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1   **Original Article**
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4   **Correlations between peripheral parasite load and common clinical and laboratory alterations in**
5   **dogs with visceral leishmaniasis**
6

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24 **Abstract**

25   Intensity of peripheral parasite infection has an important role in the transmission of

26 *Leishmania spp.* from one host to another. As parasite load quantification is still an expensive

27 procedure to be used routinely in epidemiological surveillance, the use of surrogate predictors may be

28 an important asset in the identification of dogs with high transmitting ability. The present study

29 examined whether common clinical and laboratory alterations can serve as predictors of peripheral

30 parasitism in dogs naturally infected with *Leishmania spp*. Thirty-seven dogs were examined in order

31 to establish correlations between parasite load (PL) in multiple peripheral tissues and common clinical

32 and laboratory findings in canine visceral leishmaniasis (CVL). Quantitative polymerase chain reaction

33 was employed to determine PL in conjunctival swabs, ear skin, peripheral blood and buffy coat.

34 Additionally, a series of hematological, biochemical and oxidative stress markers were quantified.

35 Correlations between net peripheral infection and severity of clinical alterations and variation in

36 laboratory parameters were assessed through a new analytical approach, namely Compressed Parasite

37 Load Data (CPLD), which uses dimension reduction techniques from multivariate statistics to

38 summarize PL across tissues into a single variable. The analysis revealed that elevation in PL is

39 positively correlated with severity of clinical sings commonly observed in CVL, such as skin lesions,

40 ophthalmic alterations, onycogriphosis, popliteal lymphadenomegaly and low body mass. Furthermore,

41 increase in PL was found to be followed by intensification of non-regenerative anemia, neutrophilia,

42 eosinopenia, hepatic injury and oxidative imbalance. These results suggest that routinely used clinical

43 and laboratory exams can be predictive of intensity of peripheral parasite infection, which has an

44 important implication in the identification of dogs with high transmitting ability.

45

46 Keywords: *Canis lupus familiaris*; *Leishmania spp.*; Hematology; Biochemistry; Oxidative stress;

47 Principal components analysis

1. Introduction

Visceral leishmaniasis is an anthropozoonosis caused by parasites of the *Leishmania* genus (WHO, 2010)☐ (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; Werneck et al., 2007)☐.

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 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

72   infection of an animal.

73

74   **2. Material and Methods**

75   2.1. Ethics statement

76   The study was carried out in strict accordance with the recommendations in the Ethical

77   Principles of the Brazilian College of Animal Experimentation (COBEA - http://www.cobea.org.br).

78   The protocol was approved by the UNESP Ethics Committee on Animal Experimentation (CEUA-FOA

79   permit number: FOA- 01984/12).

80

81   2.2. Data collection

82   Thirty seven adult mixed-breed dogs from the Center for Zoonosis Control of Araçatuba (Sao

83   Paulo, Brazil) were evaluated to determine the severity of common clinical alterations found in

84   leishmaniasis, especially tegumentary (including dermatitis, body alopecia, periocular alopecia or

85   lunettes, hyperkeratosis, ulcerative lesions and onychogryphosis) and ophthalmic alterations (including

86   uveitis and conjunctivitis), lymphadenomegaly, emaciation (evaluated by visual inspection of

87   prominent ribs and lumbar vertebrae, as well as atrophy of the temporal muscle) and mucosa paleness

88   (as a proxy for anemia). All dogs had infection status predetermined by the detection of antibodies anti-

89   *Leishmania spp*. with the enzyme-linked immunosorbent assay technique (Lima et al., 2003). Severity

90   of clinical alterations was assigned in a discrete ordered scale and based on consensus scoring by two

91   veterinary experts.

92

93   Blood samples were obtained via jugular venipuncture and used for PL estimation, complete

94   blood cell count, biochemical analysis and quantification of oxidative stress markers, following

95   protocols described elsewhere (Almeida et al., 2013b; Aycicek et al., 2005; Erel, 2005, 2004; Francino

4

96 et al., 2006; Hunter et al., 1985; Jain, 1986; Kaneko et al., 1997)□. Bilateral conjunctiva swabs and

97 skin biopsies (i.e., ear punching) were also collected for PL quantification. For details concerning the

98 procedures used here, please see the *Extended Methods* section in the Supplementary Information file.

