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

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Abstract

Objectives The aims of this study were 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 randomly selected cats. *Leishmania* species infection was evaluated using the indirect fluorescent antibody test (IFAT), setting a cut-off value of 1:80, and using 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 χ^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 an IFAT titre of ⩾1:80 and 1/152 [0.7%] was real-time PCR-positive from a hair sample); 106/152 (69.7%) cats were negative; and 27/152 (17.8%) cats were unconfirmed for *Leishmania* species. Total proteins, beta$_2$-globulin and gamma-globulin were significantly increased in the positive *Leishmania* group compared with the negative group.

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

Keywords: Feline leishmaniosis; indirect immunofluorescence test; real-time PCR; clinicopathological abnormalities

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Introduction

To date, in Italy, canine leishmaniosis (CanL) is considered to be endemic throughout most of the national territory, with a variable prevalence. Recent studies have detected *Leishmania* species infection in domestic cats, as well in areas where CanL is endemic,[1] with seroprevalence ranging from 0% to 68.5% and molecular prevalence ranging from 0% to 60.7%.[2] Cats have a neglected and controversial role in the cycle of *Leishmania infantum*,[3] but whether they serve as primary, secondary or accidental hosts is currently unknown.[4] Furthermore, only a few studies have been carried out regarding clinical and clinicopathological abnormalities in cats with feline leishmaniosis (FeL).[2]

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The majority of the diagnostic techniques for *Leishmania* species infection available for dogs are also used for cats. The most common serological test used is the indirect fluorescent antibody test (IFAT).[2,4–7] Of the molecular methods, real-time PCR (qPCR) allows quantitative and fast detection of *Leishmania* species DNA.[8,9] Detection using DNA can be applied to a variety of biological matrices, including bone marrow, skin biopsy and lymph node aspirate. These samples are difficult to obtain and, in some cases, sedation of the animal is required.[2,10] Still little is known regarding the use of non-invasive biological matrices for the molecular detection of *Leishmania* species DNA in cats, such as peripheral blood, conjunctival swabs and hair.[6,11–13]

The aims of this study were: (1) to investigate the presence and distribution of *Leishmania* species infection in cats in Northern Italy using IFAT and qPCR assays applied to different biological samples (serum, blood, conjunctival swabs and hair); and (2) to evaluate possible associations between infection by *L infantum* and signalment and clinicopathological data.

Materials and methods

Study design, inclusion criteria, sampling and groups

This was a prospective study carried out in a veterinary university hospital in Northern Italy (Department of Veterinary Medical Sciences, University of Bologna, Bologna, Italy). Client-owned cats were included in the study during the sandfly activity season (from June to November 2017) after random selection and owner consent. Cats were included if they: (1) were living in the Emilia-Romagna region; and (2) had undergone a blood sample after clinical examination. Signalment, lifestyle (indoor or outdoor) and clinical data were recorded for each cat sampled.

Blood sampling was carried out by venepuncture, and samples were collected using a vacuum system (Vacutest Kima). The K_3EDTA samples were used for a complete blood count (CBC) and molecular analyses. Serum samples underwent a chemistry profile and serum protein electrophoresis. The urine samples were collected by spontaneous voiding, cystocentesis or urethral catheterisation, when required, by the attending clinicians, and underwent urinalysis. Conjunctival swabs from both eyes and hair samples (plucked) were also collected from each cat. Clinicopathological evaluation was carried out within 1 h from the sampling and the samples were stored at –80°C after examination. *Leishmania* species infection was investigated using IFAT on serum and qPCR on blood, conjunctival swabs and hair samples.

Cats with a positive IFAT titre (≥1:80)[14] and/or positive qPCR on blood and/or conjunctival swabs and/or hair samples were included in the positive *Leishmania* (PL) group; cats with a negative IFAT titre (<1:40) and negative qPCR results were included in the negative *Leishmania* (NL) group; cats with an IFAT titre equal to 1:40 and negative qPCR results were included in the unconfirmed *Leishmania* (UL) group.

Furthermore, the medical records of the PL group were analysed to evaluate signalment, history, and clinical and clinicopathological data.

