymerase chain reaction was employed to determine PL in conjunctival swabs, ear skin, peripheral blood and buffy coat. Additionally, a series of hematological, biochemical and oxidative stress markers were quantified. Correlations between net peripheral infection and severity of clinical alterations and variation in laboratory parameters were assessed through a new analytical approach, namely Compressed Parasite Load Data (CPLD), which uses dimension reduction techniques from multivariate statistics to summarize PL across tissues into a single variable. The analysis revealed that elevation in PL is positively correlated with severity of clinical sings commonly observed in CVL, such as skin lesions, ophthalmic alterations, onycogriphosis, popliteal lymphadenomegaly and low body mass. Furthermore, increase in PL was found to be followed by intensification of non-regenerative anemia, neutrophilia, eosinopenia, hepatic injury and oxidative imbalance. These results suggest that routinely used clinical and laboratory exams can be predictive of intensity of peripheral parasite infection, which has an important implication in the identification of dogs with high transmitting ability.

1. Introduction

Visceral leishmaniasis is an anthropozoonosis caused by parasites of the *Leishmania* genus (Solano-Gallego et al., 2011). As the parasite is transmitted from one host to another by the bites of *Lutzomiya longipalpis* sandflies, the intensity of peripheral parasitism is an important contributor to the transmitting ability of the host. The main urban reservoir of the disease is the domestic dog (*Canis lupus familiaris*), and the presence of infected dogs in the vicinity of humans has been incriminated as a risk factor for human leishmaniasis (Solano-Gallego et al., 2009).

Clinical staging in canine visceral leishmaniasis (CVL) has been shown to be associated with parasite density in specific tissue compartments, such as skin (de Almeida Ferreira et al., 2012), lymph nodes (Reale et al., 1999), spleen (Solcà et al., 2012) and bone marrow (Reis et al., 2006). *Leishmania* spp. infection has also been found to alter markers of hematological, biochemical and oxidative homeostasis in dogs (Almeida et al., 2013b; Nicolato et al., 2013; Reis et al., 2006). However, it remains unclear at what extent the

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http://dx.doi.org/10.1016/j.prevetmed.2016.08.006
0167-5877/

occurrence of clinical signs and variation in laboratory parameters translates into peripheral parasite load and consequently to transmission risk. This information is a key first step in the identification of highly infected reservoirs in endemic areas, as peripheral parasite load (PL) quantification techniques are still costly to be routinely used in epidemiological surveillance.

Here, we aimed at contributing to the further elucidation of correlations between parasite load and laboratory/clinical alterations in dogs naturally infected with *Leishmania* spp. Additionally, as the independent analysis of PL in specific compartments can be misleading, we developed a strategy to compile PL quantified in multiple tissues to have a general picture of the intensity of peripheral infection of an animal.

2. Material and methods

2.1. Ethics statement

The study was carried out in strict accordance with the recommendations in the Ethical Principles of the Brazilian College of Animal Experimentation (COBEA – http://www.cobea.org.br). The protocol was approved by the UNESP Ethics Committee on Animal Experimentation (CEUA-FOA permit number: FOA- 01984/12).

2.2. Data collection

Thirty seven adult mixed-breed dogs from the Center for Zoonosis Control of Araçatuba (Sao Paulo, Brazil) were evaluated to determine the severity of common clinical alterations found in leishmaniasis, especially tegumentary (including dermatitis, body alopecia, periocular alopecia or lunettes, hyperkeratosis, ulcerative lesions and onychogryphosis) and ophthalmic alterations (including uveitis and conjunctivitis), lymphadenomegaly, emaciation (evaluated by visual inspection of prominent ribs and lumbar vertebrae, as well as atrophy of the temporal muscle) and mucosa paleness (as a proxy for anemia). All dogs had infection status predetermined by the detection of antibodies anti-*Leishmania* spp. with the enzyme-linked immunosorbent assay technique (Lima et al., 2003). Severity of clinical alterations was assigned in a discrete ordered scale and based on consensus scoring by two veterinary experts.

