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SHORT  
COMMUNICATION



# Serum protein electrophoretogram profile detected in apparently healthy cats infected with *Leishmania infantum* – Short communication

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## ABSTRACT

The information about the clinical features of *Leishmania infantum* infection in cats is scarce. In this study, we evaluated the serum protein electrophoresis of samples from 19 infected but apparently healthy cats. To detect *L. infantum* infection, two serological tests, i.e. western blot (WB) and enzyme-linked immunosorbent assay (ELISA) as well as quantitative polymerase chain reaction (qPCR) on the blood samples were performed. Eventual infection by several selected bacterial and viral pathogens was also tested. All but one of the cats were found positive with WB. The WB-negative cat was positive by ELISA only. From the 18 WB-positive cats, only three were positive also by ELISA and eight with qPCR, including the only animal which was positive in all the three tests. No concomitant infections were detected in any of the cats. The main alteration of the proteinogram was characterised by an increase of the  $\alpha$ -2 fraction. In the five cats with hypergammaglobulinaemia, the pattern detected was polyclonal. None of the cats were seropositive to any other pathogens tested. The presence of polyclonal gammopathy and elevation of the  $\alpha$ -2 fraction could suggest the presence of active infection. In contrast, the only detection of an increase of the  $\alpha$ -2 fraction alone with the presence of positive serological result could be associated by immune response activation against *L. infantum*.

## KEYWORDS

agarose gel electrophoresis, cat, *Leishmania infantum*, PCR, serology

Feline leishmaniosis (FeL) is a disease caused by *Leishmania infantum* and transmitted by the bite of an infected female phlebotomine sand fly under natural conditions (Pennisi et al., 2015). In cats, this disease has often been underestimated since clinical illness is uncommon and the cats can be infected for years without exhibiting clinical signs. The most typical clinical signs associated with the disease include enlargement of peripheral lymph nodes, as well as skin, ocular and oral lesions (Pennisi and Persichetti, 2018).

Different techniques to confirm *L. infantum* infection are available including parasitological methods such as cytology, histology, specific immunohistochemistry and parasite culture; molecular methods to detect parasitic nucleic acids by polymerase chain reaction (PCR) including conventional PCR, nested PCR and quantitative PCR; and finally serological methods based on the detection of a specific IgG response against *L. infantum*, including enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence antibody test (IFAT) and western blot (WB) (Pennisi et al., 2013). Although the same confirmatory

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techniques to detect *L. infantum* infections are available in dogs and cats, the validation of each serological technique is necessary to confirm that the technique is able to detect the presence of anti-*Leishmania* antibodies in the serum samples (Giner et al., 2020a).

Serum protein electrophoresis provides a measure of serum proteins in fractions (albumin fraction,  $\alpha$ -1 globulin fraction,  $\alpha$ -2 globulin fraction,  $\beta$ -1 globulin fraction,  $\beta$ -2 globulin fraction and  $\gamma$  globulin fraction). In this sense, limited data are available on serum protein electrophoresis in apparently healthy cats demonstrated to be infected by FeL by results obtained by different confirmatory techniques to detect *L. infantum* infection.

Feline serum samples were submitted to the Clinical Immunology Laboratory, Veterinary Faculty, University of Zaragoza, Spain for testing for the most prevalent feline vector-borne pathogens (*Dirofilaria immitis* and *L. infantum*), associated with an annual screening examination. The sample submission document included information about the initial examination and the cat's health status. All cats were of the short-haired type, including 6 females and 13 males (all cats were intact), and all were adults of more than one year of age (the mean age was 8.9 years, with a range of 3–16 years). On the initial physical examination, all cats were in good condition, active and alert, normothermic and properly hydrated. In all of these cats, cardiac auscultation was within normal limits and respiratory sounds were also normal. None of the cats had evidence of inadequate body condition score, signs of dehydration or showed any signs of disease such as lymph node enlargement, ocular or skin lesions. Routine laboratory tests such as a complete blood count and biochemistry profile were recorded by a retrospective review of sample files through the laboratory and hospital database.

