

Are dogs with clinical leishmaniosis co-infected with other vector-borne zoonotic and non-zoonotic pathogens?



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A handwritten signature in blue ink, consisting of the initials 'MB' followed by a long horizontal stroke.

Memoria presentada por Marta Baxarias Canals

A handwritten signature in blue ink, featuring a large, stylized 'L' and 'S'.

Directora: Laia Solano-Gallego

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Abbreviations

| | |
|------------------------------------|---------------------------------------|
| % | Percentage |
| °C | Celsius |
| µg | Microgram |
| µl | Microliter |
| ALT | Alanine transaminase |
| APTT | Activated partial thromboplastin time |
| bp | Base-pair |
| CA | California |
| CBC | Complete blood count |
| CI | Confidence interval |
| Ct | Cycle treshold |
| CanL | Canine leishmaniosis |
| cor | Correlation |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| EU | ELISA unit |
| h | Hour |
| H₂O | Water |
| H₂SO₄ | Sulfuric acid |
| IFAT | Immunofluorescence antibody test |
| IgG | Immunoglobulin G |
| M | Molarity |
| MA | Massachusetts |
| MCH | Mean corpuscular haemoglobin |
| MD | Maryland |

| | |
|---------------|---------------------------|
| min | Minute |
| ml | Milliliter |
| MO | Missouri |
| nM | Nanomolar |
| nm | Nanometer |
| NTC | Non-template control |
| OD | Optical density |
| OR | Odds ratio |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| RBC | Red blood cells |
| RT-PCR | Real-time PCR |
| s | Second |
| SD | Standard deviation |
| SFG | Spotted fever group |
| SPF | Specific pathogen free |
| UK | United Kingdom |
| USA | United States of America |
| UV | Ultraviolet |

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1. Abstract

Introduction: Canine leishmaniosis (CanL) due to *Leishmania infantum* is a zoonotic protozoan disease endemic in Spain, where other vector-borne pathogens are quite common (*Rickettsia conorii*, *Ehrlichia canis*, *Anaplasma* spp, *Hepatozoon canis*, *Babesia* spp). Dogs are considered the main peridomestic reservoir for *L. infantum* infection and its clinical manifestation can vary from a total absence of clinical signs and clinicopathological abnormalities to a severe fatal clinical illness. Furthermore, there is evidence that other vector-borne organisms might affect the severity of CanL or mimic its clinical signs and/or clinicopathological abnormalities. The aim of this study was to determine co-infections with other vector-borne pathogens based on serological and molecular techniques in dogs with clinical leishmaniosis living in the Mediterranean basin and to associate them with clinical signs and clinicopathological abnormalities as well as disease severity.

Materials and methods: Sixty-two dogs with clinical leishmaniosis and sixteen apparently healthy dogs were tested for *Rickettsia conorii*, *Ehrlichia canis* and *Anaplasma phagocytophilum* by the immunofluorescence antibody test (IFAT) and for *Ehrlichia canis*, *Anaplasma* spp, *Hepatozoon* spp. and *Babesia* spp. by polymerase chain reaction (PCR).

Results: Among the dogs examined by IFAT, the seroprevalences were: 69.3% for *R. conorii*, 57.3% for *E. canis* and 44.0% for *A. phagocytophilum*; while the prevalences found by PCR were: 7.9% for *E. canis/Anaplasma*, 2.6% for *Anaplasma platys* and 1.3% for *H. canis*. Statistical association was found between dogs with clinical leishmaniosis and seroreactivity to *R. conorii* antigen ($P = 0.025$; OR = 4.09) and *A. phagocytophilum* antigen ($P = 0.002$; OR = 14.34) and being positive to more than one serological or molecular tests (co-infections) ($P = 0.013$) when compared with healthy dogs. Interestingly, a statistical association was found between the presence of *R. conorii*, *E. canis* and *A. phagocytophilum* antibodies in sick dogs and some clinicopathological abnormalities such as a decrease of albumin and albumin/globulin ratio associated to the presence of *R. conorii*, *A. phagocytophilum* and *E. canis* high antibody titers, and an increase in serum globulins associated to the presence of *A. phagocytophilum* and *E. canis* high antibody titers. Furthermore, seroreactivity with *A.*

phagocytophilum antigens was statistically associated with LeishVet clinical stages III and IV.

Conclusion: This study demonstrates that dogs with clinical leishmaniosis from the Barcelona and Tarragona area have a higher rate of co-infections with other vector-borne pathogens when compared with healthy controls. Furthermore, positivity to other vector-borne pathogens was associated with more pronounced clinicopathological abnormalities as well as disease severity with canine clinical leishmaniosis.

Keywords: Canine leishmaniosis; Spain; *Leishmania infantum*; *Rickettsia conorii*; *Ehrlichia canis*; *Anaplasma phagocytophilum*; *Anaplasma platys*; *Hepatozoon canis*; Co-infection.

2. Introduction

Canine leishmaniosis (CanL) is a zoonotic and infectious disease caused by the protozoan *Leishmania infantum*. *Phlebotomus* spp. sand flies are the only arthropods adapted to the biologic transmission of this parasite in Europe (Petrella et al. 2015). Dogs (*Canis familiaris*) are considered the main peridomestic reservoir of this parasite in endemic areas, and owning a dog infected by this parasite could be a risk factor for members of the household (Gavgani et al. 2002). CanL is endemic in the Mediterranean basin, where its prevalence can be as high as 67 per cent in selected populations (Solano-Gallego et al. 2001), but the prevalence of the clinical disease can be lower than 10 per cent (Solano-Gallego et al. 2009). The most useful diagnosis of CanL includes serology by quantitative techniques and PCR, although the direct observation of amastigote forms of *Leishmania* spp. are also useful in the clinical setting (Solano-Gallego et al. 2009; Zanette et al. 2014; Pennisi 2015).

The clinical manifestation of CanL can vary from a total absence of clinical signs and clinicopathological abnormalities to a severe fatal clinical illness. The most common clinical signs are skin lesions, progressive weight loss, generalized lymphadenomegaly, muscular atrophy, exercise intolerance, decreased appetite, lethargy, splenomegaly, polyuria and polydipsia, ocular lesions, epistaxis, onychogryphosis, lameness, vomiting and diarrhea (Baneth et al. 2008; Solano-Gallego et al. 2009; Pennisi 2015).

In Spain, other vector-borne diseases are quite common. Some studies have documented *Ehrlichia canis* (Roura et al. 2005; Solano-Gallego et al. 2006; Amusatogui et al. 2008; Tabar et al. 2009; Couto et al. 2010; Miró et al. 2013), *Anaplasma platys* (Tabar et al. 2009; Miró et al. 2013) and *Rickettsia conorii* (Solano-Gallego et al. 2006; Amusatogui et al. 2008; Ortuño et al. 2009; Espejo et al. 2016) infections in dogs, which are intracellular Gram-negative bacteria that appear to be transmitted by *Rhipicephalus sanguineus* ticks (Beugnet and Marié 2009; Little 2010; Chomel 2011; Dantas-Torres et al. 2012; Solano-Gallego et al. 2015; Espejo et al. 2016). It has been reported that the prevalence of these vector-borne infections is higher in communal shelter dogs and dogs that live outdoors (Amusatogui et al. 2008; Miró et al. 2013). These clinical characteristics of rickettsial disease in dogs can be similar to those caused by *L. infantum*. *Anaplasma phagocytophilum* (Solano-Gallego et al. 2006; Amusatogui et al. 2008; Couto et al. 2010; Miró et al. 2013) is another pathogen transmitted by *Ixodes*

ricinus ticks that can also infect dogs and humans causing acute febrile illness or transient subclinical infections (Doudier et al. 2010; Miró et al. 2013). Other protozoan pathogens such as *Babesia vogeli* (Tabar et al. 2009) and *Hepatozoon canis* (Tabar et al. 2009) infect dogs in the Mediterranean basin and they are also transmitted by *R. sanguineus* ticks (Beugnet and Marié 2009; Chomel 2011; Dantas-Torres et al. 2012).