Blood samples were obtained via jugular venipuncture and used for PL estimation, complete blood cell count, biochemical analysis and quantification of oxidative stress markers, following protocols described elsewhere (Almeida et al., 2013b; Aycicek et al., 2005; Erel, 2005, 2004; Francino et al., 2006; Hunter et al., 1985; Jain, 1986; Kaneko et al., 1997). Bilateral conjunctiva swabs and skin biopsies (i.e., ear punching) were also collected for PL quantification. For details concerning the procedures used here, please see the *Extended Methods* section in the Supplementary information file.

2.3. Statistical analyses

Parasite load data was transformed to $\log10(PL+1)$ prior to the statistical analyses. Additionally, in order to facilitate the multivariate analysis of PL across multiple tissues, we developed a new approach based on Principal Components Analysis to summarize all data into a single representative synthetic variable, namely Compressed Parasite Load Data (CPLD). Briefly, a singular value decomposition of the input matrix of PL across tissues was performed in order to obtain the leading principal component (i.e., PC1), which was used as a proxy for the intensity of net peripheral infection. This simplified our statistical assessment and allowed for obtaining insights about the correlations between peripheral parasitism and routinely collected exams.

Correlations between clinical and laboratory parameters and PL across tissues were tested using a permutation test for Spearman's correlations with 10,000 randomizations each. For all analyses, we considered significant $P<0.05$, whereas $0.05 \leq P<0.10$ were considered suggestive correlations. For details concerning the statistical analyses, as well as *R* v3.2.1 (available at: http://www.r-project.org/) scripts for the computation of CPLD and permutation tests, please see the *Extended Methods* section in the Supplementary information file.

3. Results

3.1. Clinical findings

Clinical examinations revealed presence of skin lesions in 75.7% of the animals (28 out of 37 dogs), which included dermatitis (71.4%, 20 dogs), body alopecia (64.3%, 18 dogs), lunettes (32.1%, 9 dogs), hyperkeratosis (39.3%, 11 dogs) and ulcerative lesions (78.6%, 22 dogs). Among the ophthalmic alterations (67.6%), we observed 20 dogs with conjunctivitis (80.0%) and five with uveitis (20.0%). Other frequent alterations included onycogriphosis (73%, 27 dogs), popliteal lymphadenomegaly (70.3%, 26 dogs) and low body mass (59.5%, 22 dogs). All animals (100%, 37 dogs) had at least one clinical sign.

3.2. Descriptive statistics of hematological, biochemical and oxidative stress markers

A complete summary of the clinical, hematological, biochemical and oxidative stress markers is provided in Supplementary Table 1. All samples exhibited at least one hematological alteration compared to reference values. All examined dogs had non-regenerative normocytic hypocromic anemia, except for a single dog that presented macrocytic hypochromic anemia. All animals presented leukogram abnormalities. Two animals with leukocytosis, neutrophilia and lymphopenia had suppurative skin lesions. Only one dog presented eosinophilia, and the other dogs had at least one of the following alterations: monocytopenia, lymphopenia or eosinopenia.

The biochemical analysis showed that all dogs had hypoalbuminemia. Hyperglobulinemia and hyperproteinemia were observed in 31 (86.5%) and 21 (56.75%) dogs, respectively. Biochemical profiles compatible with hepatic injury (considered here as alterations in two or more of the following biochemical markers: ALT, AST, alkaline phosphatase, GGT and albumin) was found in 31 (83.8%) dogs. Evidences of kidney alterations (considered here as increased creatinine and/or urea) were found in 13 dogs (35.1%). Importantly, no dog presented signs of dehydration such as bilateral enophthalmos and decreased skin turgor, which could affect the interpretation of the biochemical markers.

We observed reduced oxidant and antioxidant status in 73.3% and 82.6% of the examined samples, respectively. Accordingly, a large number of samples presented decreased values for other endogenous antioxidants, including albumin (100%) and total bilirrubin (54.5%) in comparison to reference values. Only three and one sample presented increased TAC and TOC, respectively. Except for one animal, all dogs presented decreased lipid peroxidation in the plasma.

3.3. Descriptive statistics of parasite load

Supplementary information (Supplementary Table 2) presents summary statistics for the log-transformed PL across different tissues. The highest average PL was observed in the skin (29,511 parasites/mL). Absence of parasites was found in 54.9%, 24.32%, 13.51% and 12.5% of the peripheral blood, buffy coat, conjunctivas