IFAT

Anti-*Leishmania* IgG antibodies were investigated in serum samples using IFAT according to the CanL diagnosis described in the World Organisation for Animal Health manual of diagnostic tests and vaccines for terrestrial animals.[15] Slides coated with 30 µl of an in-house antigen consisting of promastigotes of *L infantum* (strain MHOM/TN/80/IPT1)[16] were probed with sera serially diluted in phosphate-buffered saline (PBS) starting with a concentration of 1:40 until reaching a concentration of 1:5120, incubated for 30 mins at 37°C and washed three times with PBS. Internal feline positive (1:160) and negative sera controls were included on each slide. The slides were probed with 30 µl of fluorescein isothiocyanate (FITC) conjugated anti-cat IgG antibody diluted in PBS at a concentration of 1:32 (Anti-Cat IgG-FITC antibody; Sigma-Aldrich) for 30 mins at 37°C and were washed three times with PBS and examined under a fluorescent microscope. The highest dilution showing fluorescent promastigotes was the final antibody titre.

Molecular diagnosis

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

Leishmania species DNA detection was carried out using SYBR Green qPCR as reported by Ceccarelli et al.[17] A reaction was carried out using a commercially available kit (PowerUp SYBR Green Master Mix Kit; Life Technologies) and the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Life Technologies). Serial 10-fold dilutions of a plasmid (pCR4 plasmid; Invitrogen, Life Technologies) containing one copy of the target sequence was used as an external standard for the construction of the assay standard curve. Melting experiments were carried out after the last extension step with a continuous increment from 60°C to 95°C; the specific melting temperature was 80.7°C. The limit of detection (LOD) of the assay was assessed by testing serial 10-fold

dilutions of the recombinant plasmid and was found to be one copy/µl. The DNA samples and standards were repeated within each run in duplicate. A no template control underwent analysis simultaneously. The specimens were considered positive if the fluorescence curve in the amplification plot showed an exponential increase, a specific melting peak was observed and the mean of the DNA copy number obtained from the replicates was greater than the LOD.

Clinicopathological investigations

A CBC was carried out using an automated haematology analyser (ADVIA 2120; Siemens Healthcare Diagnostics). The haematology was completed with a microscopic blood smear examination using May-Grünwald Giemsa staining. A serum chemistry profile, including creatinine, urea, phosphate, total protein, albumin, albumin to globulin ratio (A:G), alanine transaminase, aspartate transaminase, alkaline phosphatase, gamma (γ)-glutamyltransferase, total bilirubin, cholesterol, total calcium, sodium, potassium, chloride, glucose and serum amyloid A (SAA), as reported previously,[18] was determined. Urinalysis included urine specific gravity, dipstick (Combur10TestUX; Roche) and microscopic sediment examination, and a urine protein:creatinine ratio (UPC). Urine samples with a visible red colour and/or >250 red blood cells in a high-power field were excluded from the UPC analysis. Serum and urine chemical analysis were carried out using an automated analyser (AU480; Beckman Coulter-Olympus).

Serum protein electrophoresis was carried out on agarose gel using a semi-automated system (Hydragel protein kit, Hydrasys; Sebia).

Statistical analysis

The data were evaluated using standard descriptive statistics and reported as mean ± SD or median and range, based on their distribution. The cats included in the UL group were excluded from all analyses. The categorical data were analysed using the χ^2 test. The upper limits of the reference interval used in the university laboratory for cats were used as cut-off values for hyperproteinaemia and hypergammaglobulinaemia and were 8.8 g/dl and 1.30 g/dl, respectively. The continuous data (age and clinicopathological results) were compared among the study groups using the Mann–Whitney U-test with compensated post-hoc analysis. A P value <0.05 was considered significant. An inter-rater agreement statistic (Cohen's kappa [κ] coefficient) was calculated to compare the results obtained by IFAT and qPCR, and between the qPCR carried out on three different biological samples. Statistical analysis was carried out using commercially available software (MedCalc Statistical Software version 16.8.4).

Results

Study groups and diagnosis of L infantum infection

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

Thirteen of 19 (68.4%) cats included in the PL group were male, of which 12/13 (92.3%) were castrated, and 6/19 (31.6%) were female, of which 5/6 (83.3%) were spayed. Seventeen of 19 (89.5%) were >24 months of age and 2/19 (10.5%) were between 6 and 24 months of age, with a median age of 8 years (range 9 months to 15 years). Fourteen of 19 (73.7%) were domestic shorthair cats and 5/19 (26.3%) were from other various breeds (Maine Coon, n = 1; Bengal, n = 1; Birman, n = 1; Persian, n = 1; Chartreux, n = 1); 12/19 cats (63.2%) were indoor/outdoor, while 7/19 (36.8%) were exclusively indoor. Sixteen of 19 (84.2%) cats were from the province of Bologna. The highest frequencies of positivity were detected in July (n = 5/35; 14.3%) and August (n = 7/22; 31.8%), while the frequency decreased in the other months of sampling.