It has been detected that infections with other vector-borne organisms can affect the severity of the disease or mimic its clinical signs and/or clinicopathological abnormalities (Tuttle et al. 2003; De Tommasi et al. 2013; Baneth et al. 2015). Some studies (Cringoli et al. 2002; Roura et al. 2005; Tabar et al. 2009; Cardinot et al. 2016) have described co-infection of *L. infantum* with other vector-borne disease in dogs that showed the typical signs of leishmaniosis. Other authors (Mekuzas et al. 2009; Couto et al. 2010; De Tommasi et al. 2013; Mylonakis et al. 2014) have demonstrated co-infections of *L. infantum* with *E. canis*, *A. phagocytophilum* and *Bartonella* spp. in the Mediterranean area. Mekuzas et al. (2009) found that clinical signs were more frequent in dogs with dual infection than dogs with single infection. Roura et al. (2005) stated that simultaneous infection with 2 or more pathogens should be expected in dogs living in areas which are highly endemic for several vector-borne pathogens, in dogs maintained predominantly outdoors and dogs that have not been treated with ectoparasitides.

We hypothesized that dogs with leishmaniosis living in the Mediterranean basin might be co-infected with other pathogens that could mimic the clinical signs and/or clinicopathological abnormalities or might influence the severity of the disease.

The aim of this study was to determine co-infections with other vector-borne pathogens in dogs with clinical leishmaniosis living in the Mediterranean basin and to associate with clinical signs and clinicopathological abnormalities as well as with disease severity. These dogs were compared with healthy control dogs living in the same geographical area.

3. Materials and methods

Dogs

The dogs included in this study were from Catalonia (Spain), an area endemic for canine leishmaniosis and other vector-borne diseases. Sixty-two sick dogs were diagnosed with clinical leishmaniosis based on physical examination, a complete blood count (CBC) (System Siemens Advia 120), a biochemical profile including creatinine, urea, total proteins, alanine transaminase (ALT) and total cholesterol (Analyzer Olympus AU 400), urinalysis with urinary protein creatinine ratio, and serum electrophoresis, a medium or high antibody levels in a quantitative ELISA for the detection of *L. infantum*-specific antibodies and/or cytology or histology. The dogs were examined at different veterinary centers: 34 were from *Fundació Hospital Clínic Veterinari* (Bellaterra, Barcelona), 15 were from *Hospital Ars Veterinaria* (Barcelona, Barcelona), 7 were from *Hospital Mediterrani Veterinari* (Reus, Tarragona) and 6 were from *Consultori Montsant* (Falset, Tarragona). Furthermore, the dogs were classified according to the LeishVet clinical staging system (Solano-Gallego et al. 2009). Blood real-time PCR (RT-PCR) was also performed in all of these dogs.

Sixteen apparently healthy dogs, based on clinical history and physical examination, were also studied. All healthy dogs were also seronegative and blood RT-PCR negative for *Leishmania*.

Samples

Blood samples of all dogs were collected as described previously (Solano-Gallego et al. 2016). Six millilitres of blood were collected from the respective dogs by jugular or metatarsian venipuncture for the routine laboratory tests described above. Blood was transferred immediately into different tubes: ethylenediaminetetraacetic acid (EDTA) tubes for hematology and molecular testing and plain serum tubes. Once collected, samples were left at 4°C overnight and then frozen at minus 80°C until further use.

All dogs enrolled in the study were privately owned pets for whom client informed consent was obtained. Residual samples from blood EDTA tube and serum were used in this study; therefore, ethical approval was not required. All serum and whole blood extractions were performed between 2014 and 2016 and stored at minus 80°C until use for this study. The samples were taken at the time of diagnosis.

Serological test

*Quantitative ELISA for the detection of *L. infantum*-specific antibodies¹*

The in-house ELISA was performed on sera of all dogs studied as previously described (Solano-Gallego et al. 2014) with some modifications. The samples were diluted to 1:800 in Phosphate buffered saline (PBS)-Tween containing 1% dry milk and incubated in *L. infantum* antigen-coated plates (20 µg/ml) for 1 h at 37°C. Then, the plates were washed three times with PBS-Tween and once with PBS alone and incubated with Protein A conjugated to horseradish peroxidase (Thermo Scientific, dilution 1:30000) for 1 h at 37°C. After that, the plates were washed again as described above. The plates were developed by adding the substrate solution *o*-phenylenediamine and substrate buffer (SIGMAFAST OPD, Sigma Aldrich). The reaction was stopped with 50 µl of 2.5M H₂SO₄. Absorbance values were read at 492 nm by an automatic reader (ELISA Reader Anthos 2020). All plates included the serum from a sick dog with confirmed infection as positive control and serum from a healthy dog as a negative control and all samples were analysed in duplicate. The result was quantified as ELISA units (EU) related to a positive canine serum used as a calibrator and arbitrarily set at 100 EU.

Two-fold serial dilution ELISA²

All samples with an optical density (OD) equal or higher to three were studied using a two-fold serial dilution ELISA. Sera two-fold dilutions were started at 1:800 and continued for 9 to 11 further dilutions. All samples were analysed on the same day and in the same ELISA plate to avoid variability. The result was quantified as ELISA units (EU) related to a calibrator arbitrary set at 100 EU, with an OD value of one at the 1:800 dilution. The mean values of the dilutions at which the optical density (OD) were close to one were chosen for the calculation of the positivity % using the following formula: (Sample OD/Calibrator OD) x 100 x dilution factor. Sera were classified as: very high positive, when having a positivity percentage equal or higher than 40000 EU; high positive, when having a positive percentage equal or higher than 9000 EU and less than 40000 EU; medium positive, when having a positivity percentage equal or higher than 500 EU and less than 9000 EU; low positive, when having a positivity percentage lower than 500 EU and equal or higher than 100 EU; very low positive, when having a

¹ Performed in a previous study (Solano-Gallego et al. 2016).

² Performed in a previous study (Solano-Gallego et al. 2016).

positivity percentage lower than 100 EU and equal or higher than 35 EU. Sera with percentage lower than 35 EU were classified as negative. The cut-off was established at 35 EU (mean + 4 SD of values from 80 dogs from non-endemic area) as previously described (Solano-Gallego et al. 2014).

IFAT for Rickettsia conorii, Ehrlichia canis and Anaplasma phagocytophilum antigens³

An indirect immunofluorescence assay for the detection of specific IgG antibody against *R. conorii* antigen (MegaFLUO® RICKETTSIA conorii, Diagnostik Megacor, Hörbranz, Austria) was performed on serum of all dogs studied. The samples were diluted to 1:64 with PBS and 20 µl of every serum dilution were applied per well. The slides were incubated for 30 min at 37°C. After that, a washing procedure was performed. The slides were washed twice with PBS for 5 min and once with distilled water. After the washing procedure described, we dropped 15 µl of FLUO FITC anti-dog IgG conjugate onto each used well. The slides were incubated for another 30 min at 37°C in the dark to protect the photosensitive conjugate. The washing procedure described above was repeated. After the second washing procedure, we added some drops of mounting medium on the cover slips and removed the possible bubbles. We evaluated the slides using a fluorescence microscope (Leica DM6000 B) at 400x magnification and compared each well to the fluorescence pattern seen in the positive and negative controls. All samples were examined by three persons (Álvarez, Alejandra; Baxarias, Marta; Colvin, Maria Elisa) to avoid errors of observation. Each person examined the samples and wrote if they observed a positive or negative result. To reduce bias, the observers did not know which samples were examined. After the three people examined all the samples, the results were shared with the others. If the results obtained were different, an average of three observations was made, so if two people observed a positive result and the third person a negative one, it was considered positive. All samples negative at 1:64 were considered negative and no further dilutions were done.