99

100 2.3 Statistical analyses

101   Parasite load data was transformed to $\log10(PL + 1)$ prior to the statistical analyses.

102 Additionally, in order to facilitate the multivariate analysis of PL across multiple tissues, we developed

103 a new approach based on Principal Components Analysis to summarize all data into a single

104 representative synthetic variable, namely Compressed Parasite Load Data (CPLD). Briefly, a singular

105 value decomposition of the input matrix of PL across tissues was performed in order to obtain the

106 leading principal component (i.e., PC1), which was used as a proxy for the intensity of net peripheral

107 infection. This simplified our statistical assessment and allowed for obtaining insights about the

108 correlations between peripheral parasitism and routinely collected exams.

109

110   Correlations between clinical and laboratory parameters and PL across tissues were tested using

111 a permutation test for Spearman's correlations with 10,000 randomizations each. For all analyses, we

112 considered significant $P < 0.05$, whereas $0.05 \leq P < 0.10$ were considered suggestive correlations. For

113 details concerning the statistical analyses, as well as *R* v3.2.1 (available at: http://www.r-project.org/)

114 scripts for the computation of CPLD and permutation tests, please see the *Extended Methods* section in

115 the Supplementary Information file.

116

117

118 **3. Results**

119 3.1. Clinical findings

Clinical examinations revealed presence of skin lesions in 75.7% of the animals (28 out of 37 dogs), which included ~~variable extension and severity of~~ 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.

144

145   We observed reduced oxidant and antioxidant status in 73.3% and 82.6% of the examined

146   samples, respectively. Accordingly, a large number of samples presented decreased values for other

147   endogenous antioxidants, including albumin (100%) and total bilirrubin (54.5%) in comparison to

148   reference values. Only three and one sample presented increased TAC and TOC, respectively. Except

149   for one animal, all dogs presented decreased lipid peroxidation in the plasma.

150

151   3.3. Descriptive statistics of parasite load

152   Supplementary Information (Supplementary Table 2) presents summary statistics for the log-

153   transformed PL across different tissues. The highest average PL was observed in the skin (29,511

154   parasites/mL). Absence of parasites was found in 54.9%, 24.32%, 13.51% and 12.5% of the peripheral

155   blood, buffy coat, conjunctivas and skin samples, respectively. However, all dogs presented parasite

156   infection in at least one tissue, confirming that all dogs were infected. Parasite load across tissues were

157   moderately to highly correlated, with an average correlation of $r = 0.400$ (Supplementary Information).

158   An exception was observed for blood, which was poorly correlated with all tissues (average correlation

159   of $r = 0.069$).

160

161   In order to have a representation of all tissues simultaneously, we created a new compressed variable,

162   namely CPLD, based on a principal component analysis of the PL matrix. For that matter, we required

163   complete observations of PL across tissues, which reduced the data from 37 to 31 samples. The first

164   leading principal component explained 49.1% of the total variance contained in the original matrix, and

165   therefore was adopted as the CPLD . The relative contributions of each tissue to the CPLD were 22.7%

166   for skin, 28.5% for right conjunctiva, 29.6% for left conjunctiva, 12.3% for buffy coat and 6.9% for

167   blood.

168

3.4. Correlations for peripheral parasite load

As the permutation test is conservative, we decided to discuss suggestive $(0.05 \leq P < 0.10)$ and significant $(P < 0.05)$ correlations. All clinical alterations were positively correlated $(P < 0.05)$ with parasite load in two or more tissues (Fig 1). Ophthalmic alterations were only associated with PL in the conjunctivas. Parasite load in the blood did not present associations with clinical alterations. The CPLD variable successfully captured the correlatons of the individual tissues, and only failed to predict ophthalmic alterations.

The following laboratory markers presented correlations with parasite load in at least one tissue (Fig 1): WBC (positive), PCV (negative), MCV (negative), RDW (positive), neutrophil (positive), lymphocytes (negative), eosinophil (negative), triglycerides (negative), uric acid (positive), albumin (negative), total bilirrubin (positive), AST (positive), creatinine (negative), alkaline phosphatase (positive), TAC (positive), TOC (negative), oxidative index (negative) and TBARS plasma (negative). Parasite load in total blood was not correlated with any given analyzed marker (see Supplementary Table 3).

Correlations between results for CPLD and individual tissues were 0.972, 0.936, 0.773, 0.543 and 0.251 for left conjunctiva, right conjunctiva, skin, buffy coat and blood, respectively. The compressed variable was able to capture the majority of the significant and suggestive correlations exhibited by the individual tissues, and properly conserved the magnitude and direction of the correlation coefficients.

4. Discussion

To date, no existing report in the literature has yet assessed the simultaneous correlation of peripheral parasite load in multiple tissues with severity of clinical signs or variation in biochemical,

192 hematological and, especially, oxidative stress markers in dogs naturally infected with *Leishmania spp*.

