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Serum IFAT and real-time PCR results in dogs affected by Leishmania infantum: evaluation before and after treatment at different clinical stages

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1 **Serum IFA and real-time PCR results in dogs affected by *Leishmania infantum*: evaluation**
2 **before and after treatment at different clinical stages**

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14 Running head: Changes of IFAT and real-time PCR in dogs with leishmaniasis

15

Abstract. We compared results of a serum immunofluorescence assay (IFA) and lymph node quantitative PCR (qPCR) in dogs classified as exposed, infected, or sick because of leishmaniasis. We also determined how IFA or qPCR changed in response to treatment and reflected different clinical and clinicopathologic improvement of dogs. We included 108 dogs in our retrospective study: 12 exposed, 25 infected, and 71 sick, as classified according to Canine Leishmaniasis Working Group standards. Between-group comparison showed higher IFA values ($p < 0.01$) for sick dogs; qPCR values were higher for sick than infected dogs ($p < 0.01$). A novel clinical and clinicopathologic score was created and applied to 50 sick dogs. Using this score, 41 were reclassified as partially recovered (PR) within 3 mo, and 37 as totally recovered (TR) 3-6 mo after presentation. Statistically significant differences in IFA values were found between the sick and TR dogs ($p < 0.01$), but not between sick and PR dogs ($p = 0.98$). During follow-up, qPCR revealed a progressive decrease in parasite load, with a statistically significant difference in sick versus PR ($p < 0.01$), sick versus TR ($p < 0.01$), and PR versus TR ($p < 0.01$) dogs. A decrease of 1 point in the clinical score corresponded to 1.3 *Leishmania*/µL qPCR decrease ($p < 0.01$) and decrease of 1:42 in IFA ($p < 0.01$). Our findings confirm that the clinical status of dogs affected by leishmaniasis is closely related to parasite load and antibody level, both before and after treatment.

Key words: Clinical score; dog; IFA; leishmaniasis; qPCR.

36 Canine leishmaniasis (CanL) is a zoonotic disease caused by the protozoan parasite *Leishmania*
37 *infantum*. Dogs are the main peridomestic reservoir for this organism in the Mediterranean basin.
38 Host immune response is the most important factor for efficiently controlling parasite infection.
39 Clinical features of infection vary widely because of the various pathogenic mechanisms involved
40 in the disease process. Diagnosing CanL is extremely challenging as a result of nonspecific clinical
41 presentations.[3,11]

42 Two guidelines for clinical classification of infected dogs, proposed by LeishVet[16] and by the
43 Canine Leishmaniasis Working Group (CLWG),[9] can aid in establishing a diagnosis, correlating
44 infection classes with treatment and prognosis.[13,15] The CLWG classification[9] divides dogs into 3
45 classes (exposed, infected, and sick) based on positive serology, parasitologic analysis, and the
46 presence or absence of clinical signs, including laboratory abnormalities suggestive of
47 leishmaniasis. The guidelines indicate therapeutic protocols and follow-up evaluations but are less
48 clear about how to interpret laboratory test results for monitoring response to therapy and
49 progression of associated chronic diseases such as proteinuric nephropathy and chronic renal
50 failure.[8,16] Currently, an objective method does not exist to evaluate the clinical and laboratory
51 improvements of dogs after therapy, and to identify early changes compatible with disease
52 recurrence.

53 The techniques used most commonly in the diagnosis of *Leishmania* infection are the indirect
54 immunofluorescent assay (IFA), which measures the humoral antibody response, and PCR for
55 direct identification of protozoal DNA in tissues.[9,10] Studies have shown correlation of humoral
56 response, clinical status, and tissue parasite density,[1,2,5,12,14,18] and have evaluated the ability of
57 ELISA, IFA, or PCR to monitor therapeutic response.[2,14,17] If treatment is successful, antibody titer
58 and parasite loads decrease with time; however, contradictory results are reported for early or late
59 declines in antibody titer and its usefulness to monitor treatment.[14,17]

