1 **Experimental infection course of canine leishmaniasis: follow-up and usefulness of non-**
2 **invasive diagnostic techniques**

3 Leticia Hernández [a], Ana Montoya [a], Rocío Checa [a], Diana Dado [a], Rosa Gálvez [a], Domenico
4 Otranto [b], Maria Stefania Latrofa [b], Gad Baneth[c] & Guadalupe Miró [a] *.

5 [a] Department of Animal Health, Veterinary Faculty, Universidad Complutense de Madrid, Avda. Puerta
6 de Hierro s/n, 28040 Madrid, Spain

7 [b] Department of Veterinary Medicine, University of Bari, 70010 Valenzano, Bari, Italy

8 [c] School of Veterinary Medicine, Hebrew University, P.O. Box 12, Rehovot 76100, Israel

9 * Corresponding author. Tel.: +34 91 3943711; Fax: +34 91 3943708; Email: gmiro@ucm.es

10

11 **Abstract**

12 Canine leishmaniosis (CanL) caused by *Leishmania infantum* is a zoonotic disease transmitted
13 by the bite of phlebotomine sand flies and *Canis familiaris* is the main reservoir for human
14 visceral infection. However, there is currently no gold standard diagnostic technique to detect
15 this infection in dogs.

16 Our aim was to compare the utility of a molecular diagnosis of experimental CanL on non-
17 invasive samples (urine, conjunctival (CS), oral (OS) and vulvar (VS) swabs) with that of
18 traditional invasive techniques throughout the course of infection. Eight beagle dogs were
19 intravenously infected with promastigotes of *Leishmania infantum* and monitored monthly for
20 12 months to assess clinical, clinicopathological, immunological and parasitological variables.
21 Active infection was produced in 100% of the dogs. The animals showed positive bone marrow
22 (BM) cytologies and cultures, clinical signs, clinicopathological abnormalities and a high specific
23 humoral immune response. The technique that detected the infection earliest, at 60 days post-
24 infection (p.i.), was real-time quantitative PCR (rtQ-PCR) on BM, while anti-*L. infantum*
25 antibody seroconversion occurred between Days 120 and 180 days p.i. BM was the organ with
26 the highest load of *L. infantum* kDNA, as detected by rtQ-PCR (range 381.5-70000 parasites/ml
27 at the study end) and showed greater sensitivity than peripheral blood (PB). The vulvar swabs
28 used here for the first time to quantify parasite loads in the dogs revealed a greater load than

oral and conjunctival swabs at one year p.i. Urine samples showed the lowest concentrations of *L. infantum* DNA (maximum: 8.57 parasites/ml), which could not be detected in all animals (2 out 8). This sample type may therefore not be the best for diagnosing clinical CanL. Our results suggest that for the early detection of infection, adding to serology a test such as rtQ-PCR on OS or VS improves sensitivity and specificity. We recommend that conjunctival, oral and vulvar mucosae obtained noninvasively should be included as alternative samples in protocols for the detection of *L. infantum* infection in dogs.

Keywords: *Leishmania infantum*; experimental infection; dog; diagnosis; non-invasive; real-time PCR; conjunctival, oral and vulvar swab

1. Introduction

49 Canine leishmaniosis (CanL) caused by *Leishmania* is a worldwide zoonosis, present in Europe,
50 Asia, North Africa and South America and more recently also North America (Duprey et al.,
51 2006; Esch and Petersen, 2013; Petersen and Barr, 2009; Solano-Gallego et al., 2011) Dogs are
52 both the natural hosts and main reservoir of human infection (Alvar et al., 2004; Gramiccia and
53 Gradoni, 2005).

54 CanL is a systemic disease that may potentially involve any organ, tissue and biological
55 fluid and produces many non-specific clinical signs, making the diagnosis fairly difficult (Solano-
56 Gallego et al., 2011). Although after treatment clinical signs may improve, parasitological cure
57 is never achieved in dogs and relapses occur frequently (Amusategui et al., 1998; Noli and
58 Auxilia, 2005; Oliva et al., 2010; Solano-Gallego et al., 2011).