Seroreactivity (IFAT titre ⩾1:40) to *L infantum* was found in 45/152 (29.6%) cats tested. Of the 18 seropositive cats (IFAT titre ⩾1:80), 11 (61.1%) had an antibody titre equal to 1:80, five (27.8%) to 1:160 and two (11.1%) to 1:320 (Tables 2 and 3).

The cat identified as positive from the hair sample using qPCR showed eight copies of *Leishmania* kinetoplast DNA/µl of DNA extract and was seronegative to *L infantum* (Table 2). This cat was a 10-year-old domestic shorthair castrated male that lived indoors and was sampled in August.

Forty-nine of 106 cats included in the NL group were male, of which 43/49 (87.8%) were castrated, while the remaining 57 were female, of which 45/57 (78.9%) were spayed. Ninety-two (86.8%) were >24 months old, with a median age of 7 years (range 4 months to 20 years); 92/106 (86.8%) were domestic shorthair cats and 93/106 (87.7%) were from the province of Bologna. Forty-nine of 106 (46.2%) were indoor cats and 47/106 (44.3%) were indoor/outdoor cats.

No statistical association was found regarding positivity to *L infantum* and sex, age, breed, geographical origin, access to outdoor environment and month of sampling. Although not significant, an increased frequency in positivity among cats sampled in July and August was observed ($P = 0.071$) (Table 1).

No agreement between the results obtained by IFAT and qPCR and between the qPCR carried out on three

Table 1 Descriptive statistics and prevalence of infection among the cats included in the study groups

Variables	Total (n = 152)	PL group (n = 19)	NL group (n = 106)	*P* value	UL group (n = 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 (63.2)	43 (40.6)		10 (37)
Female	77 (50.7)	6 (31.6)	57 (53.8)		14 (51.9)
Intact	16 (10.5)	1 (5.3)	12 (11.3)		3 (11.1)
Spayed	61 (40.1)	5 (26.3)	45 (42.5)		11 (40.7)
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					
DSH	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 (0)	2 (1.9)		1 (3.7)
Lifestyle					
Indoor/outdoor	74 (48.7)	12 (63.2)	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 (9.4)		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 (25.9)

The χ^2 test was carried out on the positive *Leishmania* (PL) and negative *Leishmania* (NL) groups. Data are n (%)
**P* value referred to male/female
†*P* value referred to male (intact or castrated)/female (intact or spayed)
UL = unconfirmed *Leishmania*; NA = not available; DSH = domestic shorthair

Table 2 Results obtained using the indirect immunofluorescence antibody test (IFAT) and real-time PCR (qPCR) in blood, conjunctival swabs and hair samples

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

Data are n unless otherwise indicated

different biological samples was shown by the value of Cohen's κ coefficient ($P = 0.309$).

Clinical and clinicopathological findings

The results of the CBC, serum chemistry, protein electrophoresis and urinalysis, including UPC, obtained in the PL and NL groups are shown in Table 4. Total proteins, beta $(\beta)_2$-globulin and γ-globulin fractions were significantly increased in the PL group (Table 4). Hyperproteinaemia and hypergammaglobulinaemia were detected in 3/19 (15.8%) and 11/19 (57.9%) PL cats, respectively. The frequency of hyperproteinaemia and

Table 3 Prevalence of *Leishmania* species infection in cats detected by indirect fluorescent antibody test (IFAT)

	IFAT			
Variables	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 (27.8)
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				
DSH	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 (27.8)
August	3 (27.3)	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)

Data are n (%)
NA = not available; DSH = domestic shorthair

hypergammaglobulinaemia were significantly increased in the PL group when compared with the NL group ($P = 0.028$ and $P = 0.038$, respectively). No other significant differences between the groups selected were detected.