60 We compared IFA and quantitative PCR (qPCR) values in dogs in the 3 classification groups:
61 exposed, infected, or sick. We also determined how IFA or qPCR results changed in response to
62 treatment and reflected different degrees of clinicopathologic improvement of dogs.
63 We reviewed the medical records of all dogs examined at our Veterinary Hospital at Turin
64 University, Grugliasco, Torino, Italy, between 2010 and 2015. Dogs included in our study were
65 privately owned, brought to the hospital for suspected *Leishmania infantum* infection or routine
66 checkup, and had both IFA and qPCR performed.
67 Our Institutional Ethical and Animal Welfare Committee did not require study approval given
68 the observational nature of the study. The owners of all dogs examined at our hospital had
69 consented to diagnostic procedures proposed by the attending veterinarians.
70 The clinicopathologic data collected included: physical examination findings, complete blood
71 count (ADVIA 120 Hematology, Siemens Healthcare Diagnostics, Tarrytown, NY), biochemical
72 profile and urine protein:creatinine ratio (ILAB 300 plus, Clinical Chemistry System,
73 Instrumentation Laboratories, Milan, Italy), serum protein electrophoresis, urinalysis (Multistix 10
74 SG Reagent Strips, Siemens Healthcare Diagnostics), and urine sediment analysis.
75 Testing for *Leishmania infantum*, including serologic IFA and qPCR assay, was carried out
76 by an external laboratory (Istituto Zooprofilattico Sperimentale of Piemonte, Liguria and Valle
77 d'Aosta, Torino, Italy). Samples for qPCR and cytologic evaluation were obtained by fine-needle
78 lymph node aspiration upon presentation. We included dogs testing positive by IFA or qPCR at the
79 first visit in our study. Based on these test results, the dogs were classified as *exposed* if they had
80 negative qPCR findings, positive antibody titer, and were clinically normal or did not have clinic
81 signs associated with leishmaniasis. Dogs were classified as *infected* if they had positive qPCR
82 findings, a positive antibody titer, and were healthy or did not have clinical signs associated with
83 leishmaniasis. *Sick* dogs had positive qPCR findings, a positive antibody titer, and one or more
84 clinical signs common to leishmaniasis were present.[9]

85 IFA was performed as described in the World Organization for Animal Health Manual of
86 Diagnostic Tests and Vaccines for Terrestrial Animals.[20] Anti-leishmania antibodies were detected
87 with the use of anti-dog IgG conjugated to fluorescein isothiocyanate (Sigma-Aldrich, St. Louis,
88 MO).
89 For the qPCR assay, 1 mL of each lymph node aspirate was centrifuged at 16,000 $\times$ *g* for 5 min.
90 The pellet was resuspended in 100 µL of lysis kit buffer and then processed for total genomic DNA
91 extraction (Illustra tissue and cells genomic Prep Mini Spin Kit, GE Healthcare Bio-Sciences,
92 Pittsburgh, PA) according to the manufacturer's instructions. Three replicates of 6 DNA
93 concentrations (10^3/µL to 10^9/µL) in 10-fold serial dilutions obtained from a culture of *L. infantum*
94 (MON-1 IPT1; provided by the National Reference Centre for Leishmaniasis, Istituto
95 Zooprofilattico Sperimentale della Sicilia, Palermo, Italy) were used to evaluate the qPCR assay
96 sensitivity and efficiency. The purified DNA concentration was determined by UV
97 spectrophotometer (GeneQuant Pro, Amersham Biosciences, Buckinghamshire, UK) and
98 normalized to 40 ng/µL. TaqMan probe, PCR primers, master mix concentrations, and thermal
99 profile were used as described previously.[19] DNA samples were amplified in a thermocycler
100 (CFX96 Touch Real-Time PCR Detection System, Bio-Rad, Hercules, CA). All samples were
101 tested in triplicate. A negative control (DNA-free water) and the 6 DNA concentrations were
102 included in each run. The results were expressed as parasites per µL.
103 All laboratory analyses performed at the initial diagnosis (CBC, biochemical profile, urine
104 protein:creatinine ratio and urinalysis, serum protein electrophoresis, IFA, and qPCR) were repeated
105 at subsequent follow-up visits. A novel clinicopathologic score (Table 1) was created and used to
106 score each patient before initiation of therapy and at each visit.[9] This score was based on previously
107 reported scores, and was created to provide a more comprehensive clinical-pathologic assessment.[4-
108 7,12] The most common clinical signs reported in the literature and laboratory alterations useful for
109 the monitoring and prognosis were included, and were assigned a value that increased depending on
110 the severity of change. The score was created to be an objective value at each follow-up, creating a

