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Original Article

Serological, molecular and clinicopathological findings associated with *Leishmania infantum* infection in cats in Northern Italy

Lorenza Urbani*, Alessandro Tirolo*, Daniela Salvatore, Michele Tumbarello, Sofia Segatore, Mara Battilani, Andrea Balboni, Francesco Dondi.

Department of Veterinary Medical Sciences, Alma Mater Studiorum–University of Bologna, Ozzano dell'Emilia, Bologna, Italy

*These authors contributed equally

Corresponding author:

Francesco Dondi DVM, PhD, Department of Verinary Medical Sciences, Alma Mater Studiorum–University of Bologna, Via Tolara di Sopra 50, Ozzano dell'Emilia, Bologna 40064, Italy

Email: f.dondi@unibo.it

Abstract

Objectives To investigate the prevalence of *Leishmania* species infection in cats in Northern Italy and to evaluate the associations between infection, and signalment and clinicopathological data.

Methods The study was carried out in a veterinary university hospital from June to November 2017. Blood, urine, conjunctival swabs and hair were collected from all cats which had been randomly selected. *Leishmania* species infection was evaluated using the indirect fluorescent antibody test (IFAT), setting a cut-off value of 1:80 and real-time PCR on blood, conjunctival and hair samples. A complete blood count, serum chemistry profile, serum electrophoresis and urinalysis were also carried out. The cats were grouped on the basis of the results of the diagnostic criteria adopted in positive, negative and unconfirmed *Leishmania* cases. Non-parametric variables and continuous data were compared among the study groups using the $\chi 2$ test and the Mann–Whitney U-test, respectively.

Results One hundred and fifty-two cats were included. Nineteen of the 152 (12.5%) cats were positive: 18/152 (11.8%) showed IFAT titre ≥1:80 and 1/152 (0.7%) was real-time PCR-positive in a hair sample; 106/152 (69.7%) cats were negative, and 27/152 (17.8%) cats were unconfirmed for *Leishmania*. Total proteins, $beta_2$-globulin and gamma-globulin were significantly increased in the positive *Leishmania* group when compared to the negative group.

Conclusions and relevance The results of the present study demonstrated the spread of *L infantum* infection in cats in Northern Italy. Hyperprotidaemia and hypergammaglobulinaemia appeared to be significant clinicopathological abnormalities in this population of cats with *L infantum* infection.

58 **Keywords:** feline leishmaniosis; indirect immunofluorescence test; real-time PCR;

59 clinicopathological abnormalities

60 **Introduction**

61 To date, in Italy, canine leishmaniosis (CanL) is considered to be endemic throughout
62 most of the national territory having a variable prevalence. Recent studies have detected
63 *Leishmania* species infection in domestic cats as well in areas where CanL is endemic,[1]
64 with seroprevalence ranging from 0 to 68.5% and molecular prevalence ranging from 0
65 to 60.7%.[2] Cats have a neglected and controversial role in the cycle of *L infantum*[3] but
66 whether they serve as primary, secondary or accidental hosts is currently unknown.[4]
67 Furthermore, only a few studies have been carried out regarding clinical and
68 clinicopathological abnormalities in cats with feline leishmaniosis (FeL).[2]
69 The majority of the diagnostic techniques for *Leishmania* species infection available for
70 dogs are also employed for cats. The most common serological test used is the indirect
71 fluorescent antibody test (IFAT).[2,4-7] Of the molecular methods, the real-time PCR
72 (qPCR) allows quantitative and fast detection of *Leishmania* species DNA.[8,9] Detection
73 using DNA can be applied to a variety of biological matrices, including bone marrow,
74 skin biopsy and lymph node aspirate. These samples are difficult to obtain and, in some
75 cases, sedation of the animal is required.[2,10] Little is still known regarding the use of
76 non-invasive biological matrices for the molecular detection of *Leishmania* species
77 DNA in cats, such as peripheral blood, conjunctival swabs and hair.[6,11-13]
78 The aims of this study were (a) to investigate the presence and diffusion of *Leishmania*
79 species infection in cats in Northern Italy using IFAT and qPCR assays applied to

80 different biological samples (serum, blood, conjunctival swabs and hair) and (b) to
81 evaluate possible associations between infection by *L infantum* and signalment and
82 clinicopathological data.
83
84 **Materials and Methods**
85 **Study design, inclusion criteria, sampling and groups**
86 This was a prospective study carried out in a veterinary university hospital in Northern
87 Italy (Department of Veterinary Medical Science, University of Bologna). Client-owned
88 cats were included in the study during the sandfly activity season (from June to
89 November 2017) after random selection and owner consent. Cats were included if they
90 had the following requisites: (1) living in the Emilia-Romagna region and (2) had
91 undergone a blood sample after clinical examination. Signalment, lifestyle (indoor or
92 outdoor) and clinical data were recorded for each cat sampled.
93 Blood sampling was carried out by venepuncture, and samples were collected using a
94 vacuum system (Vacutest Kima). The K_3EDTA samples were used for a complete blood
95 count (CBC) and molecular analyses. Serum samples underwent a chemistry profile and
96 serum protein electrophoresis. The urine samples were collected by spontaneous
97 voiding, cystocentesis or urethral catheterisation, when required, by the attending
98 clinicians, and underwent urinalysis. Conjunctival swabs from both eyes and hair
99 (pulling) samples were also collected from each cat. Clinicopathological evaluation was