Indirect immunofluorescence assays for the detection of specific IgG antibody against *E. canis* (MegaFLUO® EHRLICHIA canis, Diagnostik Megacor, Hörbranz, Austria) and *A. phagocytophilum* (MegaFLUO® Anaplasma phagocytophilum, Diagnostik

³ Performed in this study by Alejandra Álvarez, Marta Baxarias and Maria Elisa Colvin.

Megacor, Hörbranz, Austria) were also performed. The same protocol explained in the previous paragraph was used in the *E. canis* IFAT and the *A. phagocytophilum* IFAT. The slides were also observed by three people to avoid errors of observation and all samples negative at 1:64 were considered negative and no further dilutions were done.

Two-fold serial dilution of IFAT (Antibody titration)⁴

All samples with a positive result were further investigated using a two-fold serial dilution IFAT. The samples were diluted to 1:128 and 1:256. The same IFAT protocol explained above was performed. All samples were analysed by three people on the same day and in the same slide to avoid variability using the same protocol explained before.

If a high positive result was still observed, the samples were diluted to 1:512 for *R. conorii*, and to 1:512 and 1:1024 for *E. canis* and *A. phagocytophilum* using the same protocol. At this point, if the samples had not reached a dilution with a negative result, the samples were classified as a high positive for *R. conorii* (>1:512) or as a high positive for *E. canis* or *A. phagocytophilum* antigens (>1:1024).

Blood DNA extraction and polymerase chain reaction (PCR)

Blood DNA extraction and Leishmania real-time PCR⁵

Both, blood DNA extraction and *Leishmania* real time PCR were performed as previously described (Montserrat-Sangrà et al. 2016; Solano-Gallego et al. 2016) in the samples from sick dogs with leishmaniosis and in samples from apparently healthy dogs. Total DNA was extracted from EDTA whole blood using the DNA Gene extraction kit (Sigma Aldrich) following the manufacturer's instructions with slight modifications. Forty µl of proteinase K solution were added to all samples. Four hundred µl of whole blood were used for all the samples. The other steps were performed as described in the protocol. Blood from a clinically healthy non-infected dog was used as a control for DNA contamination in every DNA extraction performed.

Real-time PCR (RT-PCR) was performed with an absolute quantification as previously described (Montserrat-Sangrà et al. 2016; Solano-Gallego et al. 2016). Briefly, PCR mix reaction was prepared with 4 µl of DNA, 10 µl of master mix (TaqMan® Fast

⁴ Performed in this study by Alejandra Álvarez, Marta Baxarias and Maria Elisa Colvin.

⁵ Performed in a previous study (Solano-Gallego et al. 2016).

Advanced Master Mix, Life Technologies), 1 µl of *Leishmania* primers and probes [Eukaryotic 18S rRNA Endogenous Control (VIC™/MGB Probe, Primer Limited)] and 5 µl of H₂O. PCR reaction was performed in duplicates for each sample and for each target gene.

In order to verify that the PCR was done successfully, a positive control for *Leishmania*, a negative control from non-infected clinically healthy dog and a blank (well without DNA sample) were included in all the plates. PCR was carried out in a QuantStudio Flex™ 7 Real-Time PCR system (Life Technologies). Thermal cycling profile consisted of 50°C for 2 min in order to activate the enzyme called amperase and 20 s at 95°C followed by 40 cycles of 1 s at 95°C and 20 s at 60°C (Montserrat-Sangrà et al. 2016; Solano-Gallego et al. 2016).

Absolute quantification was carried out by the interpolation of the unknown samples to the standard curve generated from a negative sample spiked with different quantities of *Leishmania* promastigotes. Depending on the value of parasitic load, the samples were classified as negative (0 parasites/ml), low positive (< 10 parasites/ml), medium positive (10-100 parasites/ml), high positive (100-1000 parasites/ml) or very high positive (< 1000 parasites/ml) (Martínez et al. 2011).

***PCR for the detection of Ehrlichia canis and Anaplasma spp.*⁶**

All samples were sent to the Koret School of Veterinary Medicine at the Hebrew University in Israel to be tested by PCR under the supervision of professor Gad Baneth. All samples were screened in duplicates for the presence of *E. canis* DNA using the real-time PCR assay as described below. A 123 base-pair (bp) segment of the 16S ribosomal RNA gene of *E. canis* was amplified using primers E.c 16S-fwd (5'-TCGCTATTAGATGAGCCTACGT-3') and E.c 16S-rev (5'-GAGTCTGGACCGTATCTCAG-3') as previously described (Peleg et al. 2010). The real-time PCR reaction was performed in a total volume of 20 µl containing 10 µl Maxima Hot Start PCR Master Mix (2X) (Thermo Scientific, Epsom, Surrey, UK), 0.6 µl of 50 µM SYTO 9 solution (Invitrogen, Carlsbad, CA, USA), 1 µl of 8 µM solution of each primer, 5 µl of DNA and 2.4 µl sterile DNase/RNase-free water (Sigma, St. Louis, MO, USA), using the StepOnePlus real-time PCR thermal cycler (Applied

⁶ This part of the study was performed at the Koret School of Veterinary Medicine of Hebrew University.

Biosystems, Foster City, CA, USA). Initial denaturation for 5 min at 95°C was followed by 45 cycles of denaturation at 95°C for 15 s, annealing and extension at 60°C for 30 s, and final extension at 72°C for 20 s. Amplicons were subsequently subjected to a melt step with the temperature being raised to 95°C for 10 s and then lowered to 60°C for 1 min. The temperature was then raised to 95°C at a rate of 0.3°C/s. Amplification and melt profiles were analysed using the StepOnePlus software v2.2.2 (Applied Biosystems, Foster City, CA, USA). Samples were considered positive for *E. canis* DNA when the cycle threshold (Ct) values were in the range of 25-42 and the melting curves were identical to that of the positive control.

Positive samples from this reaction were further analysed by conventional PCR using primers EHR16SD and EHR16SR which amplify a 345-bp fragment of the *16S rRNA* gene of the genera *Anaplasma* and *Ehrlichia* (Parola et al. 2000). The PCR was performed in a total volume of 25 µl using PCR-ready High Specificity mix (Syntezza Bioscience, Jerusalem, Israel) with 500 nM of each primers and sterile DNase/RNase-free water (Sigma, St. Louis, MO, USA). Amplification was performed using a programmable conventional thermocycler (Biometra, Göttingen, Germany). Initial denaturation was at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing and extension at 55°C for 30 s, and final extension at 72°C for 30 s. After the last cycle, the extension step was continued for a further 5 min. PCR products were electrophoresed on 1.5% agarose gels stained with ethidium bromide and checked under UV light for the size of amplified fragments by comparison to a 100 bp DNA molecular weight marker.