59 For a diagnosis of CanL, parasitological (cultures, cytology or histology
60 immunohistochemistry), serological (IFAT, ELISA) and molecular (conventional, nested and real
61 time PCR) procedures are used individually or in combination (Solano-Gallego et al., 2011).
62 IFAT is today the gold standard method of detecting anti-*L. infantum* antibodies (Alvar et al.,
63 2004; Maia and Campino, 2008) due to its high sensitivity and specificity (Ciaramella et al.,
64 1997; Mancianti et al., 1995). However, epidemiological studies in endemic areas of CanL has
65 shown higher prevalence of infection by means of molecular techniques than seroprevalence
66 (Baneth and Aroch, 2008).

67 Real-time Q-PCR both detects the presence of *Leishmania* DNA and accurately
68 determines the parasite load in the sample, with the advantage of speed and reduced risk of
69 sample contamination. The different biological samples used for the PCR detection of
70 *Leishmania* DNA have shown varying degrees of sensitivity. These include samples of bone
71 marrow (BM), lymph node, spleen, skin, peripheral blood (PB), buffy coat and other fluids
72 (Maia et al., 2009; Solano-Gallego et al., 2009). Although several authors have reported better
73 results using BM, lymph node and skin samples in subclinical and clinical CanL (Maia and
74 Campino, 2008; Mimori et al., 2002; Miró et al., 2008; Quaresma et al., 2009), there is still no

75 general consensus regarding the standard sample of choice (Almeida et al., 2013; Manna et al.,
76 2004). The use of non-invasive sampling techniques could serve to simplify diagnostic
77 procedures, epidemiological studies, field trials, reduce pain and thus would be more readily
78 accepted by owners. The non-invasive samples tested so far have been urine, hair, conjunctival
79 (CS), oral (OS), nasal and ear swabs (Belinchón-Lorenzo et al., 2013; Carvalho Ferreira et al.,
80 2014; Ferreira et al., 2013; Gramiccia et al., 2010; Lombardo et al., 2012; Manna et al., 2008a;
81 Muñoz-Madrid et al., 2013; Solano-Gallego et al., 2007).

82 In the present study, clinical, immunological and parasitological factors were assessed
83 monthly in 8 Beagle dogs experimentally infected with *L. infantum* during 12 months of follow-
84 up. The main objective was to compare *L. infantum* presence and load data obtained in
85 invasive (PB and BM) and non-invasive samples (urine, CS, OS and vulvar swabs (VS)) with the
86 information provided by other diagnostic techniques throughout the course of infection.

87

88 **2. Material and methods**

89 Eight healthy female intact Beagle dogs (8 months old, body weight 10.5 to 16.6 kg)
90 were purchased from Isoquimen S.L. (St. Feliu de Codres, Spain). Each dog was identified by a
91 microchip and assigned an identification number (1-8). No dog had detectable levels of
92 *Leishmania*-specific antibodies or *Leishmania* DNA in blood.

93 All dogs had been previously vaccinated against rabies, canine distemper,
94 hepatitis/adenovirus type 2, leptospirosis and parvovirus, and correctly dewormed. The dogs
95 were kept in indoor kennels and fed a standard diet of puppy food pellets with water given *ad*
96 *libitum*. The kennel's nested windows were sprayed with deltamethrin to avoid natural
97 *Leishmania* infection. The study protocol followed International guidelines for the Care and
98 Use of Experimental Animals and Spanish legislation guidelines (RD 1201/2005) and received
99 Ethics Committee approval (Universidad Complutense de Madrid, Spain).

100 Dogs were experimentally infected with the *L. infantum* MON-1 strain
101 MCRI/ES/06/BCN-721, kindly provided by Dr. Portús (Universidad Autónoma de Barcelona,
102 Spain), isolated from a dog that had acquired the infection naturally in the Priorat region
103 (Catalonia, Spain). Each dog was intravenously inoculated with $5x10^7$ promastigotes per
104 milliliter in a total volume of 0.5 ml.
105
106 During the one-year course of infection, the dogs were subjected to monthly physical,
107 clinicopathological, immunological and parasitological exams at 13 time points from Days 0 to
108 360 postinfection (D0, D30,…, D360). At each time point, all sample types were collected from
109 each dog, previous sedation with medetomidine (Domtor®, Pfyzer S.A.) at a dose of 0.02
110 mg/kg i.v. Atipamezole (Antisedan®, Pfyzer S.A.) was given for the reversal of sedation.