No clinical signs associated with leishmaniosis were observed in any cat included in this study, and leishmaniosis was never suspected by the attending clinicians. In the PL group, cats with an antibody titre equal to 1:80 (n = 11/18) had gastroenteritis (n = 6), neurological disorders (n = 1) and chronic kidney disease (CKD; n = 2). Moreover, 2/11 in the PL group tested positive for feline calicivirus (FCV) and 1/11 for feline immunodeficiency virus. Cats with an antibody titre >1:80 (n = 7/18) had more severe diseases, including autoimmune thrombocytopenia (n = 1), hypertrophic cardiomyopathy (n = 1), hyperthyroidism (n = 2), neoplasia (n = 2), CKD (n = 3) and Addison's disease (n = 1). One of the seven presented with coinfection with feline herpesvirus and FCV, and was treated with glucocorticoids. The only cat identified as positive by qPCR had CKD.

Six months after serological and molecular positivity, 8/19 (42.1%) cats included in the PL group died from causes not apparently attributable to leishmaniosis. Two of them had an IFAT titre of 1:80 upon admission, four had 1:160 and one had 1:320. The qPCR-positive cat was euthanased within 6 months of inclusion in the study owing to CKD progression.

Discussion

The primary aim of this study was to investigate the prevalence of *Leishmania* species infection in cats in

Table 4 Clinicopathological results of the cats included in the positive *Leishmania* (PL) and negative *Leishmania* (NL) groups

Variables	NL group (n = 106)	PL group (n = 19)	RI	*P* value
Haematology				
RBCs (cells/mm^3)	7,449,505 ± 2,268,986	7,473,333 ± 2,055,798	7,000,000–11,000,000	0.962
WBCs (cells/mm^3)	10,200 (950–77,480)	10,545 ± 4196	4800–14,930	0.705
Hb (fl)	11 ± 3	11 (7–15)	10–16	0.879
Hct (g%)	32 ± 10	35 (32–44)	32–48	0.982
MCV (fl)	43 (33–57)	44 ± 4	36–55	0.810
MCH (pg)	14.7 (10.8–19.7)	14.7 ± 1.6	12.3–16.2	0.672
MCHC (g%)	34 (32–47)	34 ± 1	31–36	0.259
MPV (fl)	17 (9–32)	18 ± 5	8–26	0.727
RDW (%)	15 (12.7–27.1)	15.37 ± 1.84	13.17–17	0.781
Platelets (cells/mm^3)	217,000 (1000–644,000)	252,278 ± 148,735	150,000–500,000	0.609
Reticulocytes (cells/mm^3)	33,000 (2200–162,100)	28,078 ± 14,184	0–80,000	0.106
Neutrophils (cells/mm^3)	7110 (390–76,050)	7943 ± 4176	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 ± 821	900–5600	0.750
Basophils (cells/mm^3)	10 (0–900)	10 (0–40)	0–60	0.096
Eosinophils (cells/mm^3)	290 (0–2430)	499 ± 400	60–1470	0.439
Serum chemistry				
ALT (U/l)	64 (3–743)	60 ± 38	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)	0.2 (0.04–3.23)	0–0.3	0.544
Cholesterol (mg/dl)	160 ± 53	151 (72–325)	64–229	0.775
Glucose (mg/dl)	127 (57–591)	121 ± 34	63–148	0.114
Albumin (g/dl)	3.02 ± 0.5	3.01 ± 0.54	2.6–4	0.997
Total protein (g/dl)	7.17 ± 0.9	7.93 ± 0.95	6.5–8.8	**0.005**
A:G (g:g)	0.76 ± 0.19	0.65 ± 0.21	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)	63 (13–533)	59 (26–600)	30–65	0.773
Phosphate (mg/dl)	4.8 (1.8–15.8)	5.1 (3.4–15.2)	2.5–6.2	0.547
Potassium (mEq/l)	4.3 (2.6–7.4)	4.4 ± 0.6	3.4–5.1	0.418
Sodium (mEq/l)	152 (136–168)	151 ± 4	145–155	0.720
Chloride (mEq/l)	117 (97–127)	116 ± 4	110–123	0.561
Magnesium (mg/dl)	2.3 (1.5–4.6)	2.5 ± 0.3	1.9–2.8	0.259
Total calcium (mg/dl)	9.8 (6.7–13.1)	9.8 ± 0.7	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)	2.80 ± 0.61	2.91 ± 0.66	2.10–3.30	0.443
Albumin (%)	39 ± 8	37 ± 9	–	0.492
α_1-globulin (g/dl)	1.18 (0.02–3.62)	1.16 (0.09–1.63)	0.1–0.8	0.710
α_1-globulin (%)	16 (0–54)	15 (1–20)	–	0.057
α_2-globulin (g/dl)	0.83 (0.33–2.87)	0.81 (0.61–3.3)	0.58–1.05	0.573
α_2-globulin (%)	12 (6–39)	10 (7–40)	–	0.557
β_1-globulin (g/dl)	0.56 (0.21–1.29)	0.59 ± 0.12	0.45–0.80	0.507
β_1-globulin (%)	8 (3–20)	7 ± 2	–	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 (2–25)	7 ± 2	–	0.143
γ-globulin (g/dl)	1.03 (0.02–2.79)	1.37 (0.82–3.44)	0.60–1.30	**0.003**
γ-globulin (%)	15 (0–33)	20 ± 7	–	**0.014**
A:G (g:g)	0.67 ± 0.2	0.63 ± 0.23	–	0.489
Urinalysis				
USG	1026 (1008–1084)	1039 ± 23	>1040	0.428
Urine creatinine (mg/dl)	105 (14–437)	246 ± 186	–	**0.042**
Urine protein (mg/dl)	46 (2–2188)	94 (8–565)	0–0.4	0.114
UPC (mg:mg)	0.35 (0.04–38.58)	0.28 (0.12–10.63)	<0.2	0.750