Negative and non-template controls (NTC) as well as the positive control were included in the reaction in duplicates. DNA from blood of a specific pathogen free (SPF) dog was used as a negative control. Non-template control reactions were done using the same procedure and reagents described above but without DNA added to the PCR reaction to rule out PCR contamination and nonspecific reactions.

PCR for the detection of Hepatozoon spp. and Babesia spp.⁷

All samples were sent to the Koret School of Veterinary Medicine at the Hebrew University to be tested by PCR under the supervision of professor Gad Baneth. Molecular detection of *Babesia* spp. and *Hepatozoon* spp. was performed by screening all DNA samples by a conventional PCR assay targeting a 400 bp fragment of the 18S rRNA gene: Piroplasmid-F (3'-CCAGCAGCCGCGGTAATTC-5') and Piroplasmid-R (3'-CTTTCGCAGTAGTTYGTCTTTAACAAATCT-5') (Tabar et al. 2008). In order to identify cases of co-infection, positive samples were tested by additional PCRs using primers specifically designed for the detection of a fragment of the 18S rRNA gene of *Babesia* spp. (PIROA/PIROB) (Olmeda et al. 1997). DNA extracted from a dog infected with *H. canis* and from another dog infected with *B. vogeli* confirmed by PCR and sequencing were used as positive controls.

Conventional PCR was performed in a total volume of 25 µl using the PCR-ready High Specificity mix (Syntezza Bioscience, Jerusalem, Israel) with 500 nM of each primers and sterile DNase/RNase-free water (Sigma, St. Louis, MO, USA). Amplification was performed using a programmable conventional thermocycler (Biometra, Göttingen, Germany). Initial denaturation at 95°C for 5 min was followed by 35 cycles of denaturation at 95°C for 30 s, annealing and extension at 64°C for 30 s (for Piroplasmid-F/Piroplasmid-R), 58°C for 30 s (for PIROA/PIROB), and final extension at 72°C for 30 s. After the last cycle, the extension step was continued for a further 5 min. PCR products were electrophoresed on 1.5% agarose gels stained with ethidium bromide and evaluated under UV light for the size of amplified fragments by comparison to a 100 bp DNA molecular weight marker. Negative uninfected dog DNA, and non-template DNA controls were used in each run for all pathogens.

Sequencing PCR products⁸

Samples that were positive in PCR were purified using a PCR purification kit (ExoSAP; New England BioLabs, Inc., Ipswich, MA) and subsequently sequenced with sense and antisense primers using BigDye Terminator cycle sequencing chemistry by Applied Biosystems ABI 3700 DNA analyser (Sanger) and evaluated by the ABI's data collection and sequence analysis software V5.4 (ABI, Carlsbad, CA) at The Center for

⁷ This part of the study was performed at the Koret School of Veterinary Medicine of Hebrew University.

⁸ This part of the study was performed at the Koret School of Veterinary Medicine of Hebrew University.

Genomic Technologies (The Hebrew University, Jerusalem). Further analyses were done by the Chromas (V2.6) (www.techneleysium.com.au; techneleysium pty, Woollahra, Australia) and MEGA6 (Tamura et al. 2013) softwares. Clean Sequences were compared to other sequences deposited in GenBank® using NCBI Blastn software (National Center for Biotechnology Information, Bethesda, MD, USA). Only sequences with identity between 97%-100% and coverage above 99% were considered as positive for an organism.

Statistical analysis

A descriptive study of the detection of antibodies, the number of co-infections detected in each dog (depending on the results of the IFATs and PCRs performed) and the level of antibodies detected for the different pathogens was performed, and the medians were compared using a Mann-Whitney U test, a chi square test or a Fisher's exact test depending on the distribution, the type of variable (quantitative or qualitative) and the number of samples. Shapiro-Wilk test was performed to detect the normality of the distribution of the samples. Spearman's correlation was used to associate the number of co-infections and the clinical data of dogs that consisted on CBC, biochemical profile including creatinine, urea, total proteins, ALT and total cholesterol, urianalysis with urinary protein creatinine ratio, and serum electrophoresis, the antibody levels in a quantitative ELISA for the detection of *L. infantum*-specific antibodies and the result for *Leishmania* real time PCR. Logistic regression and Kruskal-Wallis test were also used to associate the detection of antibodies, the number of co-infections and the level of antibodies detected for the different pathogens with sex, age and season at time of diagnosis and the clinical data of dogs. A *P*-value < 0.05 was considered statistically significant. The statistical analysis was performed using the R program i386 version 3.3.1 (<https://www.r-project.org/>) and the DeduceR program version 1.7-16 (<http://www.deducer.org>) for Windows software.

4. Results

Signalment and clinical data

Both sexes were represented in the sick group with 37 males (59.7%) and 25 females (40.3%). Forty-two out of 62 were intact, 30 males and 12 females. The median of age

at diagnosis was 5 years with a range from 5 months to 13 years. Forty-one were purebred (66%) and 21 were classified as mixed breed (34%); the most frequent purebreds were German shepherd (n=5; 8.1%), Boxer (n=4; 6.5%), Labrador retriever (n=3; 4.8%), French bulldog (n=3; 4.8%), Golden retriever (n=2; 3.2%), Dachshund (n=2; 3.2%), Doberman (n=2; 3.2%), American Staffordshire terrier (n=2; 3.2%) and Breton (n=2; 3.2%). Other breeds were represented only once.

Fifty-nine of the 62 dogs were classified in different stages of leishmaniosis. Five (8.5%) were classified in stage I with mild disease, 41 (69.5%) were classified in stage II with moderate disease (29 classified in substage IIa and 12 classified in substage IIb), 9 (15.2%) were classified in stage III with severe disease and 4 (6.8%) were in stage IV with very severe disease.

Both sexes were also represented in the healthy group with 5 males (31.2%) and 6 females (37.5%). Gender was not recorded in 5 dogs (31.2%). The median of age at diagnosis was 7 years with a range from 15 months to 13 years. Seven were purebred (43.4%) and 4 were classified as mixed breed. Breed was not recorded in 5 dogs. All recorded breeds were represented only once.

No statistical differences were found between sick and apparently healthy dogs.

IFAT

The results of IFAT for *R. conorii*, *E. canis* and *A. phagocytophilum* antigens in sick and healthy dogs studied as well as molecular results are shown in the **Table 1**. Of the total 75 assessed by IFAT, 22 (29.3%) seroreacted with the three pathogens, 18 (24%) seroreacted with two of the pathogens screened and 26 (34.5%) seroreacted with at least one pathogen. Serum from 9 (12%) of the tested dogs did not react to any IFAT test performed. The most frequent seropositive serology were for *R. conorii* (52/75; 69.3%), followed by *E. canis* (43/75; 57.3%) and *A. phagocytophilum* (33/75; 44%) antigens.

Fifty-six of the 61 (91.8%) dogs with clinical leishmaniosis had a positive result to at least one of the IFAT tests performed while 10 of the 14 (71.4%) dogs in the healthy group had also a positive result. No statistical difference was found when comparing the two groups of dogs ($P = 0.057$). As shown in **Table 1**, the most frequent seropositivity in dogs with clinical leishmaniosis were for *R. conorii* while *E. canis* antibodies were the most frequent in the healthy group.

Dogs with clinical leishmaniosis were more likely to have a positive result to more than one test (IFAT and PCR) ($P = 0.013$) (**Fig. 1**), to be seroreactive to *R. conorii* ($P = 0.025$; OR = 4.09) and to *A. phagocytophilum* ($P = 0.002$; OR = 14.34) antigens (**Table 1**) when compared with healthy dogs. No difference was found between seroreactivity to *E. canis* or being positive in the PCRs performed.