111 Peripheral blood samples (5 ml) collected by cephalic venipuncture were distributed
112 into four tubes containing: (a) lithium heparin, for biochemical tests, (b) ethylene diamine
113 tetraacetic acid (EDTA) for complete blood counts (CBC), (c) EDTA for rtQ-PCR *Leishmania*
114 detection, and (d) no additives for antibody testing (IFAT). Complete blood counts included red
115 blood cell count (RBCC), packed cell volume (PCV), white blood cell count (WBCC), and platelet
116 count. The biochemical variables determined to assess renal and hepatic function were urea,
117 creatinine, alanine aminotransferase (ALT), alkaline phosphatase (ALP), total plasma protein,
118 albumin/globulin ratio, and serum protein electrophoresis.

119 Bone marrow aspirates (0.5 ml) were obtained from the fifth-sixth costo-condral
120 junction for cytological examination and culture on NNN specific medium. An aliquot was
121 stored in 200 µl of buffer NET 10 (NaCl 10 mM, EDTA 10 mM, Tris 10 mM) for DNA extraction.
122 Urine was collected by catheterization for a complete urinalysis in which the urinary protein
123 creatinine ratio (UPC) was monitored. An aliquot of urine (1.5 ml) was stored for DNA
124 extraction. Sterile cotton swabs were used to remove exfoliative cells from the conjunctiva,
125 oral and vulvar mucosae. Swab tips were broken off and placed in DNAse-free tubes. All
126 samples for DNA purification were stored at -20˚C until further analysis.

127 Each dog was scored monthly for 23 clinical signs assessed in a physical examination
128 using a categorized scoring system from 0 to 3 (from low to high severity), as previously
129 described (Miró et al., 2009), obtaining an overall monthly clinical score (maximum possible
130 score = 52). In a similar way, dogs were scored for 11 clinicopathological variables (CBC,
131 biochemical profile, urinalysis and UPC) using a categorized scoring system from 0 to 2
132 (maximum possible score of 16).

133 Anti-*L. infantum* immunoglobulin G (IgG) antibodies were detected by an
134 immunofluorescence antibody test (IFAT) against in-house cultured promastigotes as
135 described elsewhere (Mancianti and Meciani, 1988) on serial dilutions from 1/50 to 1/12800.
136 Seropositivity was defined by a cut-off ≥ 1/100.

137 The presence of *Leishmania* amastigotes was assessed by the same operator monthly
138 by microscopy examination of three Giemsa-stained BM smears and BM cultures in biphasic
139 NNN medium (WHO, 2010). Cultures were scored as negative after four consecutive negative
140 results over a 4-week period (Dedet et al., 1999).

141 The QuiAamp® DNA Micro Kit (50) (QIAGEN®) was used to obtain DNA from peripheral
142 whole blood (100 µl), BM aspirates (100 µl) and urine (800 µl) according to the manufacturer's
143 instructions. For the CS, OS and VS samples the protocol was modified as follows: each swab
144 was re-suspended in 290 µl of lysis buffer ATL, 20 µl of proteinase K and 200 µl of buffer AL (to
145 which "carrier RNA" had been previously added to increase DNA binding to the eluted column
146 membrane) and incubated at 56°C for 2 h under agitation to promote protein digestion. Next,
147 after perforating each extreme (bottom and lid), the Eppendorf containing the swabs was
148 placed in another Eppendorf and centrifuged at 3500 r.p.m. for 3 min. 50 µl of ethanol (100%)
149 were then added and mixed for 15 seconds followed by vortexing and spin off for 5 s. The final
150 steps were as described by the manufacturer. The extracted DNA was eluted in sterilized water
151 (70 µl) and stored at -20°C until use.