Anti-*Leishmania* antibodies were detected by WB and ELISA using a whole antigen of *L. infantum* promastigotes (MHOM/FR/78/LEM75 zymodeme MON-1) (Alcover et al., 2021). Briefly, each plate was coated with 20  $\mu\text{g mL}^{-1}$  of crude antigen obtained from *L. infantum* promastigote forms (MHOM/MON-1/LEM 75) in 0.1 M carbonate/bicarbonate buffer (pH 9.6), and incubated overnight at 4 °C. A 100- $\mu\text{L}$  aliquot of cat sera, diluted 1:200 in PBS containing 0.05% Tween 20 (PBST) and 1% dry skimmed milk (PBST-M), was added to each well. The plates were then incubated for 1 h at 37 °C in a moist chamber, after which they were washed and 100  $\mu\text{L}$  of Protein A conjugated to horseradish peroxidase (Thermo Fisher Scientific) diluted 1:20,000 in PBST-M was added. The plates were incubated for 1 h at 37 °C in a moist chamber and were washed again with PBST and PBS as described above. The substrate solution (ortho-phenylenediamine) and stable peroxide substrate buffer (Thermo Fisher Scientific) were added to each well and the reaction was allowed to develop for 20  $\pm$  5 min at room temperature in the dark. The reaction was stopped by adding 2.5 M  $\text{H}_2\text{SO}_4$  to each well. Absorbance values were read at 492 nm in an automatic microELISA reader (ELISA Reader Labsystems Multiskan, Midland, ON, Canada). As a positive control (calibrator), each plate included serum from a cat from Spain, diagnosed with FeL,

and confirmed by isolation of *L. infantum* in NNN culture medium. A negative control serum from a healthy, non-infected cat was also included. The same calibrator serum was used for all assays and plates, with a constant inter-assay variation of <10%. Plates with an inter-assay variation of >10% were discarded. All samples and controls were run in duplicate. The results were quantified as ELISA units (EU) compared to the positive control serum used as a calibrator and arbitrarily set at 100 EU. The cut-off was established at 13 EU (mean +4 standard deviations [SD] of values from 50 indoor cats from northern Spain) and the results above this value were considered to be positive.

Antigen electrophoresis in 1% sodium dodecyl sulfate/15% polyacrylamide gels together with molecular mass protein standards (Standard Low Range; Bio-Rad, Hercules, CA, USA) was performed on a Mini-Gel AE 6400 Dual Mini Slab Kit (ATTO Corp., Tokyo, Japan). The gels were run at 100 V for 1 h at room temperature.

In the case of the WB technique, polypeptides were blotted onto nitrocellulose sheets (0.45-mm pore size, HAWP 304 FO; Millipore Corp., Bedford, MA, USA), which were blocked with 20 mM Tris, 0.13 mM NaCl, pH 7.6 (TS) and 5% skimmed milk, overnight at 4 °C. The sheets were washed in TS and introduced into a multiscreen apparatus (Mini Protean II, Multiscreen Apparatus; Bio-Rad). Sera were diluted 1:200 in TS/1% skimmed milk and 0.2% Tween 20. Then 500  $\mu\text{L}$  of each sample was introduced into each channel of the multiscreen apparatus and incubated for 2 h at 37 °C. Bound immunoglobulins were developed by incubation with a 1:1,000 dilution of Protein A peroxidase conjugate (Thermo Fisher Scientific) for 1 h. After the sheets were washed three times with TST and a final time with TS, colour was developed with 4-chloro-1-naphthol (Thermo Fisher Scientific) and  $\text{H}_2\text{O}_2$ , and the reaction was stopped with tap water after 30 min. According our experience, the sera were considered to be positive when immunoreactivity against the 14 to 16 kDa *L. infantum* antigen fraction was observed. In the case of serum with immunoreactivity against some bands considered non-diagnostic of low molecular weight between 20 and 46 kDa, these bands were classified as indeterminate.

A quantitative real-time PCR (qPCR) was used. DNA was extracted from 200  $\mu\text{L}$  of blood by the isolation of nucleic acids according to the protocol of the Quick-DNA Miniprep Plus Kit (ZymoResearch, California, USA), and eluted in 50  $\mu\text{L}$  of elution buffer, following the manufacturer's instructions. *Leishmania* spp. DNA was detected and quantified by amplification of a kinetoplast minicircle DNA sequence by qPCR (Alcover et al., 2021). The qPCR was considered positive for *Leishmania* when the cycle threshold (Ct) was lower than 40 and when the amplification was detected in all the replicates (Mary et al., 2004).