111 continuous variable based on clinical evaluation. Response to therapy consistent with current
112 guidelines was evaluated for the sick dogs that began treatment.[8] Prophylaxis for sandflies with
113 synthetic pyrethroids (spot-on or collar) applied every month, was prescribed for all dogs. Treated
114 dogs with partial improvement in clinical signs and laboratory abnormalities, defined as a decrease
115 of 1 score point on ≥ 50% of the parameters in the novel scoring system, were classified as *partially*
116 *recovered* (PR). Dogs with complete regression of clinical signs and laboratory abnormalities
117 defined as when all score parameters in the novel scoring system returned to 0, were classified as
118 *totally recovered* (TR). Dogs with chronic diseases (e.g., proteinuric nephropathy, chronic renal
119 failure, arthropathy, or permanent ocular disease), were classified as TR although the
120 clinicopathologic score was > 0. In these latter cases, the residual clinical and laboratory
121 abnormalities composing the clinicopathologic score were related to irreversible injury resulting
122 from chronic illness.

123 Statistical analysis was performed (Stata v.14, StataCorp, College Station, TX). An ANOVA
124 was used to compare the results of initial IFA (exposed vs. infected vs. sick) and qPCR values
125 (infected vs. sick), or a Mann-Whitney test when underlying assumptions for an ANOVA were
126 absent. Bonferroni correction was applied to correct for multiple comparisons. To evaluate the
127 effect of treatment and because of repeated measures on the same patient, a quantile multivariate
128 regression model was used to identify a correlation between IFA values and clinical status (i.e.,
129 sick, PR, TR), and a random-effects model for panel data was used to identify a correlation between
130 qPCR values and clinical status. A random-effects model for panel data, if the underlying
131 assumptions were met, or a quantile multivariate regression model if not, was used to identify
132 differences in scores between the sick, PR, and TR dogs. A linear regression model was applied to
133 identify correlations between IFA and qPCR in comparison to the clinicopathologic score, without
134 considering the clinical status or patient age (covariates). To compare the IFA and qPCR values
135 before and after treatment in sick dogs, an ANOVA with Bonferroni multiple-comparison

136 correction was used if underlying assumptions were met, and if not the Mann-Whitney test was
137 applied. Statistical significance was set at 5% ($p \leq 0.05$).
138 There were 108 dogs with positive IFAT and/or qPCR that qualified for inclusion in our study.
139 There were 60 males (36 intact, 24 neutered) and 48 females (33 intact,15 spayed), 1-15-y-old
140 (median 7-y-old). Breed distribution was 45% mixed-breed, 6% Segugio Italiano, 5% Labrador
141 Retriever, and 44% other breeds. Based on IFA, qPCR, clinical score, and laboratory evaluation at
142 first visit, 12 dogs were classified as exposed, 25 as infected, and 71 as sick (Table 2). There was no
143 significant difference in IFA values between exposed and infected dogs ($p = 0.09$), whereas IFAT
144 values were significantly higher for the sick dogs ($p < 0.01$) (Table 2, Fig. 1). qPCR values were
145 significantly higher in sick dogs compared to infected dogs ($p < 0.01$) (Table 2, Fig. 1).
146 Fifty of the 71 sick dogs received treatment: allopurinol alone in 8 of 50, allopurinol and
147 meglumine antimoniate combined in 5 of 50, and allopurinol and miltefosine combined in 37 of 50.
148 Of these 50 sick dogs, 41 were classified as PR on follow-up visit 30-90 d after the end of
149 treatment, and 37 as TR on follow-up visit 90-180 d after completion of treatment (Table 3).
150 The quantile multivariate regression model showed statistically significant differences in IFAT
151 values among the 3 groups, with lower values for TR dogs than the sick ($p < 0.01$) and the PR ($p <$
152 0.01) dogs, but no differences between the PR and the sick dogs ($p = 0.98$). Analysis of qPCR
153 results by a random-effects model showed a progressive decrease in parasitic load from sick to TR
154 dogs, with a statistically significant difference at every follow-up assessment visit [sick versus PR
155 ($p < 0.01$), sick versus TR ($p < 0.01$), and PR versus TR ($p < 0.01$)].
156 The linear regression model used to test the relationship between the total score and the IFA and
157 qPCR value showed an inverse linear relation such that a decrease of 1 score point corresponded to
158 an average decrease of 1.3 *Leishmania*/µL qPCR ($p < 0.01$) and an average decrease of 1:42 in IFA
159 ($p < 0.01$). IFA values for the PR and the TR dogs were higher than those for the exposed and the
160 infected dogs (PR vs. exposed: $p < 0.01$; PR vs. infected: $p < 0.01$; TR vs. exposed: $p < 0.05$; TR vs.
161 infected: $p < 0.01$). qPCR values for the TR dogs were lower than those for the infected dogs ($p <$