152 *Leishmania infantum* DNA in tissue samples was detected by RtQ-PCR on D0, D120, 153 D270 and D360 p.i., as described previously (Francino et al., 2006). The final 20 µl volume of 154 the reaction mixture contained 10 µl of iQ™ Supermix (Bio-Rad Laboratories, Hercules CA, 155 USA), each primer at a concentration of 900 nM, the probe at a concentration of 200 nM, and 156 2 µl of template DNA. The PCR conditions were a hot start at 95°C for 3 min and 42 cycles of 157 denaturation (95°C for 10 s) and annealing-extension (60°C for 30 s).

158 The rtQ-PCR procedure was carried out in a CFX96™ Real-Time System (Bio-Rad 159 Laboratories, Inc., Hercules CA, USA) and data analyzed with CFX Manager™ Software Version 160 1.6 (Bio-Rad).

161 *Leishmania* kDNA was quantified by the absolute quantification method whereby a 162 standard curve is constructed using a 10-fold dilution series of standard DNA from 163 promastigotes (log phase concentration, 1.7 x 10^6 parasites/ml) of *L. infantum* (zymodeme 164 MON-1) as calibrators, testing each dilution in triplicate (Dantas-Torres et al., 2011). The limit 165 of detection for the rtQ-PCR was assessed using a serial dilution from 1.7 x 10^{-1} to 1.7 x 10^{-8} 166 parasites per reaction. Results are expressed as absolute numbers of parasites present in 1 ml 167 of PB, BM, urine, or per swab.

168 Statistical tests were carried out with SAS software package version 9.2. The 169 nonparametric Wilcoxon signed rank test was used to compare data between two time points 170 and correlations among the different variables were examined by Spearman's rank correlation 171 analysis (ρ). Significance was set at a p value ≤ 0.05.

172

173 **3. Results and discussion**

174 The incubation period was between D120 and D180, being the first clinical sign 175 observed lymphadenomegaly among a broad range of clinical signs, reflecting the wide

176 spectrum of clinical manifestations produced during natural infection (Hommel et al., 1995).
177 Five dogs presented exfoliative dermatitis associated with diffuse to general non inflammatory
178 alopecia, which is the most prevalent cutaneous manifestation of CanL (Carcelén et al., 2009;
179 Fernández-Cotrina et al., 2013; Rodríguez-Cortés et al., 2007). After D300 and D360, the main
180 systemic signs of CanL were observed, such as asthenia (n=2), splenomegaly (n=8),
181 conjunctivitis (n=5), generalized muscular atrophy (n=3) temporal muscles atrophy (n=2),
182 onychogryphosis (n=5), alopecia (n=6) and plantar hyperkeratosis (n=5). Dogs 2, 3 and 7
183 progressed to a more advanced clinical state (scoring 23, 17 and 14, respectively on D360). By
184 the end of the study, dogs 1, 2 and 3 showed slight weight loss (0.75, 0.75 and 0.65 kg
185 respectively).

186 Our hematological, biochemical and urinalysis data revealed that infected dogs
187 manifested the classic alterations observed in naturally infected dogs, including non-
188 regenerative anemia, which is the most common hematological disorder in CanL
189 (Saridomichelakis, 2009) and was observed in six out of the eight present dogs (lowest
190 hematocrit 27.7% dog 4). This sign reflects the involvement of BM, which is highly parasitized
191 in clinical CanL. Hematocrits varied significantly between D120 and D240 (p=0.015) and D120
192 and D360 (p=0.007). After D150, three dogs showed thrombocytopenia, which persisted on
193 D360 in half the dogs. Dysproteinemia was present on D120 in three dogs, and all dogs
194 developed hyperproteinemia associated with hypergammaglobulinemia from D210. At one
195 year p.i., total serum proteins ranged from 8.2 to 11.9 g/dl and γ-globulin was higher than 4
196 g/dl in six dogs. An inverted albumin/globulins ratio (A/G ratio) associated with
197 hypergammaglobulinemia and hypoalbuminemia was observed in all dogs on D120 (dogs with
198 a lower A/G ratio showed a higher clinical score).