The diagnosis of feline leishmaniosis is based on serological, cytological, histological and molecular methods (Pennisi et al., 2015). In our study, a cat was regarded as infected based on a positive result obtained by a confirmatory *L. infantum* technique including qPCR, WB, ELISA alone or by a combination of these diagnostic tests.



Samples of blood with EDTA were subjected to a haematological study, using an automatic haematological counter Vet-ABC (DIVASA-FARMAVIC S.A., Barcelona, Spain). Measured parameters were haematocrit, haemoglobin, RBC (red blood cell count), MCV (mean corpuscular volume), MCH (mean corpuscular haemoglobin, 13.5–20.8 pg), MCHC (mean corpuscular haemoglobin concentration), platelets, WBC (white blood cell count), lymphocytes, monocytes and granulocytes. Clinical biochemistry values composed of a panel with 6 parameters were analysed with the automatic analyser Catalyst Chemistry Analyzer (IDEXX, USA) including total protein concentration (TP), creatinine (CREA), blood urea nitrogen (BUN), alanine aminotransferase (ALT), alkaline phosphatase (ALKP) and gamma glutamyl transferase. Serum protein electrophoresis was also performed by agarose gel electrophoresis (AGE) system (with HYDRAGEL Kit 1–2, Sebia, Issy-les-Moulineaux, France). Serum was electrophoresed for 21 min at 92 V h and stained with diluted Amidoschwarz dye at pH 2 (4 g L<sup>-1</sup> Amidoschwarz dye and 6.7% ethylene glycol). The AGE procedure was conducted according to the manufacturer's instructions, and commercial human serum was used as the control (normal control serum, Sebia, Evry, France). The electrophoretic curve for each sample was displayed and read with a Shimadzu CS-9000 photodensitometer (Kyoto, Japan). Protein fractions were determined as the percentage optical absorbance, and the absolute concentration in g dL<sup>-1</sup> was automatically calculated from the total serum protein concentration.

Cats included in this study were tested for co-infections with other infectious disease pathogens. This was performed either by testing the presence of specific antibodies (*D. immitis*, *Toxoplasma gondii*, *Bartonella henselae*, feline immunodeficiency virus), or the presence of antigens from the pathogen (feline leukaemia virus) or by the detection of the genomic DNA of the pathogen (*Mycoplasma haemofelis*, *Candidatus Mycoplasma haemominutum*, *Bartonella* spp., *Ehrlichia* spp., *Anaplasma* spp.) by molecular tests performed in a private laboratory. The specific tests and methods are available upon request.

The apparently healthy cats were enrolled in this study based on the absence of any clinical signs and laboratory abnormalities (the haematological results can be seen in [Supplementary Table 1](#)) and the presence of a positive result obtained by a confirmatory *L. infantum* technique. Among the 19 cats, one was positive by all the three techniques, seven were positive by WB and qPCR, two were positive by serological test and nine out of these were positive by serology alone ([Table 1](#)). An increase in protein concentration was detected in four cats, whilst all cats showed increased  $\alpha$ -2 globulin fractions ([Table 2](#)). The presence of polyclonal gammopathy was observed on serum protein electrophoresis in six cats ([Table 3](#)). Co-infection with any other pathogens besides *L. infantum*, analysed by specific serological and molecular tests, was not detected.

This study shows that the most common electrophoretic pattern detected was polyclonal gammopathy, however  $\alpha$ -2 fraction elevation was observed in all infected cats. In dogs,

Table 1. The individual results of the different tests for the detection of *Leishmania infantum* infection in each cat