100 carried out within 1 h from the sampling and the samples were stored at –80°C after
101 examination. *Leishmania* species infection was investigated using IFAT and qPCR on
102 serum and blood, conjunctival swabs and hair samples, respectively.
103 Cats with a positive IFAT titre (≥1:80)[14] and/or positive qPCR on blood and/or
104 conjunctival swabs and/or hair samples were included in the positive *Leishmania* (PL)
105 group; cats with a negative IFAT titre (<1:40) and negative qPCR results were included
106 in the negative *Leishmania* (NL) group; cats with a IFAT titre equal to 1:40 and
107 negative qPCR results were included in the unconfirmed *Leishmania* (UL) group.
108 Moreover, the medical records of the PL group were analysed to evaluate signalment,
109 history, and clinical and clinicopathological data.
110 **Indirect fluorescent antibody test**
111 Anti-*Leishmania* IgG antibodies were investigated in serum samples using IFAT
112 according to the CanL diagnosis described in the World Organisation for Animal Health
113 (OIE) manual of diagnostic tests and vaccines for terrestrial animals.[15] Slides coated
114 with 30 µl of an in-house antigen consisting of promastigotes of *L infantum* (strain
115 MHOM/TN/80/IPT1)[16] were probed with sera serially diluted in phosphate-buffered
116 saline (PBS) starting with a concentration of 1:40 until reaching a concentration of
117 1:5120, incubated for 30 mins at 37°C and washed three times with PBS. Internal feline
118 positive (1:160) and negative sera controls were included on each slide. The slides were
119 probed with 30 µl fluorescein isothiocyanate (FITC) conjugated anti-cat IgG antibody

120 diluted in PBS at a concentration of 1:32 (Anti-Cat IgG-FITC antibody; Sigma-Aldrich)
121 for 30 mins at 37°C, were washed three times with PBS, and examined under the
122 fluorescent microscope. The highest dilution showing fluorescent promastigotes was the
123 final antibody titre.

124 **Molecular diagnosis**

125 Genomic DNA was extracted from blood, conjunctival swabs and hair samples using a
126 commercially available kit (NucleoSpin Tissue Kit; Macherey-Nagel) according to the
127 manufacturer's protocol. Before extraction, for each cat sampled, the two conjunctival
128 swab samples (right and left eyes) were resuspended together in 250 µl of PBS. Instead,
129 the hair samples were cut into three segments (proximal, central and distal) and lysed at
130 56°C overnight with 250 µl of lysis buffer (Buffer T1) and 50 µl of proteinase K
131 supplied in the extraction kit.

132 *Leishmania* species DNA detection was carried out using SYBR Green qPCR as
133 reported by Ceccarelli and collaborators.[17] A reaction was carried out using a
134 commercially available kit (PowerUp SYBR Green Master Mix Kit, Life Technologies)
135 and the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Life
136 Technologies). Serial 10-fold dilutions of a plasmid (pCR4 plasmid; Invitrogen; Life
137 Technologies) containing one copy of the target sequence was used as an external
138 standard for the construction of the assay standard curve. Melting experiments were
139 carried out after the last extension step with a continuous increment from 60 to 95°C;

140 the specific melting temperature (T*m*) was 80.7°C. The limit of detection (LOD) of the
141 assay was assessed by testing serial 10-fold dilutions of the recombinant plasmid and
142 was found to be 1 x 10^0 copy/µl. The DNA samples and standards were repeated within
143 each run in duplicate. A no template control underwent analysis simultaneously. The
144 specimens were considered positive if the fluorescence curve in the amplification plot
145 showed an exponential increase, a specific melting peak was observed and the mean of
146 the DNA copy number obtained from the replicates was greater than the LOD.

147 **Clinicopathological investigations**

148 A CBC was carried out using an automated haematology analyser (ADVIA 2120;
149 Siemens Healthcare Diagnostics). The haematology was completed with a microscopic
150 blood smear examination using May-Grünwald Giemsa staining. A serum chemistry
151 profile, including creatinine, urea, phosphate, total protein, albumin, the albumin to
152 globulin ratio (A:G), alanine transaminase, aspartate transaminase, alkaline
153 phosphatase, gamma(γ)-glutamyltransferase, total bilirubin, cholesterol, total calcium,
154 sodium, potassium, chloride, glucose, and serum amyloid A (SAA), as reported
155 previously,[18] was determined. Urinalysis included urine specific gravity (USG), dipstick
156 (Combur[10]TestUX; Roche) and microscopic sediment examination, and the urine
157 protein:creatinine ratio (UPC). Urine samples with a visible red colour and/or >250 red
158 blood cells (RBCs) in a high power field (hpf) were excluded from the UPC analysis.

159 Serum and urine chemical analysis were carried out using an automated analyser
160 (AU480; Beckman Coulter-Olympus).
161 Serum protein electrophoresis was carried out on agarose gel using a semi-automated
162 system (Hydragel protein kit; Hydrasys; Sebia).

163 **Statistical analysis**

164 The data were evaluated using standard descriptive statistics and reported as mean ±
165 standard deviation or median and range (min–max), based on their distribution. The cats
166 included in the UL group were excluded from all analyses. The categorical data were
167 analysed using the $\chi 2$ test. The upper limits of the reference interval used in the
168 university lab for cats were used as cut-off values for hyperproteinaemia and
169 hypergammaglobulinaemia and were 8.8 g/dl and 1.30 g/dl, respectively. The
170 continuous data (age and clinicopathological results) were compared among the study
171 groups using the Mann–Whitney U-test with compensated post-hoc analysis. A *P* value
172 <0.05 was considered significant. An inter-rater agreement statistic (Cohen's kappa
173 coefficient) was calculated to compare the results obtained by IFAT and qPCR, and
174 between the qPCR carried out on three different biological samples. Statistical analysis
175 was carried out using commercially available software (MedCalc Statistical Software
176 version 16.8.4).
177

178 **Results**

179 **Study groups and diagnosis of *L infantum* infection**

180 During the study period, 152 cats met the inclusion criteria. Nineteen of the 152
181 (12.5%) cats were included in the PL group: 18/152 (11.8%) showed IFAT titre ≥1:80
182 and 1/152 (0.7%) was tested qPCR-positive in hair sample. The NL group included
183 106/152 (69.7%) cats and the UL group included 27/152 (17.8%). Table 1 shows the
184 descriptive statistics results and data regarding the prevalence of infection in the study
185 population.