199 A transient decrease in urine specific gravity (≤ 1010) was detected in 50% of the dogs
200 on D360 and 75% showed transient proteinuria (100-2000 mg/dl) between D180 and D360.

201 Despite no significant differences detected in the UPC results during the study period, dogs 1
202 and 3 showed UPC values of above 0.5 on D360 (0.97 and 0.66 respectively).

203 At the end of the study, overall clinicopathological scores ranged from 2 to 6
204 (median=5.1).

205 According to these results, all dogs except dogs 1 and 3 were classified as Stage II of
206 clinical leishmaniosis using the clinical staging system proposed by the LeishVet group to
207 manage CanL (Solano-Gallego et al., 2011). Dogs 1 and 3 were proteinuric and were
208 consequently classified as Substage II b.

209 An intense early humoral response was observed in all dogs (Table 3). Seroconversion
210 occurred between D120 and D180, similar to that noted in other trials (Fernández-Cotrina et
211 al., 2013; Paranhos-Silva et al., 2003; Rodríguez-Cortés et al., 2007), while others have
212 reported seroconversion on Days 30-45 (Carrera et al., 1996; Leandro et al., 2001; Maia et al.,
213 2010; Martínez-Moreno et al., 1995). On D360, all dogs showed higher antibody titers (range
214 1/3200-1/12800) than those found in other experimental infections, where the method's
215 detection limit was over 1/400 (Rosypal et al., 2005) or 1/640 (Carcelén et al., 2009;
216 Fernández-Cotrina et al., 2013). Although IFAT titers and clinical signs could not be correlated,
217 highest antibody levels (1/12800) were recorded in the two dogs showing the highest clinical
218 scores, as previously described by Abranches et al. (1991), Pinelli et al. (1994) and Reis et al.
219 (2006).

220 No *Leishmania* promastigotes or DNA were detected on D0.

221 According to both microscopy observations and BM aspiration specimen cultures in
222 NNN medium, the prepatent period was estimated at between D90 and D180 (Table 4).

223 The BM culture was more sensitive than cytology, which sensitivity can be reduced by
224 a lower parasite load present in some samples (Moreira et al., 2007; Solano-Gallego et al.,
225 2011). Most positive cultures were detected in the first week, and less frequently cultures

226 became positive in the second and third weeks. As recommended by other authors, this
227 supports the need for subculture at least up to week 3 p.i. (Maia et al., 2009).
228

229 The TaqMan probe and microcircular kinetoplast DNA (kADN) were used as the
230 molecular target as described by others (Francino et al., 2006). RtQ-PCR is able to quantify 0.01
231 to 10,000 parasites in a single reaction and and detects infection even before seroconversion
232 (Martínez et al., 2011; Oliva et al., 2006), being an effective tool to diagnose CanL when the
233 dog lacks detectable antibodies, monitor and predicting relapses during follow-up (da Silva et
234 al., 2012; Hernández et al., 2013; Martínez et al., 2011; Mary et al., 2004; Miró et al., 2009;
235 Pennisi et al., 2005). However, the information provided by PCR should not be separated from
236 data obtained in clinical status, clinicopathological and serological assessments, since PCR does
237 not reveal immunological status and a single positive result by this technique confirms
238 *Leishmania* infection but not disease (Solano-Gallego et al., 2009).

239 Median *Leishmania* parasite loads for each sample analyzed by rtQ-PCR are provided in
240 Figure 1. The parasite load varied significantly (range 0.22 parasites/ml of urine to 4,800,000
241 parasites/ml of BM).