Cat	ELISA	WB (band)	qPCR
1	Positive	Negative	Negative
2	Negative	Positive (16 kDa)	Negative
3	Negative	Positive (16–18 KDa)	Negative
4	Negative	Positive (16 KDa)	Negative
5	Negative	Positive (16–18 KDa)	Negative
6	Negative	Positive (14 KDa)	Negative
7	Negative	Positive (14–20 KDa)	Negative
8	Negative	Positive (16–46 KDa)	Negative
9	Negative	Positive (16–46 KDa)	Negative
10	Positive	Positive (20–46 KDa)	Negative
11	Positive	Positive (14–16–36–46 KDa)	Negative
12	Negative	Positive (46 KDa)	Positive
13	Negative	Positive (24–46 KDa)	Positive
14	Negative	Positive (46 KDa)	Positive
15	Negative	Positive (46 KDa)	Positive
16	Negative	Positive (46 KDa)	Positive
17	Negative	Positive (46 KDa)	Positive
18	Negative	Positive (46 KDa)	Positive
19	Positive	Positive (14–16–24–36–46 KDa)	Positive

an increase of the  $\alpha$ -2 fraction is a characteristic of a dog with clinical leishmaniosis. In our study, two cats tested positive by all the diagnostic methods employed. Although the blood of asymptomatic animals is not the most suitable sample to detect *L. infantum* infection, low parasitaemia levels were found in apparently healthy cats. This circumstance, together with the presence of polyclonal gammopathy and elevation of the  $\alpha$ -2 fraction, could suggest the presence of active infection in the absence of clinical lesions. By contrast, the only detection of an increase of the  $\alpha$ -2 fraction alone with the presence of a positive serological result could be associated with immune response activation against *L. infantum* and a better control of the infection.

Serum protein electrophoresis is a common biochemical technique used for the investigation of a normal distribution of serum protein fractions, separating the proteins of the serum into albumin and globulin components ( $\alpha$ -1,  $\alpha$ -2,  $\beta$ -1,  $\beta$ -2 and  $\gamma$  fraction) ([Giordano and Paltrinieri, 2010](#)). In canine leishmaniosis, different serum protein electrophoresis patterns could be detected, polyclonal gammopathy, oligoclonal gammopathy and finally monoclonal gammopathy ([Paltrinieri et al., 2016](#)). In this sense, elevation of total protein and total globulins are the first alterations that appear during the course of the disease, showing a positive correlation between the elevations of globulins and the severity of the disease ([Proverbio et al., 2014](#)). As in infected dogs, alterations in globulin patterns can be the first alteration detected. Due to this, all cats were classified as apparently healthy following a thorough clinical examination and laboratory findings. Moreover, the complete blood count and biochemistry profile could help in the better characterisation of the clinical status.

This technique is a useful tool in the diagnosis and monitoring of a large number of diseases, especially for the response to leishmaniosis treatment in different animals



Table 2. Results deduced from the electrophoretograms (or obtained by the electrophoresis)

Parameter	Mean $\pm$ SD (range)	Minimum value	Maximum value	Reference values*
Total protein (g dL <sup>-1</sup> )	7.20 $\pm$ 1.00	5.50	9.00	5.70–7.90
Albumin (g dL <sup>-1</sup> )	3.20 $\pm$ 0.60	2.40	4.90	2.10–4.00
Alpha 1 globulins (g dL <sup>-1</sup> )	0.10 $\pm$ 0.00	0.10	0.10	0.10–1.10
Alpha 2 globulins (g dL <sup>-1</sup> )	1.50 $\pm$ 0.30	1.00	2.20	0.40–0.90
Beta globulins (g dL <sup>-1</sup> )	0.80 $\pm$ 0.20	0.50	1.20	0.90–1.90
Gamma globulins (g dL <sup>-1</sup> )	1.70 $\pm$ 0.80	0.80	3.60	1.30–2.20
A/G ratio	0.90 $\pm$ 0.30	0.40	1.30	0.45–1.30

\*Reference values. A/G: albumin/globulin ratio; SD: standard deviation.

Table 3. Distribution of the *Leishmania infantum*-infected cats according to the characteristic serum protein profile alterations