186 Thirteen of the 19 (68.4%) cats included in the PL group were male of which 12/13
187 (92.3%) were castrated, and 6/19 (31.6%) were female of which 5/6 (83.3%) were
188 spayed. Seventeen of the 19 (89.5%) were more than 24 months of age and 2/19
189 (10.5%) were between 6 and 24 months of age, with a median age of 8 years (9 months-
190 15 years). Fourteen of the 19 (73.7%) were European domestic shorthair cats and 5/19
191 (26.3%) were from other various breeds (Maine Coon = 1; Bengal = 1; Birman = 1;
192 Persian = 1; Chartreux = 1); 12/19 cats (63.2%) were indoor/outdoor while 7/19
193 (36.8%) were exclusively indoor. Sixteen of the 19 (84.2%) cats were from the province
194 of Bologna. The highest frequency of positivity was detected in July (5/35; 14.3%) and
195 August (7/22; 31.8%) while the frequency decreased in the other months of sampling.
196 Seroreactivity (IFAT titre ≥1:40) to *L infantum* was found in 45 of the 152 (29.6%) cats
197 tested. Of the 18 seropositive cats (IFAT titre ≥1:80), 11 (61.1%) had an antibody titre
198 equal to 1:80, five (27.8%) 1:160 and two (11.1%) 1:320 (Tables 2 and 3).

199 The cat identified as positive on hair using qPCR showed 8 copies of *Leishmania*
200 kinetoplast DNA/µl of DNA extract and was seronegative to *L infantum* (Table 2). This
201 cat was a 10-year-old European domestic shorthair castrated male which lived indoors
202 and was sampled in August.
203 Forty-nine of the 106 cats included in the NL group were male of which 43/49 (87.8%)
204 were castrated while the remaining 57 were female of which 45/57 (78.9%) were
205 spayed. Ninety-two (86.8%) were more than 24 months old, with a median of 7 years (4
206 months–20 years); 92/106 (86.8%) were European domestic shorthair cats and 93/106
207 (87.7%) were from the province of Bologna. Forty-nine of the 106 (46.2%) were indoor
208 cats and 47/106 (44.3%) were indoor/outdoor cats.
209 No statistical association was found among positivity to *L infantum* and sex, age, breed,
210 geographical origin, access to outdoor environment and month of sampling. Although
211 not significant, an increased frequency in positivity among cats sampled in July and
212 August was observed ($P = 0.071$) (Table 1).
213 No agreement between the results obtained by IFAT and qPCR and between the qPCR
214 carried out on three different biological samples was shown by the value of Cohen's
215 kappa coefficient ($P = 0.309$).
216 **Clinical and clinicopathological findings**
217 The results of the CBC, serum chemistry, protein electrophoresis, and urinalysis
218 including UPC obtained in the PL and NL groups are shown in Table 4. Total proteins,

219 beta(β)$_2$-globulin and gamma(γ)-globulin fractions were significantly increased in the
220 PL group (Table 4). Hyperproteinaemia and hypergammaglobulinaemia were detected
221 in 3/19 (15.8%) and 11/19 (25.6%) PL cats, respectively. The frequency of
222 hyperproteinaemia and hypergammaglobulinaemia were significantly increased in the
223 PL group when compared to the NL group ($P = 0.028$ and $P = 0.038$, respectively). No
224 other significant differences among the groups selected were detected.
225 No clinical signs associated with leishmaniosis were observed in any cat included in this
226 study, and leishmaniosis was never suspected by the attending clinicians. In the PL
227 group, cats with antibody titre equal to 1:80 (11/18) had gastroenteritis (n = 6),
228 neurological disorders (n = 1) and chronic kidney disease (CKD) (n = 2). Moreover,
229 2/11 in the PL group tested positive for feline *calicivirus* (FCV) and 1/11 for feline
230 immunodeficiency virus. Cats with an antibody titre greater than 1:80 (7/18) had more
231 severe diseases including autoimmune thrombocytopenia (n = 1), hypertrophic
232 cardiomyopathy (n = 1), hyperthyroidism (n = 2), neoplastia (n = 2), CKD (n = 3) and
233 Addison's disease (n = 1). One out of the seven presented coinfection with feline
234 herpesvirus and FCV, and was treated with glucocorticoids. The only cat identified
235 positive by qPCR was affected by CKD.
236 Six months after serological and molecular positivity, 8/19 (42.1%) cats included in the
237 PL group died from causes not apparently attributable to leishmaniosis. Two of them
238 had an IFAT titre of 1:80 upon admission, four had 1:160 and one had 1:320. The

239 qPCR-positive cat was euthanised within six months after inclusion in the study due to
240 CKD progression.
241
242 **Discussion**
243 The primary aim of this study was to investigate the prevalence of *Leishmania* species
244 infection in cats in Northern Italy, an area which is becoming endemic for CanL. The
245 secondary aim was to determine whether there was an association between *L infantum*
246 positivity, and signalment and clinicopathological data.
247 Two different diagnostic methods, IFAT and qPCR, were used to evaluate the presence
248 of *Leishmania* species infection in cats, and the overall prevalence detected was 12.5%.
249 Recent studies carried out in Italy have reported a similar (12.2%) or a greater (25.8%)
250 prevalence of infection.[5,6] In the present survey, the use of a molecular test allowed the
251 identification of infected subject which had not developed a detectable antibody
252 response, suggesting that serology may underestimate the real number of infected
253 animals.[19]
254 No standardised IFAT assay for the detection of antibodies against *Leishmania* species
255 is currently available for cats, and an antibody titre value universally accepted as
256 indicative of active infection has not been identified.[20] In the present study,
257 seroreactivity (≥1:40) to *L infantum* was found in 29.6% of the cats tested. Other studies
258 have reported seroreactivity ranging from 16.3 to 30% in Italy.[5,21] However, positivity