162 0.01), whereas no statistically significant difference in qPCR values was found between the PR and
163 infected dogs ($p = 0.95$) (Fig. 1).
164 As expected, important differences in qPCR and IFA values were found between the dogs
165 classified as sick versus those classified as exposed or infected. The median IFA values were 10 and
166 30 times higher in sick dogs compared to the exposed and the infected dogs respectively, and 16
167 times higher than the maximum laboratory reference range (IFA 1:80), without overlap between
168 groups (Fig. 1). Despite the marked differences between symptomatic and asymptomatic dogs,
169 some sick dogs were noted to have very low IFA values (1 dog had 1:80 and 2 dogs had 1:160).
170 Because not all dogs seroconvert after infection,[10] a very high antibody titer could be consistent
171 with leishmaniasis, whereas a low titer may need further evaluation to confirm disease, such as the
172 tissue identification of parasites.[9,15]
173 The median qPCR value for the sick dogs was 900 times greater than that for the infected dogs,
174 with minimal overlap between groups at higher values. Dogs with clinical signs suggestive of
175 leishmaniasis have significantly higher parasite loads than infected dogs. As reported in previous
176 studies, parasite load and antibody titer are related to the severity of leishmaniasis and the degree of
177 clinical and pathologic abnormalities.[4,12]
178 For the second objective of our study, dogs were reclassified as PR or TR based on clinical and
179 laboratory improvements after therapy. To standardize and objectively evaluate dogs affected by
180 leishmaniasis, the most common clinical and laboratory alterations reported in the literature were
181 combined with a numeric value to create a score.[9,15] The advantage of this score, compared with
182 those reported previously,[4-7,12] is the combination of clinical signs and laboratory alterations typical
183 of leishmaniasis that is applicable in dogs with different clinical presentations, including those
184 without dermatologic signs or with minimal laboratory abnormalities. Reduction in clinical signs
185 corresponded to a significant decrease in parasite load, with an average decrease of 1.3
186 *Leishmania*/µL for each point decrease in score. The correlation with qPCR makes the proposed

187 clinicopathologic score useful for monitoring sick dogs, and provides the clinician with an objective
188 tool to measure improvement and suggest a trend in parasitic load.

189 The dogs with total remission of clinical and laboratory abnormalities had significantly lower
190 qPCR values than the infected dogs. After treatment, a marked decrease in parasite load usually
191 indicates effective control of disease but not parasitologic cure in *Leishmania* infection, because
192 most dogs remain infected for life. Even dogs with a qPCR of zero can have fluctuations in parasite
193 burden over time (data not shown).[2,8] However, a progressive increase in parasite load detected in
194 the follow-up visit by serial monitoring of qPCR could potentially identify a dog at risk of relapse.
195 Further studies to evaluate this possibility are warranted.