Table 1

Results of IFAT for *R. conorii*, *E. canis* and *A. phagocytophilum* antigens and PCR for *E. canis*, *Anaplasma* spp., *Hepatozoon* spp. and *Babesia* spp. in dogs with clinical leishmaniosis and healthy dogs. A comparison of the groups was made with Fisher's exact test.

| Number (% , 95% CI) of seroreactive dogs | | | | |
|---|---|-----------------------|----------------------|--------------|
| IFAT test (antibody detection) | Dogs with clinical leishmaniosis (n = 61) | Healthy dogs (n = 14) | Total dogs (n = 75) | P-value |
| <i>R. conorii</i> | 46 (75.4, 64.5-86.2) | 6 (42.9, 17.0-68.8) | 52 (69.3, 58.9-79.7) | 0.025 |
| <i>E. canis</i> | 34 (55.7, 43.2-68.2) | 9 (64.3, 39.2-89.4) | 43 (57.3, 46.1-68.5) | 0.766 |
| <i>A. phagocytophilum</i> | 32 (52.5, 40.0-65.0) | 1 (7.14, 0-20.6) | 33 (44, 32.8-55.2) | 0.002 |
| PCR | Dogs with clinical leishmaniosis (n = 60) | Healthy dogs (n = 16) | Total dogs (n = 76) | P-value |
| <i>E. canis</i> and <i>Anaplasma</i> spp. | 8 (13.3, 4.7-21.9) | 0 (0, -) | 8 (10.5, 3.6-17.4)* | 0.191 |
| <i>H. canis</i> and <i>Babesia</i> spp. | 1 (1.6, 0-4.8) | 0 (0, -) | 1 (1.3, 0-3.8)** | 0.606 |

*Only two dogs remained with a positive result after performing a conventional PCR and sequencing. *Anaplasma platys* was diagnosed.

**Only one dog with *Hepatozoon canis* infection was detected. No *Babesia* spp. was detected.

CI: confidence interval

As shown in **Table 2**, of the 66 dogs that had a positive reaction to at least one pathogen, 22 seroreacted to the three pathogens assessed, 12 to *E. canis* and *R. conorii*, 12 only to *R. conorii*, nine only to *E. canis*, six to *R. conorii* and *A. phagocytophilum* and finally only five dogs seroreacted only to *A. phagocytophilum*.

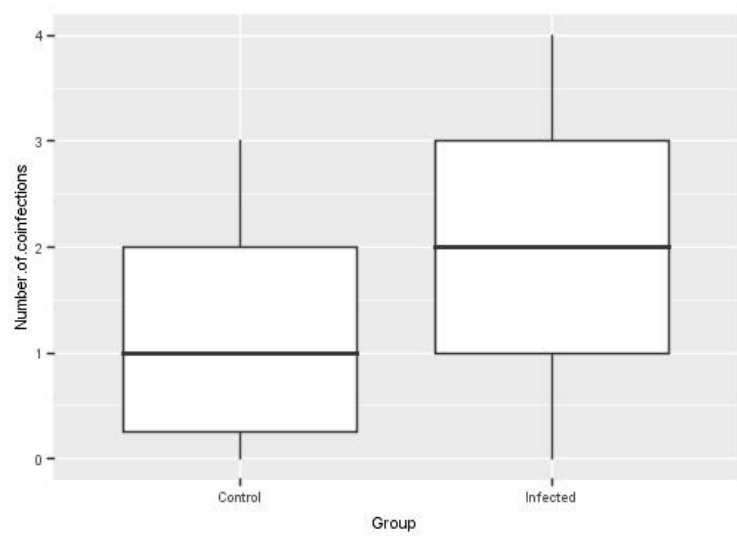


Fig. 1

Number of co-infections detected by the sum of IFAT and PCR results according to dog group: healthy dogs (Control) or dogs with clinical leishmaniosis (Infected). A comparison of the means was performed with Mann-Whitney U test ($P = 0.013$).

No statistical association was found between sex or the blood parasite load of *L. infantum* and any of the pathogens tested by IFAT. The presence of *R. conorii* antibodies was more frequent among the dogs that were older at time of diagnosis ($P = 0.0036$), dogs with a lower albumin/globulin ratio ($P = 0.0217$; OR = 0.17) (**Fig 2**), dogs with a lower count of lymphocytes ($P = 0.0309$) and a high positive antibody level by the *L. infantum* quantitative ELISA ($P = 0.005$). The presence of *E. canis* antibodies was only associated to neutered dogs ($P = 0.033$) while the presence of *A. phagocytophilum* antibodies was more frequent in dogs with an increase of total protein ($P = 0.0312$; OR = 1.31), beta globulins ($P = 0.0385$; OR = 3.61) and gamma globulins ($P = 0.0204$; OR = 1.45), a decrease of albumin ($P = 0.0017$; OR = 0.24), lower albumin/globulin ratio ($P = 0.0003$; OR = 0.04) (**Fig 2**), a high positive antibody levels by the *L. infantum* quantitative ELISA ($P = 0.003$), being classified as stage III or IV of the LeishVet clinical staging for *L. infantum* ($P = 0.042$) (**Fig 3**) and being diagnosed on spring or winter ($P = 0.014$) (**Fig 4**). Also, a significant association was found between seroreactivity to *R. conorii* and seroreactivity to *E. canis* ($P = 0.044$; OR = 2.94) or *A. phagocytophilum* ($P = 0.012$; OR = 4.2), and seroreactivity to *A. phagocytophilum* and *E. canis* high antibody titers ($P = 0.001$; OR = 0).

No significant association was found between sex, clinical stage of leishmaniosis or the blood parasite load of *L. infantum* and the number of co-infections (both with IFAT and PCR). A significant association with age at time of diagnosis ($P = 0.0222$; $cor = 0.2791$), total protein ($P = 0.0373$; $cor = 0.2531$), albumin ($P = 0.0063$; $cor = -0.3327$), albumin/globulin ratio ($P = 0.0042$; $cor = -0.3588$) and mean corpuscular haemoglobin (MCH) ($P = 0.0349$; $cor = -0.3052$) and number of co-infections detected was found by Spearman's correlation.

Of the 66 dogs that had a positive reaction to at least one pathogen, serial dilutions were performed and the results are listed in **Table 3**.

Healthy dogs were more likely to have a negative result or to have low antibody titers when compared with sick dogs (**Table 3**). Healthy dogs were commonly negative for *R. conorii* ($P = 0.025$) and *A. phagocytophilum* ($P = 0.002$) antigens while they presented higher numbers of dogs with 1:64 positive antibody titer for *E. canis* ($P = 0.014$) when compared with sick dogs.