259 with a 1:40 titre is not indicative of infection; therefore, the seroprevalence could be
260 overestimated using lower cut-offs.[4,14] Based on the cut-off value ≥1:80,[2,4,14] the
261 seroprevalence detected decreases to 11.8%. Similar results have been reported in
262 Northern Italy by Spada and colleagues,[5] suggesting the spread of feline infection in
263 recent years. In the present study, *L infantum* DNA was only identified in the hair
264 sample of one cat. *L infantum* DNA was not detected in any of the blood and
265 conjunctival swab samples, even in seropositive cats. The possible reasons for the
266 inconsistency of DNA detection in blood is the absence of parasitaemia in cats except in
267 case of severe disease or intermittent parasitaemia as described in dogs.[22,23]
268 Nevertheless, higher and variable values of molecular prevalence of *L infantum*
269 infection assessed by testing feline blood and conjunctival samples have been reported
270 in Italy;[5,6,11,13,24] and in other European countries.[12,19,25-28] These results suggest that
271 future molecular studies should be carried out to evaluate the diagnostic accuracy of
272 non-invasive samples in cats as has already been done in dogs.
273 In the present study, *L infantum* DNA was detected in the hair of one cat, confirming
274 the possible utility of this biological sample for the diagnosis of FeL as has been
275 reported for CanL.[29] The negative result obtained in the IFAT carried out on this cat
276 could reflect an ineffective immune system response or could be explained by the
277 absence of antibody production during an early stage of the infection.[25] Discordant

278 findings between the serological testing and molecular methods have also been reported
279 previously.[20,30-33]
280 No significant association was found between *L infantum* infection and signalment data
281 as has been reported in previous studies.[5,25,33,34] An interesting finding of the present
282 study, although not sustained by statistical significance, is the increased prevalence of
283 infection in the cats sampled in July and August, probably due to the increased
284 phlebotomus activity in Southern Europe during these months.[35]
285 Positivity to *L infantum* in cats was associated with a significant increase in total
286 proteins, β_2 and γ globulins and with a decrease in the A:G, although not significant
287 when compared to the negative cases. Interestingly, 15.8% and 25.6% of the PL group
288 had hyperproteinaemia and hypergammaglobulinaemia, respectively, and the frequency
289 of these abnormalities was significantly increased when compared to the NL group.
290 Hyperproteinaemia associated with hyperglobulinaemia has been commonly found in
291 FeL,[4,36,37,38] and, in a study carried out on four cats, hyperglobulinaemia was classified
292 as hypergammaglobulinaemia.[36] However, the presence of hyperproteinaemia,
293 decreased the A:G, and polyclonal β and γ hyperglobulinaemia have long been reported
294 in the course of CanL.[39] Therefore, it can be hypothesised that in cats, as in dogs, the
295 humoral immune response could also be activated by the production of
296 immunoglobulins after infection. When FeL is suspected in a feline patient, performing

297 serum electrophoresis can be recommended to support the diagnosis by means of
298 evidence of β_2 and γ polyclonal hyperglobulinaemia.
299 No other significant difference was found in the haematological and serum chemistry
300 variables between the two groups studied, although mild to severe non-regenerative
301 normocytic anaemia, thrombocytopenia, leukopenia, pancytopenia and
302 hypoalbuminemia increased liver enzyme activity, and serum creatinine and urea
303 concentrations have been the most frequent abnormalities previously reported in clinical
304 cases of FeL.[2,4,36-38,40] Proteinuria is often the only abnormal clinicopathological finding
305 in dogs with CanL[39] whereas it has been described only occasionally in cats with
306 FeL.[4,36,41] In this study, an increase in the UPC in the PL group was not observed as
307 compared to the NL group. These results could be explained by the composition of the
308 NL group. In fact, the NL group was not composed of healthy cats, but also included
309 patients suffering from other diseases not related to *L infantum* infection, including
310 numerous cats suffering from renal disease which can be accompanied by several
311 laboratory abnormalities.
312 The clinical cases of feline leishmaniosis reported have frequently been associated with
313 skin lesions as well as other less specific clinical signs.[30,34] None of the positive cats
314 included in this study showed clinical signs of leishmaniosis, and the disease had never
315 been suspected by the attending clinicians. This suggests that subclinical feline

316 infections could be common in areas which are becoming endemic for CanL and that
317 clinical illness due to *L infantum* in cats is currently rare.[2]
318 Some limitations should be acknowledged when interpreting the present results.
319 Sampling should have lasted for more than a year, and the number of cats sampled
320 within the inclusion period should have been more homogeneous in each month. A
321 second staggered sampling would have been necessary for the cats included in the UL
322 group to assess the changes in the antibody response. Moreover, the study population
323 consisted mainly of cats referred to a veterinary hospital and, consequently, the animals
324 included in the study could have presented clinicopathological abnormalities not related
325 to *L infantum* infection, affecting the comparison between the PL and NL groups.
326

327 **Conclusions**

328 The results of the present study suggested the spread of *L infantum* infection in cats in
329 Northern Italy, although in the majority of cases, the infection did not seem to lead to
330 active disease. The use of a molecular technique for the detection of the *Leishmania*
331 species does not replace the serology but may support the final diagnosis.
332 Hyperproteinaemia and hypergammaglobulinaemia appeared to be significant
333 clinicopathological abnormalities in cats with *Leishmania* species infection. Additional
334 studies are needed to better assess the role of cats in the epidemiological cycle of
335 *Leishmania* species and the pathogenic role of *L. infantum* in this host.

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Ethical approval This work involved the use of non-experimental animals only (owned or unowned) and followed established internationally recognised high standards ('best practice') of individual veterinary clinical patient care. Ethical approval from a committee was not necessarily required.