196 There was also a direct correlation between antibody level and clinicopathologic score, with an
197 average decrease of 1:42 of IFA for each score point. It has been reported that a decrease in parasite
198 load is revealed early by PCR assay in dogs responding to treatment, whereas antibody titers have
199 demonstrated contrasting results. Some studies have found that it is not useful to monitor antibody
200 levels soon after treatment because a significant decrease is seen only after 6 mo. A 2016 article
201 reported a considerable decrease in ELISA titer as early as 30 d of therapy in dogs treated with
202 meglumine antimoniate and allopurinol.[17] Our study showed no difference in IFA values between
203 the sick and the PR dogs, although the TR dogs had a significantly lower antibody level than either
204 of the other 2 groups. We also noted that the antibody level took time to decrease after treatment.
205 Our findings may differ because we used a different method to measure the IgG antibody level (IFA
206 versus ELISA), and the majority of our dogs were treated with miltefosine, which can take longer to
207 result in clinical improvement.[6]

208 Our study has several limitations, including its retrospective design with incomplete follow-up
209 data (not all sick dogs could be classified as PR or TR), multiple therapeutic regimens, and non-
210 standardized follow-up examination after completion of therapy. Another limitation is the lack of
211 long-term data on how many PR dogs reached TR status, at what time, and how many relapsed.

212 Our findings indicate that the clinical and pathologic status of dogs affected by leishmaniasis is
213 closely related to parasite load and antibody level both before and after treatment. However, a
214 decrease in the clinicopathologic score in PR was associated with a significant decrease in qPCR
215 values whereas a difference in IFA titer was not found between PR and sick dogs. This
216 clinicopathologic score could potentially provide guidance on patients' parasite burden while
217 monitoring the effect of treatment. Prospective studies that track the performance of this novel
218 clinical score, IFA, and qPCR in dogs with a relapse should be pursued.

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269

270 **Table 1.** Novel clinicopathologic scoring system for dogs with suspected *Leishmania infantum*
271 infection.

	Score			
Finding	0	1	2	3
Appetite	Normal	Dysorexia	Anorexia	Anorexia
Decreased performance	No	Mild	Moderate	Severe
Lameness	No	Occasional episodes	Recurrent episodes	Persistent
Gastrointestinal signs (vomiting or diarrhea)	No	Occasional episodes	Recurrent episodes	Persistent
Epistaxis	No	Single episode	Recurrent episodes	Persistent bleeding
Mucous membrane	Pink	Congested	Pale	White
Muscle atrophy	No	Localized	Multifocal	Generalized
Lymphadenopathy	No	Mild	Moderate	Severe
Body condition score (5= normal)	5	4	3	2
Cutaneous ulcers	No	Localized	Multifocal	Generalized
Cutaneous crusts	No	Localized	Multifocal	Generalized
Alopecia	No	Localized	Multifocal	Generalized
Onychogryphosis	No	Mild	Moderate	Severe
Dandruff	No	Localized	Multifocal	Generalized
Hyperkeratosis	No	Mild	Moderate	Severe
Cutaneous nodules	No	Localized	Multifocal	Generalized
Uveitis	No	Mild	Moderate	Severe
Conjunctivitis	No	Mild	Moderate	Severe
Hematocrit (L/L)	>0.37	0.3-0.36	0.2-0.29	<0.2
Platelets (x10^9/L)	>200	200-51	50-20	<20
Creatinine (µmol/L) (mg/dl)	<124 (<1.4)	124-168 (1.4-1.9)	169-442 (2-5)	>442 (>5)
UPC (with inactive sediment)	< 0.2	0.2- 0.49	0.5 - 2	>2
Total protein g/L (g/dL)	55-75 (5.5 – 7.5)	76-84 (7.6 – 8.4)	85-95 (8.5 – 9.5)	95 (>9.5)
Albumin g/L (g/dL)	35-30 (3.5-3)	29-25 (2.9-2.5)	24.9-20 (2.49-2.0)	<20 (<2)
Gamma globulins g/L (g/dL)	4-8 (0.4-0.8)	8.1-13 (0.81-1.3)	14-25 (1.4-2.5)	>26 (>2.6)
A/G ratio	≥0.6	0.4-0.59	0.21-0.39	<0.2

272

273 A/G = albumin to globulin ratio; Hct = hematocrit; UPC = urine protein to creatinine ratio with

274 original units as mg/dL.