193 Elucidation of these correlations may allow for accurate identification of animals with high

194 transmitting ability based on routine clinical examination and laboratory analysis. Here, we present

195 such an investigation, which was facilitated by the development of a new approach, namely CPLD,

196 which summarizes PL data across tissues into a single variable that serves as a proxy for net parasite

197 infection. For the sake of clarity, we use PL, CPLD and net peripheral parasite infection

198 interchangeably throughout the discussion, as CPLD was shown to efficiently capture the majority of

199 the tissue-specific PL correlations.

200

201   The main clinical alterations observed in our study are consistent with those commonly found in

202 CVL (Manna et al., 2009)□. We found that elevation of PL increases severity of clinical signs in

203 infected dogs, consistent with previous reports suggesting that increased parasite density is associated

204 with the severity of CVL (Manna et al., 2009; Reis et al., 2006)□. Interestingly, ophthalmic alterations

205 were associated only with PL in conjunctival tissues and buffy coat, suggesting that this clinical sign is

206 a specific indicator of local parasitism. Altogether, these results indicate that high peripheral parasitism

207 can be inferred in dogs presenting clinical alterations such as alopecia, onychogryphosis, popliteal

208 lymphadenomegaly, mucosa paleness and emaciation. Although these relationships are often speculated

209 by veterinary practitioners and health agents, we believe our study is the first to formally demonstrate

210 them empirically.

211

212   The vast majority of the dogs in our study presented non-regenerative anemia, which is

213 compatible with bone marrow injuries or impaired hormonal stimulation (Grimes and Fry, 2014)□.

214 Although non-regenerative anemia in CVL is often described as normocytic normochromic (Lafuse et

215 al., 2013; Solano-Gallego et al., 2009)□, our samples presented normocytic hypochromic anemia.

216   Decreased MCHC paired with normal MCV is usually indicative of iron deficiency (Jain, 1986)☐.

217   *Leishmania spp.* amastigotes require iron from the host in order to survive and exert their pathogenicity

218   (Flannery et al., 2013)☐, and this could be related to the low MCHC in our sample.

219

220   As we found no variation in the type of anemia across dogs, our data suggests that the presence

221   of non-regenerative anemia is independent of intensity of peripheral infection. However, by performing

222   the analysis of each blood parameter individually, we found that elevation in PL leads to increased

223   RDW and decreased PCV and MCV, which implies progression and worsening of iron deficiency

224   anemia (Jain, 1986)☐ with higher peripheral parasite density. This hypothesis is also supported by the

225   positive correlation between PL and mucosa paleness.

226

227   The strongest positive correlation ($r = 0.563$) with PL across tissues was found for neutrophils.

228   Neutrophilia may be attributed to the inflammatory response resulting from parasitism in multiple

229   organs (Amusategui et al., 2003; Mogami, 2008; Tryphonas et al., 1977)☐. Neutrophils are deemed to

230   be used as 'Trojan horses' by *Leishmania spp.* in order to invade the host for later multiplication inside

231   macrophages (Zandbergen et al., 2004)☐. Progression of CVL has been shown to lead to increased

232   apoptosis (Almeida et al., 2013a)☐ and reduced oxidation status (Almeida et al., 2013b)☐ in

233   neutrophils. We also observed that PL is positively correlated with TAC and negatively correlated with

234   TOC and oxidative index. Moreover, *Leishmania spp.* has been found to also produce antioxidants as a

235   defense mechanism against the respiratory burst (Assche et al., 2011)☐. These findings point to

236   parasitism-induced neutrophilia coupled with inhibition or over-consumption of reactive oxygen

237   species (ROS) as a key mechanism used by the parasite to evade the immune system and multiply

238   inside the host.

239

240   At the other extreme, we found the strongest negative correlation of PL with eosinophil (r = -

241   0.651). Anemia, neutrophilia and eosinopenia have been described as hallmarks of hematological

242   dysfunction in CVL, and decreased eosinophil precursors in the bone marrow has been implicated in

243   reduction of the number of peripheral blood eosinophils (Nicolato et al., 2013)□. As eosinophils are

244   also a lineage of phagocytes actively producing ROS, lower TOC in highly infected animals could also

245   be partly explained by eosinopenia.