Data are mean ± SD or median (range). Values in bold indicate statistical significance
RI = reference interval; RBCs = red blood cells; WBCs = white blood cells; Hb = haemoglobin; Hct = haematocrit; MCV = mean cell volume; MCH = mean cell haemoglobin; MCHC = mean cell haemoglobin concentration; MPV = mean platelet volume; RDW = red cell distribution width; ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase; GGT = gamma-glutamyl transferase; A:G = albumin:globulin ratio; SAA = serum amyloid A; USG = urine specific gravity; UPC = urine protein:creatinine ratio

Northern Italy, an area that is becoming endemic for CanL. The secondary aim was to determine whether there was an association between *L infantum* positivity and signalment and clinicopathological data.

Two different diagnostic methods, IFAT and qPCR, were used to evaluate the presence of *Leishmania* species infection in cats, and the overall prevalence detected was 12.5%. Recent studies carried out in Italy have reported a similar (12.2%) or a greater (25.8%) prevalence of infection.[5,6] In the present survey, the use of a molecular test allowed the identification of an infected subject that had not developed a detectable antibody response, suggesting that serology may underestimate the real number of infected animals.[19]

No standardised IFAT assay for the detection of antibodies against *Leishmania* species is currently available for cats, and an antibody titre value universally accepted as indicative of active infection has not been identified.[20] In the present study, seroreactivity (⩾1:40) to *L infantum* was found in 29.6% of the cats tested. Other studies have reported seroreactivity to range from 16.3% to 30% in Italy.[5,21] However, positivity with a 1:40 titre is not indicative of infection; therefore, the seroprevalence could be overestimated using lower cut-offs.[4,14] Based on the cut-off value of ⩾1:80,[2,4,14] the seroprevalence detected decreased to 11.8%. Similar results have been reported in Northern Italy by Spada et al,[5] suggesting that feline infection has spread in recent years. In the present study, *L infantum* DNA was only identified in the hair sample of one cat. *L infantum* DNA was not detected in any of the blood and conjunctival swab samples, even in seropositive cats. The possible reasons for the inconsistency of DNA detection in blood is the absence of parasitaemia in cats except in the cases of severe disease or intermittent parasitaemia as described in dogs.[22,23] Nevertheless, higher and variable values of molecular prevalence of *L infantum* infection assessed by testing feline blood and conjunctival samples have been reported in Italy,[5,6,11,13,24] and in other European countries.[12,19,25–28] These results suggest that future molecular studies should be carried out to evaluate the diagnostic accuracy of non-invasive samples in cats, as has already been done in dogs.