Informed consent Informed consent (either verbal or written) was obtained from the owner or legal custodian of all animals described in this work for the procedures undertaken.

Informed consent for publications No animals or humans are identifiable within this publication, and therefore additional informed consent for publication was not required.

356 **References**

357 1 Metzdorf IP, da Costa Lima-Junior MS, de Fatima Cepa Matos M, et al. **Molecular**
358 **characterization of *Leishmania infantum* in domestic cats in a region of Brazil**
359 **endemic for human and canine visceral leishmaniasis.** *Acta Trop* 2017; 166:
360 121–125.

361 2 Pennisi MG, Cardoso L, Baneth G, et al. **LeishVet update and recommendations**
362 **on feline leishmaniosis.** *Parasit Vectors* 2015; 8: 302.

363 3 Pennisi MG. **Leishmaniosis of companion animals in Europe: an update.** *Vet*
364 *Parasitol* 2015; 208: 35–47.

365 4 Pennisi MG and Persichetti MF. **Feline leishmaniosis: Is the cat a small dog?** *Vet*
366 *Parasitol* 2018; 251: 131–137.

367 5 Spada E, Canzi I, Baggiani L, et al. **Prevalence of *Leishmania infantum* and co-**
368 **infections in stray cats in northern Italy.** *Comp Immunol Microbiol Infect*
369 *Dis* 2016; 45: 53–58.

370 6 Otranto D, Napoli E, Latrofa MS, et al. **Feline and canine leishmaniosis and other**
371 **vector-borne diseases in the Aeolian Islands: Pathogen and vector circulation**
372 **in a confined environment.** *Vet Parasitol* 2017; 236: 144–151.

373 7 Dedola C, Zobba R, Varcasia A, et al. **Serological and molecular detection of**
374 ***Leishmania infantum* in cats of northern Sardinia, Italy.** *Vet Parasitol Reg Stud*
375 *Reports* 2018; 13: 120–123.

376 8 Francino O, Altet L, Sanchez-Robert E, et al. **Advantages of real-time PCR assay**
377 **for diagnosis and monitoring of canine leishmaniosis.** *Vet Parasitol* 2006; 137:
378 214–221.
379 9 Benassi JC, Benvenga GU, Ferreira HL, et al. **Detection of *Leishmania infantum***
380 **DNA in conjunctival swabs of cats by quantitative real-time PCR.** *Exp*
381 *Parasitol* 2017; 177: 93–97.
382 10 Braga ARC, Langoni H and Lucheis SB. **Evaluation of canine and feline**
383 **leishmaniasis by the association of blood culture, immunofluorescent antibody**
384 **test and polymerase chain reaction.** *J Venom Anim Toxins Incl Trop Dis* 2014; 20:
385 5.
386 11 Pennisi MG, Lupo T, Malara D, et al. **Serological and molecular prevalence of**
387 ***Leishmania infantum* infection in cats from southern Italy.** *J Feline Med*
388 *Surg* 2012; 14: 656–657.
389 12 Chatzis MK, Andreadou M, Leontides L, et al. **Cytological and molecular**
390 **detection of *Leishmania infantum* in different tissues of clinically normal and**
391 **sick cats.** *Vet Parasitol* 2014; 202: 217–225.
392 13 Persichetti MF, Pennisi MG, Vullo A, et al. **Clinical evaluation of outdoor cats**
393 **exposed to ectoparasites and associated risk for vector-borne infections in**
394 **southern Italy.** *Parasit Vectors* 2018; 11: 136.

395 14 Persichetti MF, Solano-Gallego L, Vullo A, et al. **Diagnostic performance of**
396 **ELISA, IFAT and Western blot for the detection of anti-*Leishmania infantum***
397 **antibodies in cats using a Bayesian analysis without a gold standard**. *Parasit*
398 *Vectors* 2017; 10: 119.
399 15 World Organisation for Animal Health (OIE). **Manual of Diagnostic Tests and**
400 **Vaccines for Terrestrial Animals.** 2008, pp 240–250.
401 16 Baldelli R, Piva S, Salvatore D, et al. **Canine leishmaniasis surveillance in a**
402 **northern Italy kennel.** *Vet Parasitol* 2011; 179: 57–61.
403 17 Ceccarelli M, Galluzzi L, Migliazzo A. et al. **Detection and characterization of**
404 ***Leishmania* (*Leishmania*) and *Leishmania* (*Viannia*) by SYBR green-based real-**
405 **time PCR and high resolution melt analysis targeting kinetoplast minicircle**
406 **DNA.** *PLoS One* 2014; 9: e88845.
407 18 Troìa R, Balboni A, Zamagni S, et al. **Prospective evaluation of rapid point-of-**
408 **care tests for the diagnosis of acute leptospirosis in dogs.** *Veterinary J* 2018; 237:
409 37–42.
410 19 Maia C, Gomes J, Cristóvão J, et al. **Feline *Leishmania* infection in a canine**
411 **leishmaniasis endemic region, Portugal.** *Vet Parasitol* 2010; 174: 336–340.
412 20 Spada E, Proverbio D, Migliazzo A, et al. **Serological and molecular evaluation of**
413 ***Leishmania infantum* infection in stray cats in a nonendemic area in northern**
414 **Italy.** *ISRN Parasitol* 2013; 2013:916376.