275 Occasional episodes = 1 or 2 episodes/mo; Persistent = > 1 episode/wk; Recurrent episodes = 1

276 episode/wk;

277 Generalized = >3 sites affected; Localized = 1 site affected; Multifocal = 2 or 3 sites affected.

278

279 **Table 2.** IFA and qPCR values at presentation of 108 dogs exposed to *Leishmania infantum.* Dogs

280 were classified as exposed, infected, or sick based on IFA, qPCR, and on our leishmaniasis

281 clinicopathologic score.

	Exposed (n = 12)	Infected (n = 25)	Sick (n = 71)
IFA	1:120 * (1:80-1:640)	1:40 ** (1:20-1:2,560)	1:1,280 (1:80-1:40,960)
qPCR (*Leishmania*/µL)	0	0.01 # (0.001-16)	9 (0.001-1,000)

282

283 Numbers = median (minimum-maximum). IFA = immunofluorescence assay; qPCR = quantitative

284 PCR; *comparison of IFA values between exposed and sick dogs (p = 0.0001); **comparison of

285 IFAT values between infected and sick dogs (p = 0.0001); #comparison of qPCR values between

286 infected and sick dogs (p = 0.0001).

287

288 **Table 3.** Follow-up IFA and qPCR values of 50 sick dogs infected with *Leishmania infantum* after

289 treatment. Dogs with chronic disease were classified as total remission although the

290 clinicopathologic score was > 0, because of irreversible injury resulting from chronic illness.

Treated dogs (n = 50)	Sick dogs (n = 50)	PR (n = 41)	TR (n = 37)
IFA	1:1,280 (1:80-1:40,960)	1:1,280 (1:320-1:40,960)	1:320 ** (1:20-1:5,120)
qPCR (*Leishmania*/µL)	7.25 (0.001-1,000)	0.033 * (0-109)	0.005 *# (0-2.8)

291

292 IFA = immunofluorescence assay; Numbers = median (minimum-maximum); PR = partial

293 remission (decrease of 1 score point on ≥ 50% of the parameters of scoring system recorded as

294 abnormal at the time of diagnosis); qPCR = quantitative PCR; TR = total remission (all score

295 parameters returned to 0). **comparison of IFAT values between TR and sick dogs (p = 0.006);

296 *comparison of qPCR values between TR (p = 0.0001), PR (p = 0.0001) and sick dogs;

297 #comparison of qPCR values between PR and TR dogs (p = 0.004).

298

299 **Figure 1.** Box plot of the logarithmic distribution of IFA and qPCR values in the 3 groups of 108
300 dogs at presentation: 12 exposed, 25 infected, 71 sick dogs, and in the 2 subgroups of 50 sick
301 animals following treatment at 2 different times (PR, TR). **A.** Box plot of the logarithmic
302 distribution of IFA values in dogs at presentation. **B.** Box plot of the logarithmic distribution of
303 qPCR values in dogs at presentation. **C.** Box plot of logarithmic distribution of IFAT values in dogs
304 after treatment (subgroups PR and TR). **D.** Box plot of logarithmic distribution of qPCR values in
305 dogs after treatment (subgroups PR and TR).

306 IFA = indirect immunofluorescent assay; qPCR = quantitative PCR (*Leishmania*/µL); PR = dogs
307 with partial remission; TR = dogs with total remission. Dots in qPCR figures were values of higher
308 than the upper whiskers. The upper whiskers are called "upper adjacent value" and are defined as
309 $x_{[75]} + 1.5(x_{[75]} - x_{[25]})$, where: $x_{[75]} = 3^{rd}$ quartile; $x_{[25]} = 1^{st}$ quartile. *IFA comparison: sick dogs vs
310 exposed ($p = 0.0001$), infected ($p = 0.0001$), PR ($p = 0.98$) and TR ($p = 0.006$); qPCR comparison:
311 sick dogs vs infected ($p = 0.0001$), PR ($p = 0.0001$) and TR ($p = 0.0001$).§IFA comparison: exposed
312 dogs vs PR ($p = 0.0001$) and TR ($p = 0.005$). †IFA comparison: infected dogs vs PR ($p = 0.0001$)
313 and TR ($p = 0.001$). #qPCR comparison TR vs PR ($p = 0.004$).