242 *Leishmania* kDNA was detected in PB samples from dogs 2 and 7 starting on D90
243 (mean of 32.4 parasites/ml), whereas dogs 1, 4 and 6 were positive on D120 and dogs 3 and 5
244 on D180. No parasites were detected in PB in dog 8 throughout the study period. On D360, the
245 higher parasite concentrations were recorded in six dogs, with a median of 1328 parasites/ml
246 (range 381.5-70000). It has been reported that dogs whose parasitemia range from medium to
247 high or very high positive are sick or eventually will develop leishmaniosis (Martínez et al.,
248 2011)

249 Bone marrow was the organ showing the highest parasite concentrations; and, despite
250 not detecting *Leishmania* kDNA in the PB of dog 8 during follow-up, a low parasite load was

251 observed in BM. In effect, BM is usually densilly parasitized in infected dogs (Momo et al.,
252 2014) and is the target organ for invasion and multiplication of the parasite, while blood acts
253 as a transport system and not as a reservoir organ (Momo et al., 2014; Quaresma et al., 2009).
254 Accordingly, blood is not the sample of choice for a molecular diagnosis of CanL (Miró et al.,
255 2008). Further, blood may contain a number of PCR inhibitors that can reduce the sensitivity of
256 this technique (Fisa et al., 2001; Lachaud et al., 2002; Reithinger et al., 2002b). Parasite kDNA
257 was detected in BM in six dogs on D90 (118,000 parasites/ml were detected in dog 4), while
258 the remaining two dogs (5 and 8) were positive one month later (D120). The parasite load in
259 this tissue was much higher than that found in PB, i.e. 118000 parasites/ml on D120 in dog 4,
260 and a peak was detected on D270 in dogs 1 (4,800,000 parasites/ml), 4 (139,500 parasites/ml),
261 5 (9,870 parasites/ml) and 8 (580 parasites/ml). In contrast, a peak in blood parasitemia was
262 observed on D360 in six dogs.

263 In a similar experimental CanL study (Rodríguez-Cortés et al., 2007), rtQ-PCR detected
264 parasites in blood in five out of six dogs after D120, while in BM, no parasites were detected in
265 two dogs throughout the study, although the remaining four dogs showed 100 to 5,000-fold
266 greater parasite burdens than those observed in blood samples.

267

268

269 On D120, no *Leishmania* kDNA was detected in the urine of any dog, but on D360, six
270 dogs showed low parasite loads (maximum 8.57) while dogs 4 and 8 remained negative. The
271 presence of *L. infantum* DNA in urine has been reported in naturally infected dogs (Franceschi
272 et al., 2007; Manna et al., 2008a; Solano-Gallego et al., 2007), though parasite levels were
273 lower than in BM, lymph node or blood samples. Solano-Gallego et al. (2007) and Manna et al.
274 (2008a) correlated renal disease in dogs with the presence of *Leishmania* DNA in urine
275 detected by rtQ-PCR, however, (Franceschi et al., 2007) did not observe this link using

276 conventional PCR. In our study, the parasite load found in urine was much lower than that
277 quantified by Solano-Gallego et al. (2007) and Manna et al. (2008a), what may be attributed to
278 the fact that 17% and 30%, respectively, of dogs used in the above-mentioned studies had
279 renal disease. In our study, no dog showed clinicopathological signs, nor proteinuria (UPC>1)
280 compatible with renal disease. Thus in principle, we could say that urine is not a good target
281 tissue for a diagnosis of clinical CanL, especially in dogs without kidney disease, though
282 perhaps its prognostic value for an early diagnosis of renal disease associated with CanL should
283 be addressed.

284 Exfoliative cells are constantly generated in mucosae, thus the noninvasive collection
285 of this tissue using a swab is a source of biological material for the detection and quantification
286 *L. infantum*. In our study, a higher parasite load was detected in the vulvar mucosa, followed
287 by the oral and conjunctival mucosae. No kDNA was found in any dog on D60, and only 2 dogs
288 returned positive CS samples three months later (D120), while OS and VS from six dogs were
289 positive at this time-point. In all the dogs except dog 8, *Leishmania* kDNA was present on D360
290 in CS (range 0.25-384 parasites/swab), OS (range 0.6-1060 parasites/swab) and VS (range 2-
291 3321 parasites/swab).

292 In our study, the use of OS showed greater sensitivity than PB, urine or CS. By D360,
293 with higher parasite loads (dog 4: 1060 parasites/swab) than previously reported results
294 (Lombardo et al. (2012): mean=7 parasites/swab). While Lombardo et al. (2012) obtained a
295 low sensitivity of rtQ-PCR in seropositive dogs (8.7% of positive dogs), Ferreira et al. (2013)
296 described the good diagnostic power of OS in the absence of clinical signs, testing 79% of dogs
297 positive by conventional PCR.