415 21 Vita S, Santori D, Aguzzi I, et al. **Feline leishmaniasis and ehrlichiosis:**
416 **serological investigation in Abruzzo region.** *Vet Res Commun* 2005; 29: 319–321.
417 22 Manna L, Vitale F, Reale S, et al. **Comparison of different tissue sampling for**
418 **PCR-based diagnosis and follow-up of canine visceral leishmaniosis.** *Vet*
419 *Parasitol* 2004; 125: 251–262.
420 23 Manna L, Gravino AE, Picillo E, et al. ***Leishmania* DNA quantification by real-**
421 **time PCR in naturally infected dogs treated with miltefosine.** *Ann N Y Acad*
422 *Sci* 2008; 1149: 358–360.
423 24 Persichetti MF, Solano-Gallego L, Serrano L, et al. **Detection of vector-borne**
424 **pathogens in cats and their ectoparasites in southern Italy.** *Parasit*
425 *Vectors* 2016; 9: 247.
426 25 Ayllon T, Tesouro MA, Amusategui I, et al. **Serologic and molecular evaluation**
427 **of *Leishmania infantum* in cats from central Spain.** *Ann N Y Acad Sci* 2008;
428 1149: 361–364.
429 26 Sherry K, Miró G, Trotta M, et al. **A serological and molecular study of**
430 ***Leishmania infantum* infection in cats from the Island of Ibiza (Spain).** *Vector*
431 *Borne Zoonotic Dis* 2011; 11: 239–245.
432 27 Vilhena H, Martinez-Díaz VL, Cardoso L, et al. **Feline vector-borne pathogens in**
433 **the north and centre of Portugal.** *Parasit Vectors* 2013; 6: 99.

434 28 Akhtardanesh B, Sharifi I, Mohammadi A, et al. **Feline visceral leishmaniasis in**
435 **Kerman, southeast of Iran: Serological and molecular study.** *J Vector Borne*
436 *Dis* 2017; 54: 96–102.
437 29 Belinchón-Lorenzo S, Iniesta V, Parejo JC, et al. **Detection of *Leishmania***
438 ***infantum* kinetoplast minicircle DNA by real time PCR in hair of dogs with**
439 **leishmaniosis.** *Vet Parasitol* 2013; 192: 43–50.
440 30 Miró G, Rupérez C, Checa R, et al. **Current status of *L. infantum* infection in**
441 **stray cats in the Madrid region (Spain): implications for the recent outbreak of**
442 **human leishmaniosis?** *Parasit Vectors* 2014; 7: 112.
443 31 Silaghi C, Knaus M, Rapti D, et al. **Survey of *Toxoplasma gondii* and *Neospora***
444 ***caninum*, haemotropic mycoplasmas and other arthropod-borne pathogens in**
445 **cats from Albania.** *Parasit Vectors* 2014; 7: 62.
446 32 Attipa C, Papasouliotis K, Solano-Gallego L, et al. **Prevalence study and risk**
447 **factor analysis of selected bacterial, protozoal and viral, including vector-**
448 **borne, pathogens in cats from Cyprus.** *Parasit Vectors* 2017; 10: 130.
449 33 Montoya A, García M, Gálvez R, et al. **Implications of zoonotic and vector-borne**
450 **parasites to free-roaming cats in central Spain.** *Vet Parasitol* 2018; 251: 125–
451 130.
452 34 Sobrinho LSV, Rossi CN, Vides JP, et al. **Coinfection of *Leishmania chagasi* with**
453 ***Toxoplasma gondii*, Feline Immunodeficiency Virus (FIV) and Feline Leukemia**

454 **Virus (FeLV) in cats from an endemic area of zoonotic visceral**
455 **leishmaniasis.** *Vet Parasitol* 2012; 187: 302–306.
456 35 Gaglio G, Brianti E, Napoli E, et al. **Effect of night time-intervals, height of traps**
457 **and lunar phases on sand fly collection in a highly endemic area for canine**
458 **leishmaniasis.** *Acta Trop* 2014; 133: 73–77.
459 36 Pennisi MG, Venza M, Reale S, et al. **Case report of leishmaniasis in four**
460 **cats.** *Vet Res Commun* 2004; 28: 363–366.
461 37 Richter M, Schaarschmidt-Kiener D and Krudewig C. **Ocular signs, diagnosis and**
462 **long-term treatment with allopurinol in a cat with leishmaniasis.** *Schweiz Arch*
463 *Tierheilkd* 2014; 156: 289–294.
464 38 Pimenta P, Alves-Pimenta S, Barros J, et al. **Feline leishmaniosis in Portugal: 3**
465 **cases (year 2014).** *Vet Parasitol Reg Stud Reports* 2015; 1–2: 65–69.
466 39 Solano-Gallego L, Koutinas A, Miró G, et al. **Directions for the diagnosis, clinical**
467 **staging, treatment and prevention of canine leishmaniosis.** *Vet Parasitol*
468 2009; 165: 1–18.
469 40 Marcos R, Santos M, Malhão F, et al. **Pancytopenia in a cat with visceral**
470 **leishmaniasis.** *Vet Clin Pathol* 2009; 38: 201–205.
471 41 Navarro J A, Sánchez J, Peñafiel-Verdú C, et al. **Histopathological lesions in 15**
472 **cats with leishmaniosis.** *J Comp Pathol* 2010; 143: 297–302.