In the present study, *L infantum* DNA was detected in the hair of one cat, confirming the possible utility of this biological sample for the diagnosis of FeL, as has been reported for CanL.[29] The negative result obtained in the IFAT carried out on this cat could reflect an ineffective immune system response or could be explained by the absence of antibody production during an early stage of the infection.[25] Discordant findings between the serological testing and molecular methods have also been reported previously.[20,30–33]

No significant association was found between *L infantum* infection and signalment data, as has been reported in previous studies.[5,25,33,34] An interesting finding of the present study, although not sustained by statistical significance, is the increased prevalence of infection in the cats sampled in July and August, probably owing to the increased *Phlebotomus* species activity in Southern Europe during these months.[35]

Positivity for *L infantum* in cats was associated with a significant increase in total proteins, β_2 and γ globulins, and with a decrease in A:G, although not significant when compared with the negative cases. Interestingly, 15.8% and 57.9% of the PL group had hyperproteinaemia and hypergammaglobulinaemia, respectively, and the frequency of these abnormalities was significantly increased when compared with the NL group. Hyperproteinaemia associated with hyperglobulinaemia has been commonly found in FeL,[4,36–38] and, in a study carried out on four cats, hyperglobulinaemia was classified as hypergammaglobulinaemia.[36] However, the presence of hyperproteinaemia, decreased A:G, and polyclonal β and γ hyperglobulinaemia have long been reported in the course of CanL.[39] Therefore, it can be hypothesised that in cats, as in dogs, the humoral immune response could also be activated by the production of immunoglobulins after infection. When FeL is suspected in a feline patient, performing serum electrophoresis is recommended to support the diagnosis by means of evidence of β_2 and γ polyclonal hyperglobulinaemia.

No other significant difference was found in the haematological and serum chemistry variables between the two groups studied, although mild-to-severe non-regenerative normocytic anaemia, thrombocytopenia, leukopenia, pancytopenia and hypoalbuminemia increased liver enzyme activity, and serum creatinine and urea concentrations have been the most frequent abnormalities previously reported in clinical cases of FeL.[2,4,36–38,40] Proteinuria is often the only abnormal clinicopathological finding in dogs with CanL,[39] whereas it has been described only occasionally in cats with FeL.[4,36,41] In this study, an increase in the UPC in the PL group was not observed as compared with the NL group. These results could be explained by the composition of the NL group. In fact, the NL group was not composed of healthy cats, but also included patients suffering from other diseases not related to *L infantum* infection, including numerous cats suffering from renal disease, which can be accompanied by several laboratory abnormalities.

The clinical cases of feline leishmaniosis reported have frequently been associated with skin lesions, as well as other less specific clinical signs.[30,34] None of the positive cats included in this study showed clinical signs of leishmaniosis, and the disease had never been suspected by the attending clinicians. This suggests that subclinical feline infections could be common in areas that are becoming endemic for CanL and that clinical illness owing to *L infantum* in cats is currently rare.[2]

Some limitations should be acknowledged when interpreting the present results. Sampling should have lasted for more than a year, and the number of cats sampled within the inclusion period should have been more homogeneous in each month. A second staggered sampling would have been necessary for the cats included in the UL group to assess the changes in the antibody response. Moreover, the study population consisted mainly of cats referred to a veterinary hospital and, consequently, the animals included in the study could have presented with clinicopathological abnormalities not related to *L infantum* infection, affecting the comparison between the PL and NL groups.

Conclusions

The results of the present study suggest the spread of *L infantum* infection in cats in Northern Italy, although in the majority of cases, the infection did not appear to lead to active disease. The use of a molecular technique for the detection of *Leishmania* species does not replace serology but may support the final diagnosis. Hyperproteinaemia and hypergammaglobulinaemia appeared to be significant clinicopathological abnormalities in cats with *Leishmania* species infection. Additional studies are needed to better assess the role of cats in the epidemiological cycle of *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 therefore 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. No animals or humans are identifiable within this publication, and therefore additional informed consent for publication was not required.