298 In the present study, *Leishmania* parasite loads in dogs were quantified for the first
299 time in vulvar swabs. Dog 8 remained PCR-negative for VS, as well as in urine, and this dog also
300 showed the lowest parasite load in all the PCR tested tissues. In a prior trial, an infected bitch

301 was found to show intracytoplasmic amastigotes within an inflammatory cutaneous infiltrate
302 of macrophages in the vulva which was confirmed by immunohistochemistry and PCR (Silva et
303 al., 2008). Throughout our study period, no dogs exhibited vulvar mucosal lesions, although a
304 higher parasite load was detected in this tissue compared to CS, OS and urine. This highlights
305 the important epidemiological role of infected females in the sexual transmission of CanL as
306 well as the utility of this tissue for diagnosing *L. infantum* infection.

307 Conjunctival swabs have offered promising results for a noninvasive molecular
308 diagnosis of CanL including a high sensitivity (over 90%) (Carvalho Ferreira et al., 2014; Ferreira
309 et al., 2008; Pilatti et al., 2009; Strauss-Ayali et al., 2004) and specificity (100%) (Strauss-Ayali
310 et al., 2004) even in seronegative (Leite et al., 2010; Leite et al., 2011) and clinically healthy
311 dogs (Carvalho Ferreira et al., 2014; Leite et al., 2010). Amastigotes can reach the ocular region
312 via the bloodstream (Reithinger et al., 2002a) and most likely also via the lymphatic tissue of
313 the conjunctiva, given its infiltration by tissue histiocytes harboring parasites observed in
314 histological sections (Strauss-Ayali et al., 2004). According to our results, in all dogs *Leishmania*
315 kDNA was detected in CS on D360, though parasite loads were lower than those observed in
316 OS and VS. Low parasite loads in the conjunctiva have been described by others (Ferreira et al.,
317 2013; Lombardo et al., 2012) and it is thought that amastigotes reach the conjunctival
318 epithelium and persist in a small number of infiltrating macrophages (Strauss-Ayali et al.,
319 2004). Some authors claim that the use of bilateral samples (obtained from both eyes)
320 increases the sensitivity of the assay and this practice has been strongly recommended
321 (Ferreira et al., 2013; Ferreira et al., 2008; Lombardo et al., 2012) despite no significant
322 differences observed compared to sampling both eyes separately. In our study, positive
323 correlation was detected between overall clinical score and the rtQ-PCR results for CS (p=0.016
324 and ρ=0.8).

325 Positive correlation was observed between the parasite load detected in PB and BM
326 (D120: p=0.037, ρ=0.73 and D360: p=0.004, ρ=0.874). More significant correlations were

327 detected on around D360 between the parasite loads of several samples: PB and BM (*p*=0.004,
328 ρ=0.87), PB and CS (*p*=0.01, ρ=0.79), PB and OS (*p*=0.002, ρ=0.89), PB and VS (*p*=0.0004,
329 ρ=0.94), BM and OS (*p*=0.03, ρ=0.73), BM and VS (*p*=0.006, ρ=0.85), CS and VS (*p*=0.03,
330 ρ=0.73), CS and OS (*p*=0.013, ρ=0.81), and OS and CS (*p*=0.014, ρ=0.81).

331 An ever-increasing number of studies has detected and quantified the presence of *L.*
332 *infantum* DNA in samples obtained by non-invasive procedures. While the vast majority focus
333 on natural infections, our study examines experimentally infected dogs, such that we were
334 able to monitor parasite loads in the tissues analyzed during the course of a whole year. We
335 were also able to show for the first time, the presence of *L. infantum* DNA in cells of the vulva
336 and its high burden in some dogs. We therefore recommend the use of vulvar swabs in the
337 diagnosis of CanL, although further work is needed in a larger number of dogs and under
338 natural conditions to confirm this finding.

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340

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