473 Table 1. Descriptive statistics and prevalence (%) of infection among the cats included
474 in the study groups

Variables	**Total**	**Positive *Leishmania* (PL) group**	**Negative *Leishmania* (NL) group**	***P* value**	**Unconfirmed *Leishmania* (UL) group**
n	152	19	106		27
Sex					
Male	75 (49.3)	13 (68.4)	49 (46.2)	0.125*	13 (48.1)
Intact	10 (6.5)	1 (5.3)	6 (5.7)	0.322†	3 (11.1)
Castrated	65 (42.8)	12 (63.1)	43 (40.5)		10 (37)
Female	77 (50.7)	6 (31.6)	57 (53.8)		14 (51.9)
Intact	16 (10.6)	1 (5.3)	12 (11.3)		3 (11.1)
Spayed	61 (40.1)	5 (26.3)	45 (42.5)		11 (40.8)
Age (months)					
Kittens <6	4 (2.6)	0 (0)	3 (2.8)	0.755	1 (3.7)
Young 6–24	13 (8.6)	2 (10.5)	11 (10.4)		0 (0)
Adult >24	135 (88.8)	17 (89.5)	92 (86.8)		26 (96.3)
Breed					
European	128 (84.2)	14 (73.7)	92 (86.8)	0.263	22 (81.5)
Pedigree	24 (15.8)	5 (26.3)	14 (13.2)		5 (18.5)
Geographical origin					
Bologna	133 (87.5)	16 (84.2)	93 (87.7)	0.570	24 (88.9)
Modena	5 (3.3)	0 (0)	4 (3.8)		1 (3.7)
Ravenna	5 (3.3)	1 (5.3)	3 (2.8)		1 (3.7)
Ferrara	6 (3.9)	2 (10.5)	4 (3.8)		0 (0)
Forlì-Cesena	3 (2)	0 (0)	2 (1.9)		1 (3.7)
Lifestyle					
Indoor/outdoor	74 (48.7)	12 (63.29)	47 (44.3)	0.190	15 (55.6)
Indoor	63 (41.4)	7 (36.8)	49 (46.2)		7 (25.9)
NA	15 (9.9)	0 (0)	10 (9.5)		5 (18.5)
Month of sampling					
June	13 (8.6)	1 (5.3)	10 (9.4)	0.071	2 (7.4)
July	35 (23)	5 (26.3)	26 (24.5)		4 (14.8)
August	22 (14.5)	7 (36.8)	11 (10.4)		4 (14.8)
September	24 (15.8)	2 (10.5)	14 (13.2)		8 (29.6)
October	16 (10.5)	1 (5.3)	13 (12.3)		2 (7.4)

November	42 (27.6)	3 (15.8)	32 (30.2)	7 (26)

The $\chi 2$ test was carried out on the PL and NL groups

*P value referred to male/female

†P value referred to male (intact or castrated)/female (intact or spayed)

478 Table 2. Results obtained using the indirect immunofluorescence antibody test (IFAT)
479 and qPCR in blood, conjunctival swabs and hair samples

IFAT	**Sample tested (n)**	**Positive**	**Negative**	**Unconfirmed**
n	152	18	107	27
Median and range		1:80 (1:80–1:320)	0 (0)	1:40 (1:40)
qPCR	**Sample tested (n)**	**Positive**	**Negative**	**Quantity**
Blood	146	0	146	–
Conjunctival swabs	150	0	150	–
Hair	150	1	149	8 copies DNA/µl

480 IFAT = indirect immunofluorescence antibody test; qPCR = real-time polymerase chain
481 reaction

482 Table 3. Prevalence (%) of *Leishmania* species infection in cats detected by IFAT

Variables	**IFAT**			
	1:80 (n = 11)	1:160 (n = 5)	1:320 (n = 2)	Total (n = 18)
Sex				
Male	8 (72.7)	3 (60)	1 (50)	12 (66.7)
Intact	1 (9.1)	0 (0)	0 (0)	1 (5.6)
Castrated	7 (63.6)	3 (60)	1 (50)	11 (61.1)
Female	3 (27.3)	2 (40)	1 (50)	6 (33.3)
Intact	0 (0)	0 (0)	1 (50)	1 (5.6)
Spayed	3 (27.3)	2 (40)	0 (0)	5 (27.7)
Age (months)				
Kitten <6	0 (0)	0 (0)	0 (0)	0 (0)
Young 6–24	0 (0)	1 (20)	1 (50)	2 (11.1)
Adult >24	11 (100)	4 (80)	1 (50)	16 (88.9)
Breed				
European	10 (90.9)	3 (60)	0 (0)	13 (72.2)
Pedigree	1 (9.1)	2 (40)	2 (100)	5 (27.8)
Geographical origin				
Bologna	10 (90.9)	5 (100)	1 (50)	16 (88.9)
Modena	0 (0)	0 (0)	0 (0)	0 (0)
Ravenna	0 (0)	0 (0)	0 (0)	0 (0)
Ferrara	1 (9.1)	0 (0)	1 (50)	2 (11.1)
Forlì-Cesena	0 (0)	0 (0)	0 (0)	0 (0)
Lifestyle				
Indoor/Outdoor	7 (63.6)	4 (80)	1 (50)	12 (66.7)
Indoor	4 (36.4)	1 (20)	1 (50)	6 (33.3)
NA	0 (0)	0 (0)	0 (0)	0 (0)
Month of sampling				
June	1 (9.1)	0 (0)	0 (0)	1 (5.6)
July	2 (18.2)	1 (20)	2 (100)	5 (27.7)
August	3 (27.2)	3 (60)	0 (0)	6 (33.3)
September	2 (18.2)	0 (0)	0 (0)	2 (11.1)
October	1 (9.1)	0 (0)	0 (0)	1 (5.6)
November	2 (18.2)	1 (20)	0 (0)	3 (16.7)