246

247   The liver is one of the main affected organs in CVL and liver damage has been found to be

248   intensified by increased parasitism (Giunchetti et al., 2008)□ and by the progression of the disease

249   (Giunchetti et al., 2008; Melo et al., 2009)□. Here, increase in peripheral PL was followed by decrease

250   in serum albumin and increase in AST, alkaline phosphatase, total bilirrubin and uric acid, indicating

251   intensification of liver injury (Afzali et al., 2010; Giunchetti et al., 2008; Wolf, 1999)□ as consequence

252   of increased parasitism. Additionally, liver damage can lead to impaired lipid metabolism, which could

253   justify the negative correlation between PL and triglycerides. One obvious limitation of the present

254   study is the absence of PL measurements in internal organs such as the liver, as the scope here was the

255   investigation of peripheral parasitism. However, other tissues could capture the association between PL

256   and markers of liver function, suggesting some degree of correlation between parasitism in the liver

257   and the analyzed tissues. Although examination of PL in internal organs was out of the scope of the

258   present study, future investigations regarding the interplay of PL in peripheral and internal organs are

259   required.

260

261   Albumin, uric acid and bilirrubin are not only important markers of liver function (Afzali et al.,

262   2010; Wolf, 1999)□, but they are also important endogenous antioxidants underlying the TAC marker

263   (Erel, 2004)□. This leaves us with two distinct hypotheses to explain the observed data: 1) TAC

264 increases in response to PL as a compensatory mechanism to react against systemic ROS spilled by

265 damaged tissues; or 2) elevation of TAC is a false positive oxidative imbalance, because higher levels

266 of serum uric acid and bilirrubin are in fact caused by liver damage. As tissue damage followed by

267 spilling of ROS in the plasma is expected to produce lipid peroxidation (Hunter et al., 1985; Niki,

268 2009)☐, the first hypothesis seems to be inconsistent with the non-significant correlation between PL

269 and TBARS in the plasma and in erythrocytes in our study. Only TBARS in plasma showed suggestive

270 correlation with one tissue. This also indicates that anemia is unlikely to be caused by lipid

271 peroxidation of red blood cells in CVL. Nevertheless, our data did not allow for distinguishing the

272 source of increase in TAC, and these two hypotheses should be addressed by future studies.

273

274   In spite of being lowly invasive and easy to measure, PL in peripheral blood did not present any

275 association with clinical alterations or laboratory parameters in our study, and was poorly correlated

276 with parasitism in other tissues. On the other hand, in comparison to other tissues, conjunctiva swabs

277 are the least invasive sampling method for PL quantification and presented the largest number of

278 associations here, indicating high sensitivity. This is in agreement with previous studies showing that

279 conjunctiva PL is a reliable, sensitive proxy for parasite burden (de Almeida Ferreira et al., 2012;

280 Lombardo et al., 2012)☐.

281

282   A clear limitation of the present study is the absence of historical records for the studied dogs,

283 thus not allowing us to account for potential confounding effects in our analysis, such as a putative

284 nutritional causes for the observed iron deficiency anemia and the decreased levels of triglycerides.

285 Moreover, as 59.5% of the dogs had low body mass, lower creatinine levels could be also explained by

286 loss of muscle mass. Therefore, more studies are required in order to better elucidate the dynamics

287 among parasite burden, anemia and nutrition.

5. Conclusions

We presented novel empirical evidence supporting ~~found that~~the hypothesis that severity of clinical manifestations of the disease, as well as non-regenerative anemia, oxidative imbalance and liver and bone marrow damage are correlated with increased peripheral parasitism in dogs naturally infected with *Leishmania spp*. These results ~~can help~~have direct impact in ~~veterinary practitioners and health agents to~~the identif~~y~~ication of dogs with high transmitting ability in endemic areas ~~through routinely used clinical and laboratory exams~~. Additionally, these correlations may also serve as a tool to help veterinarians take decisions regarding prognosis and therapy based on routine clinical and laboratory exams. ~~Moreover~~Finally, the new statistical tool presented here can be used to summarize parasite load data in future studies including quantification in multiple tissues, substantially simplifying the multivariate analysis of net parasite infection.

6. Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. Mention of trade name proprietary product or specified equipment in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the authors or their respective institutions.

7. Acknowledgements

317

318   **8. Appendix A: Supplementary Information**

319   Supplementary data associated with this article can be found, in online version, at doi:

320

321   **9.**   **References**

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414

10. Figure legends

417   Fig 1. Correlogram of Spearman's rank correlation coefficients between *Leishmania spp.* density

418   (horizontal axis) in different tissues and clinical alterations, blood cell count, biochemical and oxidative

419   stress markers (vertical axis). Warm colors (towards red) represent positive correlations ($r > 0$),

420   whereas cool colors (towards blue) represent negative correlations ($r < 0$). Only markers presenting at

421   least one suggestive ($0.05 \leq P < 0.10$) or significant ($P < 0.05$) correlation in a randomization test with

422   10,000 permutations are shown. For a complete summary of correlations see the Supplementary

423   Information (Supplementary Table 3 and 4).