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References

1 Metzdorf IP, da Costa Lima-Junior MS, de Fatima Cepa Matos M, et al. **Molecular characterization of *Leishmania infantum* in domestic cats in a region of Brazil endemic for human and canine visceral leishmaniasis.** *Acta Trop* 2017; 166: 121–125.
2 Pennisi MG, Cardoso L, Baneth G, et al. **LeishVet update and recommendations on feline leishmaniosis.** *Parasit Vectors* 2015; 8: 302. DOI: 10.1186/s13071-015-0909-z.
3 Pennisi MG. **Leishmaniosis of companion animals in Europe: an update.** *Vet Parasitol* 2015; 208: 35–47.
4 Pennisi MG and Persichetti MF. **Feline leishmaniosis: is the cat a small dog?** *Vet Parasitol* 2018; 251: 131–137.
5 Spada E, Canzi I, Baggiani L, et al. **Prevalence of *Leishmania infantum* and co-infections in stray cats in northern Italy.** *Comp Immunol Microbiol Infect Dis* 2016; 45: 53–58.
6 Otranto D, Napoli E, Latrofa MS, et al. **Feline and canine leishmaniosis and other vector-borne diseases in the Aeolian Islands: pathogen and vector circulation in a confined environment.** *Vet Parasitol* 2017; 236: 144–151.
7 Dedola C, Zobba R, Varcasia A, et al. **Serological and molecular detection of *Leishmania infantum* in cats of northern Sardinia, Italy.** *Vet Parasitol Reg Stud Reports* 2018; 13: 120–123.
8 Francino O, Altet L, Sanchez-Robert E, et al. **Advantages of real-time PCR assay for diagnosis and monitoring of canine leishmaniosis.** *Vet Parasitol* 2006; 137: 214–221.
9 Benassi JC, Benvenga GU, Ferreira HL, et al. **Detection of *Leishmania infantum* DNA in conjunctival swabs of cats by quantitative real-time PCR.** *Exp Parasitol* 2017; 177: 93–97.
10 Braga ARC, Langoni H and Lucheis SB. **Evaluation of canine and feline leishmaniasis by the association of blood culture, immunofluorescent antibody test and polymerase chain reaction.** *J Venom Anim Toxins Incl Trop Dis* 2014; 20: 5. DOI: 10.1186/1378-9199-20-5.
11 Pennisi MG, Lupo T, Malara D, et al. **Serological and molecular prevalence of *Leishmania infantum* infection in cats from southern Italy.** *J Feline Med Surg* 2012; 14: 656–657.
12 Chatzis MK, Andreadou M, Leontides L, et al. **Cytological and molecular detection of *Leishmania infantum* in different tissues of clinically normal and sick cats.** *Vet Parasitol* 2014; 202: 217–225.
13 Persichetti MF, Pennisi MG, Vullo A, et al. **Clinical evaluation of outdoor cats exposed to ectoparasites and associated risk for vector-borne infections in southern Italy.** *Parasit Vectors* 2018; 11: 136. DOI: 10.1186/s13071-018-2725-8.
14 Persichetti MF, Solano-Gallego L, Vullo A, et al. **Diagnostic performance of ELISA, IFAT and Western blot for the detection of anti-*Leishmania infantum* antibodies in cats using a Bayesian analysis without a gold standard.** *Parasit Vectors* 2017; 10: 119. DOI: 10.1186/s13071-0172046-3.
15 World Organisation for Animal Health. Manual of diagnostic tests and vaccines for terrestrial animals. Paris: World Organisation for Animal Health, 2008, pp 240–250.
16 Baldelli R, Piva S, Salvatore D, et al. **Canine leishmaniasis surveillance in a northern Italy kennel.** *Vet Parasitol* 2011; 179: 57–61.
17 Ceccarelli M, Galluzzi L, Migliazzo A, et al. **Detection and characterization of *Leishmania* (*Leishmania*) and *Leishmania* (*Viannia*) by SYBR green-based real-time PCR and high resolution melt analysis targeting kinetoplast minicircle DNA.** *PLoS One* 2014; 9: e88845. DOI: 10.1371/journal.pone.0088845.