483 IFAT = indirect immunofluorescence antibody test

484 Table 4. Clinicopathological results of the cats included in the PL and NL groups

Variables	**Negative *Leishmania* (NL) group (n = 106)**	**Positive *Leishmania* (PL) group (n = 19)**	**Reference interval**	***P* value**
Haematology				
RBCs (cells/mm^3)	7,449,504.95 ± 2,268,986.283	7,473,333.333 ± 2,055,798.113	7,000,000–11,000,000	0.962
WBCs (cells/mm^3)	10,200 (950–77,480)	10,545 ± 4195.617	4800–14,930	0.705
Hb (fl)	10.974 ± 3.288	11.3 (7–15.2)	10–16	0.879
Hct (g%)	32.202 ± 9.517	34.6 (32.35–44.1)	32–48	0.982
MCV (fl)	43.1 (32.5–56.9)	43.667 ± 4.047	36–55	0.810
MCH (pg)	14.7 (10.8–19.7)	14.689 ± 1.578	12.3–16.2	0.672
MCHC (g%)	34.1 (31.8–46.9)	33.606 ± 1.143	31–36	0.259
MPV (fl)	16.8 (9.2–32.1)	17.683 ± 5.385	8–26	0.727
RDW (%)	15 (12.7–27.1)	15.367 ± 1.843	13.17–17	0.781
Platelets (cells/mm^3)	217,000 (1000–644,000)	252,277.8 ± 148,734.6	150,000–500,000	0.609
Reticulocytes (cells/mm^3)	33,000 (2200–162,100)	28,077.78 ± 14,184.48	0–80,000	0.106
Neutrophils (cells/mm^3)	7110 (390–76,050)	7943.333 ± 4175.889	1600–10,000	0.807
Monocytes (cells/mm^3)	230 (30–7670)	255 (60–920)	0–650	0.940
Lymphocytes (cells/mm^3)	1560 (40–17,270)	1817.222 ± 820.701	900–5600	0.750
Basophils (cells/mm^3)	10 (0–900)	10 (0–40)	0–60	0.096

Eosinophil (cells/mm^3)	290 (0–2430)	499.412 ± 399.585	60–1470	0.439
Serum Chemistry				
ALT (U/l)	64 (3–743)	60.211 ± 38.125	20–72	0.060
AST (U/l)	39 (16–828)	29 (16–115)	9–40	0.050
ALP (U/l)	25 (5–97,772)	15 (4–133)	0–140	0.009
GGT (U/l)	0.1 (0–5.2)	0.1 (0–1.4)	0–4	0.256
Total Bilirubin (mg/dl)	0.18 (0.01–20.85)	0.195 (0.04–3.23)	0–0.3	0.544
Cholesterol (mg/dl)	159.786 ± 52.549	150.5 (72–325)	64–229	0.775
Glucose (mg/dl)	127 (57–591)	121.316 ± 34.041	63–148	0.114
Albumin (g/dl)	3.023 ± 0.502	3.012 ± 0.537	2.6–4	0.997
Total Protein (g/dl)	7.171 ± 0.897	7.933 ± 0.946	6.5–8.8	0.005
A:G (g:g)	0.758 ± 0.191	0.654 ± 0.206	0.52–1.19	0.051
Creatinine (mg/dl)	1.54 (0.41–11.77)	1.71 (0.78–31.46)	0.8–1.8	0.375
Urea (mg/dl)	62.95 (12.6–533.05)	58.52 (26.3–599.85)	30–65	0.773
Phosphate (mg/dl)	4.835 (1.77–15.76)	5.05 (3.37–15.22)	2.5–6.2	0.547
Potassium (mEq/l)	4.25 (2.6–7.4)	4.363 ± 0.585	3.4–5.1	0.418
Sodium (mEq/l)	151.5 (136–168)	151.211 ± 4.454	145–155	0.720
Chloride (mEq/l)	117.000 (96.500–126.800)	116.050 ± 4.119	110–123	0.561
Magnesium (mg/dl)	2.31 (1.52–4.63)	2.466 ± 0.332	1.9–2.8	0.259
Total Calcium (mg/dl)	9.8 (6.7–13.14)	9.822 ± 0.734	8.5–10.5	0.948

SAA (μg/dl)	8 (1–308)	12 (1–202)	0–10	0.724
Serum protein electrophoresis				
Albumin (g/dl)	2.802 ± 0.607	2.912 ± 0.655	2.10–3.30	0.443
Albumin (%)	39.274 ± 7.635	37.205 ± 9.395	–	0.492
Alfa(α)$_1$-globulin (g/dl)	1.180 (0.02–3.62)	1.16 (0.09–1.63)	0.1–0.8	0.710
α_1-globulin (%)	16.3 (0.2–53.6)	14.9 (1.1–20)	–	0.057
α_2-globulin (g/dl)	0.825 (0.33–2.87)	0.81 (0.61–3.3)	0.58–1.05	0.573
α_2-globulin (%)	11.65 (5.6–39)	10.3 (7.4–39.9)	–	0.557
Beta(β)$_1$-globulin (g/dl)	0.560 (0.21–1.29)	0.587 ± 0.122	0.45–0.80	0.507
β_1-globulin (%)	8 (2.9–20.2)	7.458 ± 1.636	–	0.183
β_2-globulin (g/dl)	0.43 (0.14–1.66)	0.55 (0.32–1.21)	0.35–0.75	0.013
β_2-globulin (%)	6.25 (2.1–24.8)	7.132 ± 1.988	–	0.143
Gamma(γ)-globulin (g/dl)	1.03 (0.02–2.79)	1.37 (0.82–3.44)	0.60–1.30	0.003
γ-globulin (%)	14.85 (0.4–33.1)	20.132 ± 7.221	–	0.014
A:G (g:g)	0.672 ± 0.204	0.625 ± 0.230	–	0.489
Urinalysis				
USG	1026 (1008–1084)	1038.75 ± 23.344	>1040	0.428
Urine creatinine (mg/dl)	105.26 (13.7–436.98)	246.207 ± 186.088	–	0.042
Urine protein (mg/dl)	45.94 (2.28–2188)	93.61 (8.19–564.52)	0–0.4	0.114

UPC (mg:mg)	0.354 (0.041–38.582)	0.281 (0.115–10.631)	<0.2	0.750

A:G = albumin to globulin ratio; ALP = alkaline phosphatase level; ALT = alanine aminotransferase; AST = aspartate aminotransferase; GGT = gamma(γ)-glutamyl transferase; Hb = hemoglobin; Hct = hematocrit value; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; MCV = mean corpuscular volume; MPV = mean platelet volume; RBCs = red blood cells; RDW = red cell distribution width; SAA = serum amyloid A; UPC = urine protein:creatinine ratio; USG = urine specific gravity; WBCs = white blood cells