Table 2

Pattern of results of IFAT in dogs with clinical leishmaniosis and healthy dogs for one or more than one antigens (*R. conorii*, *E. canis* and *A. phagocytophilum*). Fisher's exact test was performed.

| Antigens | Number (% , 95% CI) of seroreactive dogs | | | P-value |
|---|---|-----------------------|-----------------------|--------------|
| | Dogs with clinical leishmaniosis (n = 56) | Healthy dogs (n = 10) | Total dogs (n = 66) | |
| <i>R. conorii</i> alone | 11 (19.64, 9.2-30.0) | 1 (10, 0-28.6) | 12 (18.18, 8.9-27.5) | 0.675 |
| <i>E. canis</i> alone | 5 (8.93, 1.4-16.4) | 4 (40, 9.6-70.4) | 9 (13.64, 5.3-21.9) | 0.024 |
| <i>A. phagocytophilum</i> alone | 5 (8.93, 1.4-16.4) | 0 (0, -) | 5 (7.58, 1.2-14.0) | 0.326 |
| <i>R. conorii</i> and <i>E. canis</i> | 8 (14.29, 5.1-23.5) | 4 (40, 9.6-70.4) | 12 (18.18, 8.9-27.5) | 0.074 |
| <i>R. conorii</i> and <i>A. phagocytophilum</i> | 6 (10.71, 2.6-18.8) | 0 (0, -) | 6 (9.09, 2.1-15.9) | 0.278 |
| <i>E. canis</i> and <i>A. phagocytophilum</i> | 0 (0, -) | 0 (0, -) | 0 (0, -) | - |
| <i>R. conorii</i> , <i>E. canis</i> and <i>A. phagocytophilum</i> | 21 (37.5, 24.8-50.2) | 1 (10, 0-28.6) | 22 (33.33, 21.9-44.7) | 0.146 |

CI: confidence interval

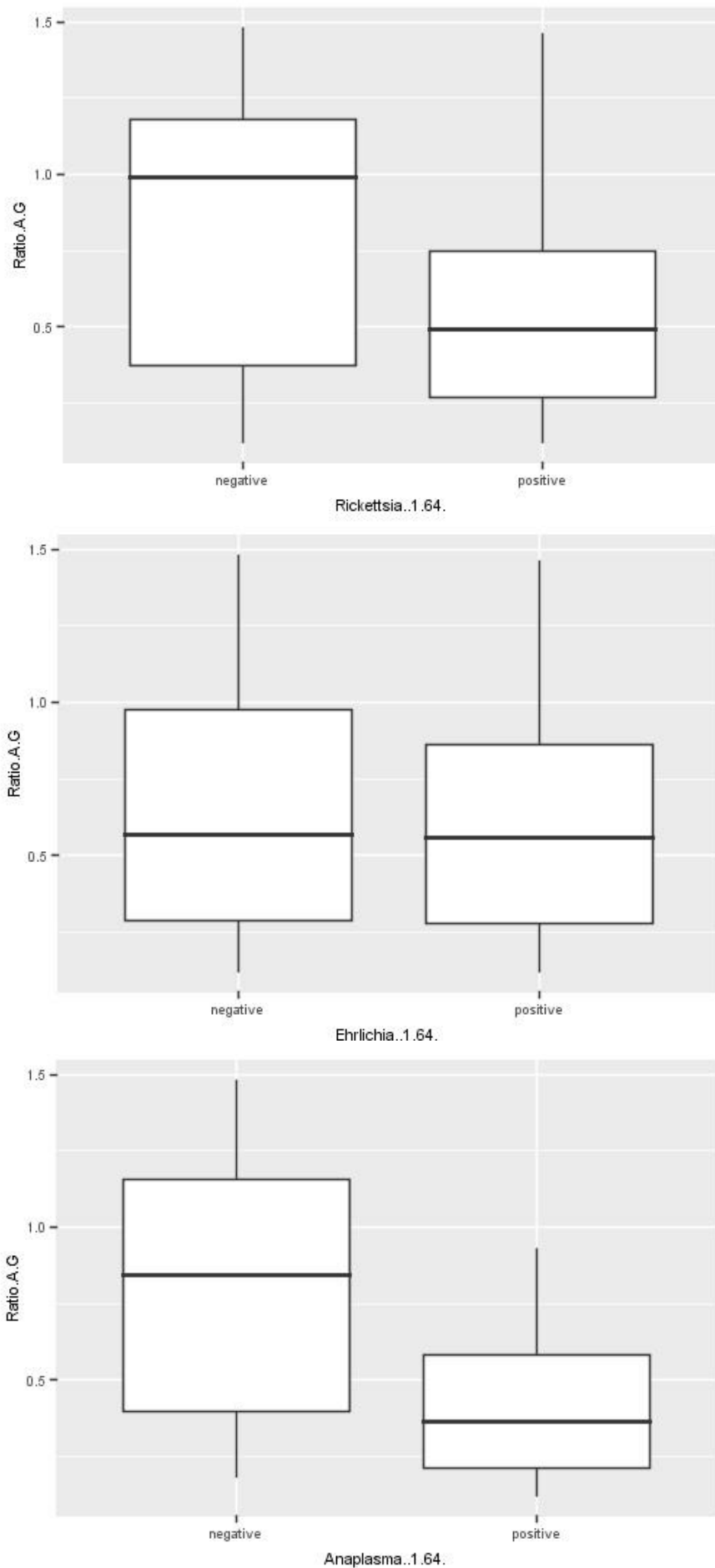
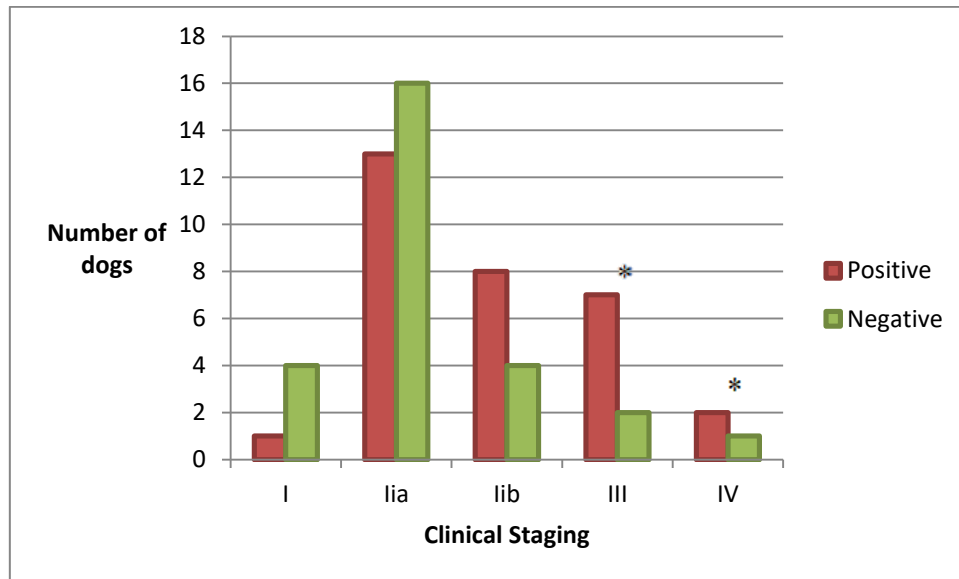
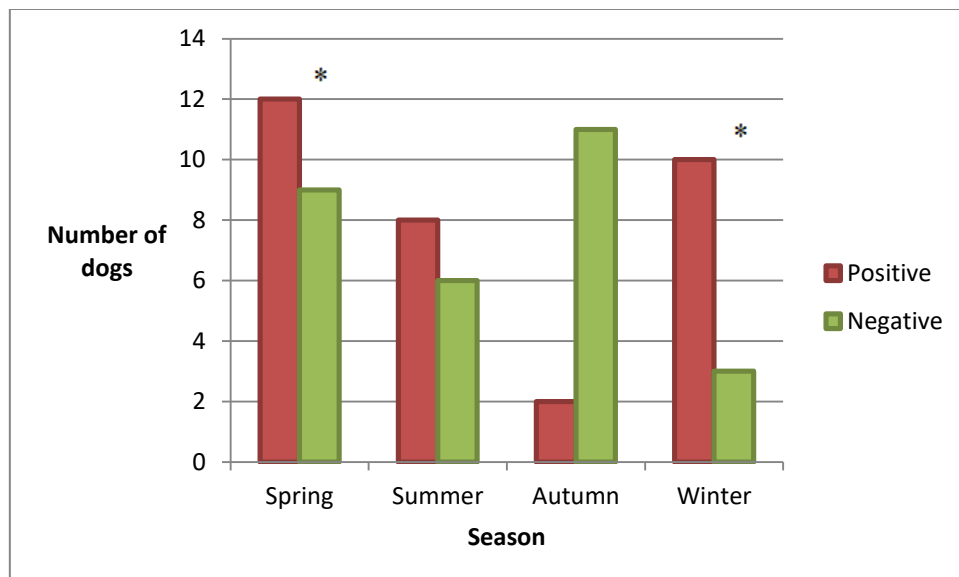


Fig 2

The albumin/globulin ratio according to the result (positive or negative) of the different IFAT performed at a dilution of 1:64. A comparison of the means was performed with logistic regression with the following results: *R. conorii* (P = 0.0217; OR = 0.17), *E. canis* (P = 0.7864; OR = 0.84) and *A. phagocytophilum* (P = 0.0003; OR = 0.04) antigens.