Positivity based on different diagnostic tests	Number of cats	Serum protein electrophoresis alterations (n)
WB+ ELISA+ qPCR+	1	Polyclonal gammopathy + $\alpha$ -2 peak increased (1)
WB indeterminate+ qPCR+	7	Polyclonal gammopathy + $\alpha$ -2 peak increased (1) $\alpha$ -2 peak increased alone (6)
WB+ ELISA+	1	Polyclonal gammopathy + $\alpha$ -2 peak increased (1)
WB indeterminate+ ELISA+	1	Polyclonal gammopathy + $\alpha$ -2 peak increased (1)
WB+	8	Polyclonal gammopathy + $\alpha$ -2 peak increased (1) $\alpha$ -2 peak increased alone (7)
ELISA+	1	$\alpha$ -2 peak increased alone (1)

such as dog (Solano-Gallego et al., 2011), cat (Brianti et al., 2019) and ferret (Giner et al., 2020b, 2021). Before anti-*Leishmania* treatment, the electrophoretic pattern can be altered, especially the  $\gamma$ -fraction; however, once an anti-*Leishmania* treatment is administered, a normal serum protein electrophoresis is observed during the follow-up in dogs (Solano-Gallego et al., 2011). In the case of cats with clinical leishmaniosis, similar circumstances are observed (Basso et al., 2016). Thus, it is necessary to highlight the importance of serum protein electrophoresis in monitoring the response to treatment in any animal infected with *L. infantum*.

In FeL, one of the most frequent laboratory alterations detected is hyperproteinaemia with hyperglobulinaemia, an alteration reported in dogs, a domestic ferret (*Mustela putorius furo*) (Giner et al., 2020a) and recently in a captive Eurasian otter (*Lutra lutra*) (Cantos-Barreda et al., 2020). However, the description of the serum protein electrophoresis and the pattern detected in cats infected with *L. infantum* is variable. Some authors detected monoclonal gammopathy in a cat with clinical disease (Pennisi et al., 2004), while other authors detected polyclonal gammopathy (Fernández-Gallego et al., 2020; Savioli et al., 2021).

A potential limitation of our study is that the cats were not tested for a panel of concurrent neoplastic and inflammatory diseases which would all induce an elevation of globulin fractions. Thus, because of the absence of positive

results in cats to selected pathogens susceptible to immunosuppression it is likely that the globulin elevation is associated with *L. infantum* infection.

In enzootic areas of this infection, the detection of a typical serum protein electrophoresis pattern, including the  $\alpha$ -2 peak and/or polyclonal gammopathy, in apparently healthy cats, should be complemented by additional tests to confirm the presence of *L. infantum*. A combination of two confirmatory techniques of different nature is recommended for a more accurate diagnosis of *L. infantum* infection.

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## APPENDIX

Supplementary Table 1. Haematochemical profile of the enrolled 19 cats

Parameter	Mean $\pm$ SD (range)	Minimum value	Maximum value	Reference values
Haematocrit (%)	36.29 $\pm$ 7.12	21.50	48.30	20.90–53.30
Haemoglobin (g dL <sup>-1</sup> )	11.04 $\pm$ 1.90	8.40	15.30	8.40–15.40
RBC (M $\mu$ L <sup>-1</sup> )	7.26 $\pm$ 1.34	4.98	10.12	4.90–10.60
MCV (fL)	50.25 $\pm$ 2.79	46	57	42–70
MCH (pg)	15.21 $\pm$ 1.08	13.70	17.20	13.50–20.80
MCHC (g dL <sup>-1</sup> )	30.35 $\pm$ 1.05	29.20	33.70	29.10–35.20
Platelets (K $\mu$ L <sup>-1</sup> )	263 $\pm$ 91.23	120	398	100–400
WBC (K $\mu$ L <sup>-1</sup> )	17.13 $\pm$ 0.80	15.50	17.90	5.40–18.00
Lymphocytes (K $\mu$ L <sup>-1</sup> )	2.31 $\pm$ 0.83	1.60	4.30	1.50–7.00
Monocytes (K $\mu$ L <sup>-1</sup> )	1.47 $\pm$ 0.42	1.00	2.50	1.00–4.00
Granulocytes (K $\mu$ L <sup>-1</sup> )	13.39 $\pm$ 0.90	12.20	15.40	12.80–35.00
CREA (mg dL <sup>-1</sup> )	1.59 $\pm$ 0.46	0.8	2.3	0.80–2.40
BUN (mg dL <sup>-1</sup> )	24.70 $\pm$ 5.29	17.	34	16–36
ALT (U/L)	39.10 $\pm$ 17.15	16	76	12–130
ALKP (U/L)	57.45 $\pm$ 26.22	16	102	14–111
GGT (U/L)	0.70 $\pm$ 0.80	0	2	0–4

SD: standard deviation.