18 Troìa R, Balboni A, Zamagni S, et al. **Prospective evaluation of rapid point-of-care tests for the diagnosis of acute leptospirosis in dogs.** *Vet J* 2018; 237: 37–42.
19 Maia C, Gomes J, Cristóvão J, et al. **Feline *Leishmania* infection in a canine leishmaniasis endemic region, Portugal.** *Vet Parasitol* 2010; 174: 336–340.
20 Spada E, Proverbio D, Migliazzo A, et al. **Serological and molecular evaluation of *Leishmania infantum* infection in stray cats in a nonendemic area in northern Italy.** *ISRN Parasitol* 2013; 2013. DOI: 10.5402/2013/916376.
21 Vita S, Santori D, Aguzzi I, et al. **Feline leishmaniasis and ehrlichiosis: serological investigation in Abruzzo region.** *Vet Res Commun* 2005; 29: 319–321.
22 Manna L, Vitale F, Reale S, et al. **Comparison of different tissue sampling for PCR-based diagnosis and follow-up of canine visceral leishmaniosis.** *Vet Parasitol* 2004; 125: 251–262.
23 Manna L, Gravino AE, Picillo E, et al. ***Leishmania* DNA quantification by real-time PCR in naturally infected dogs treated with miltefosine.** *Ann N Y Acad Sci* 2008; 1149: 358–360.
24 Persichetti MF, Solano-Gallego L, Serrano L, et al. **Detection of vector-borne pathogens in cats and their ectoparasites in southern Italy.** *Parasit Vectors* 2016; 9: 247. DOI: 10.1186/s13071-016-1534-1.
25 Ayllon T, Tesouro MA, Amusategui I, et al. **Serologic and molecular evaluation of *Leishmania infantum* in cats from central Spain.** *Ann N Y Acad Sci* 2008; 1149: 361–364.
26 Sherry K, Miró G, Trotta M, et al. **A serological and molecular study of *Leishmania infantum* infection in cats from the island of Ibiza (Spain).** *Vector Borne Zoonotic Dis* 2011; 11: 239–245.
27 Vilhena H, Martinez-Díaz VL, Cardoso L, et al. **Feline vector-borne pathogens in the north and centre of Portugal.** *Parasit Vectors* 2013; 6: 99. DOI: 10.1186/s13071-016-1534-1.
28 Akhtardanesh B, Sharifi I, Mohammadi A, et al. **Feline visceral leishmaniasis in Kerman, southeast of Iran: serological and molecular study.** *J Vector Borne Dis* 2017; 54: 96–102.
29 Belinchón-Lorenzo S, Iniesta V, Parejo JC, et al. **Detection of *Leishmania infantum* kinetoplast minicircle DNA by real time PCR in hair of dogs with leishmaniosis.** *Vet Parasitol* 2013; 192: 43–50.
30 Miró G, Rupérez C, Checa R, et al. **Current status of *L. infantum* infection in stray cats in the Madrid region (Spain): implications for the recent outbreak of human leishmaniosis?** *Parasit Vectors* 2014; 7: 112. DOI: 10.1186/1756-3305-7-112.
31 Silaghi C, Knaus M, Rapti D, et al. **Survey of *Toxoplasma gondii* and *Neospora caninum*, haemotropic mycoplasmas and other arthropod-borne pathogens in cats from Albania.** *Parasit Vectors* 2014; 7: 62. DOI: 10.1186/1756-3305-7-62.
32 Attipa C, Papasouliotis K, Solano-Gallego L, et al. **Prevalence study and risk factor analysis of selected bacterial, protozoal and viral, including vector-borne, pathogens in cats from Cyprus.** *Parasit Vectors* 2017; 10: 130. DOI: 10.1186/s13071-017-2063-2.
33 Montoya A, García M, Gálvez R, et al. **Implications of zoonotic and vector-borne parasites to free-roaming cats in central Spain.** *Vet Parasitol* 2018; 251: 125–130.
34 Sobrinho LSV, Rossi CN, Vides JP, et al. **Coinfection of *Leishmania chagasi* with *Toxoplasma gondii*, feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) in cats from an endemic area of zoonotic visceral leishmaniasis.** *Vet Parasitol* 2012; 187: 302–306.
35 Gaglio G, Brianti E, Napoli E, et al. **Effect of night time-intervals, height of traps and lunar phases on sand fly collection in a highly endemic area for canine leishmaniasis.** *Acta Trop* 2014; 133: 73–77.
36 Pennisi MG, Venza M, Reale S, et al. **Case report of leishmaniasis in four cats.** *Vet Res Commun* 2004; 28: 363–366.
37 Richter M, Schaarschmidt-Kiener D and Krudewig C. **Ocular signs, diagnosis and long-term treatment with allopurinol in a cat with leishmaniasis.** *Schweiz Arch Tierheilkd* 2014; 156: 289–294.
38 Pimenta P, Alves-Pimenta S, Barros J, et al. **Feline leishmaniosis in Portugal: 3 cases (year 2014).** *Vet Parasitol Reg Stud Reports* 2015; 1–2: 65–69.
39 Solano-Gallego L, Koutinas A, Miró G, et al. **Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis.** *Vet Parasitol* 2009; 165: 1–18.
40 Marcos R, Santos M, Malhão F, et al. **Pancytopenia in a cat with visceral leishmaniasis.** *Vet Clin Pathol* 2009; 38: 201–205.
41 Navarro J A, Sánchez J, Peñafiel-Verdú C, et al. **Histopathological lesions in 15 cats with leishmaniosis.** *J Comp Pathol* 2010; 143: 297–302.