**Fig. 3**

Results of IFAT for *A. phagocytophilum* antigen in dogs with clinical leishmaniosis based on the LeishVet clinical staging. Fisher's exact test was performed with the following result: $P = 0.042^*$.

**Fig. 4**

Results of IFAT for *A. phagocytophilum* antigen in dogs with clinical leishmaniosis when grouped based on seasonality (time of clinical presentation) and diagnosis of leishmaniosis. Fisher's exact test was performed with the following result: $P = 0.014^*$.

Table 3

IFAT antibody titers for *R. conorii*, *E. canis* and *A. phagocytophilum* antigens in dogs with clinical leishmaniosis and healthy dogs. Fisher's exact test was performed.

CI: confidence interval

| Number (% , 95% CI) of seroreactive dogs for <i>R. conorii</i> | | | | |
|--|---|-----------------------|-----------------------|--------------|
| Antibody titers | Dogs with clinical leishmaniosis (n = 61) | Healthy dogs (n = 14) | Total dogs (n = 75) | P-value |
| <64 | 15 (24.59, 13.8-35.4) | 8 (57.14, 31.2-83.0) | 23 (30.67, 20.3-41.1) | 0.025 |
| 64 | 12 (19.67, 9.7-29.7) | 2 (14.29, 0-32.6) | 14 (18.67, 9.9-27.5) | 0.641 |
| 128 | 0 (0, -) | 0 (0, -) | 0 (0, -) | - |
| 256 | 10 (16.39, 7.1-25.7) | 0 (0, -) | 10 (13.33, 5.6-21.0) | 0.192 |
| 512 | 15 (25.59, 14.6-36.6) | 4 (28.57, 4.9-52.2) | 19 (25.33, 15.5-35.1) | 0.743 |
| >512 | 9 (14.75, 5.8-23.6) | 0 (0, -) | 9 (12, 4.6-19.3) | 0.195 |

| Number (% , 95% CI) of seroreactive dogs for <i>E. canis</i> | | | | |
|--|---|-----------------------|-----------------------|--------------|
| Antibody titers | Dogs with clinical leishmaniosis (n = 61) | Healthy dogs (n = 14) | Total dogs (n = 75) | P-value |
| <64 | 27 (44.26, 31.8-56.8) | 5 (35.71, 10.6-60.8) | 32 (42.67, 31.5-53.9) | 0.766 |
| 64 | 17 (27.87, 16.6-39.2) | 9 (64.29, 39.2-89.4) | 26 (34.67, 23.9-45.5) | 0.014 |
| 128 | 3 (4.92, 0-10.3) | 0 (0, -) | 3 (4, 0-8.4) | 0.397 |
| 256 | 3 (4.92, 0-10.3) | 0 (0, -) | 3 (4, 0-8.4) | 0.397 |
| 512 | 2 (3.28, 0-7.8) | 0 (0, -) | 2 (2.67, 0-6.4) | 0.492 |
| 1024 | 8 (13.11, 4.6-21.6) | 0 (0, -) | 8 (10.67, 3.7-17.7) | 0.338 |
| >1024 | 1 (1.64, 0-4.7) | 0 (0, -) | 1 (1.33, 0-3.9) | 0.63 |

| Number (% , 95% CI) of seroreactive dogs for <i>A. phagocytophilum</i> | | | | |
|--|---|-----------------------|---------------------|--------------|
| Antibody titers | Dogs with clinical leishmaniosis (n = 61) | Healthy dogs (n = 14) | Total dogs (n = 75) | P-value |
| <64 | 29 (47.54, 35.0-60.0) | 13 (92.86, 79.4-100) | 42 (56, 44.8-67.2) | 0.002 |
| 64 | 17 (27.87, 16.6-39.2) | 1 (7.14, 0-20.6) | 18 (24, 14.3-33.7) | 0.165 |
| 128 | 4 (6.56, 0-12.8) | 0 (0, -) | 4 (5.33, 0-10.4) | 0.325 |
| 256 | 3 (4.92, 0-10.3) | 0 (0, -) | 3 (4, 0-8.4) | 0.397 |
| 512 | 4 (6.56, 0-12.8) | 0 (0, -) | 4 (5.33, 0-10.4) | 0.325 |
| 1024 | 3 (4.92, 0-10.3) | 0 (0, -) | 3 (4, 0-8.4) | 0.397 |
| >1024 | 1 (1.64, 0-4.7) | 0 (0, -) | 1 (1.33, 0-3.9) | 0.63 |

No significant association was found between sex and clinical stage of leishmaniosis and antibody titers. A comparison between means of the different antibody titers was performed. The dogs that showed a high positive ($>1:512$) antibody titer for *R. conorii* antigen were associated with a decrease in albumin ($P = 0.0113$) (**Fig 5**) while a decrease of albumin/globulin ratio was associated with an increase of antibody titers for *R. conorii* antigen ($P = 0.014$) (**Fig 6**).

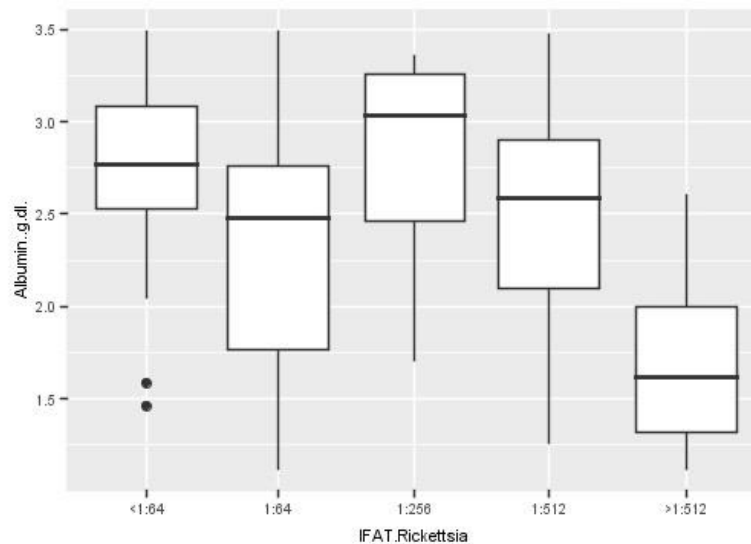


Fig. 5

Albumin concentration according to *R. conorii* antibody titer. A comparison of the means was performed with Kruskal-Wallis test ($P = 0.0113$).

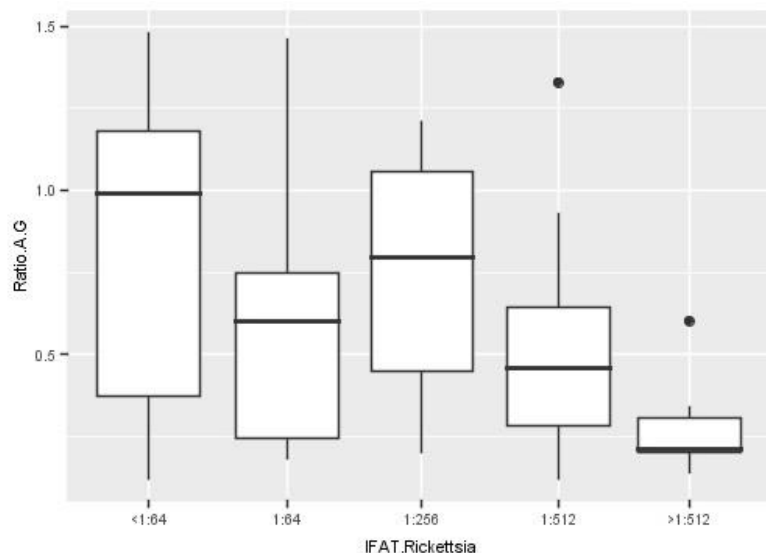


Fig. 6

Albumin/globulin ratio according to *R. conorii* antibody titers. A comparison of the means was performed with Kruskal-Wallis test ($P = 0.014$).

A significant association was found between a high *E. canis* antibody titer and a decrease of albumin ($P = 0.0087$; OR = 0.16), albumin/globulin ratio ($P = 0.0071$; OR = 0) (**Fig 7**), haematocrit ($P = 0.0048$; OR = 0.67), haemoglobin ($P = 0.0053$; OR = 0.57), RBC ($P = 0.0132$; OR = 0.07) and an increase of gamma globulins ($P = 0.0045$; OR = 2.39) (**Fig 8**) and total protein ($P = 0.0017$; OR = 3.02). Also, a significant association was found between *A. phagocytophilum* high antibody titers and a decrease of albumin ($P = 0.0014$) and albumin/globulin ratio ($P = 0.0014$).

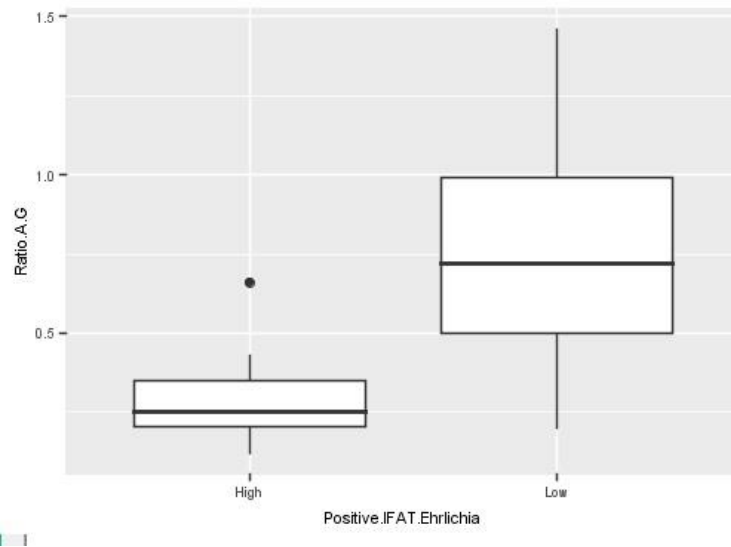


Fig. 7

Albumin/globulin ratio according to *E. canis* high antibody titers (equal or higher than 1:512) and low *E. canis* antibody titers (lower than 1:512). A comparison of the means was performed with logistic regression ($P = 0.0071$; OR = 0).

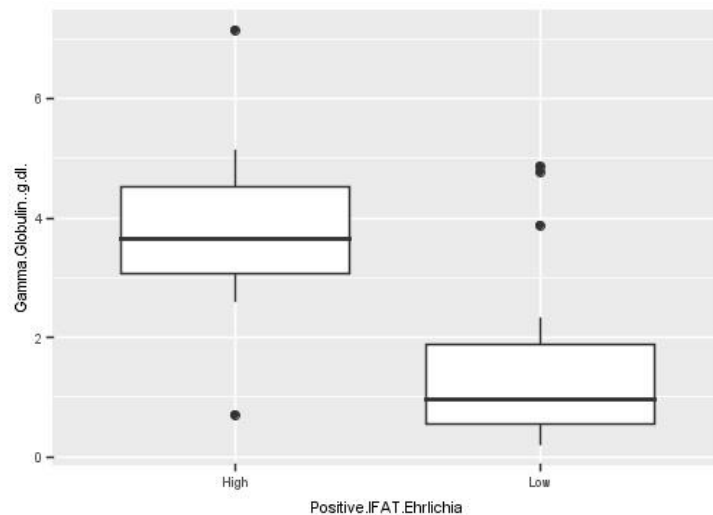


Fig. 8

Gamma globulins according to high *E. canis* antibody titers (equal or higher than 1:512) and low *E. canis* antibody titers (lower than 1:512). A comparison of the means was performed with logistic regression ($P = 0.045$; OR = 2.39).

PCR

PCR for the detection of E. canis and Anaplasma spp.

Of the 76 dogs assessed, 60 dogs with clinical leishmaniosis and 16 apparently healthy dogs, 8 (10.5%) had a positive result for *E. canis* and *Anaplasma* real-time PCR performed. All dogs with a positive result were diagnosed with clinical leishmaniosis. Of those 8 dogs, only two (2/8; 25%) maintained a positive result after performing a conventional PCR. Sequencing showed that both detected pathogens were *A. platys* (**Table 4**).

When comparing between dogs with clinical leishmaniosis and healthy dogs with Fisher's exact test, no difference was found between the groups.

A significant association was found between a positive result in the real-time PCR performed and a lower haematocrit ($P = 0.0281$; OR = 0.87), RBC ($P = 0.048$; OR = 0.42) and platelet ($P = 0.0461$; OR = 0.9) concentration.

No significant association was found between the origin of the dogs (Barcelona or Tarragona) and a positive result by PCR, although the two dogs that had a positive result in the conventional PCR were from Tarragona.

PCR for the detection of Hepatozoon spp. and Babesia spp.

Of the 77 dogs assessed, only 1 (1.3%) had a positive result in the PCR performed. The dog was diagnosed with clinical leishmaniosis. After sequencing, the pathogen found was *H. canis* (**Table 4**). *Babesia* DNA was not detected in any of samples studied.

No statistically significant association was found between the positive result in the PCR performed and any of the clinical characteristics of the dogs assessed.

Table 4

Dogs which had a positive result in the PCRs performed and the corresponding results for IFAT for different antigens studied.

| Dog ID | IFAT <i>R. conorii</i> | IFAT <i>E. canis</i> | IFAT <i>A. phagocytophilum</i> | Real-time PCR <i>Ehrlichia/Anaplasma</i> | Conventional PCR <i>Ehrlichia/Anaplasma</i> | PCR <i>Hepatozoon/Babesia</i> | Sequencing |
|--------|------------------------|----------------------|--------------------------------|--|---|-------------------------------|-------------------------|
| HCV-7 | 512 | 1024 | 1024 | Positive | Negative | Negative | - |
| HCV-9 | <64 | <64 | <64 | Positive | Negative | Negative | - |
| HCV-11 | >512 | <64 | 64 | Positive | Negative | Negative | - |
| HCV-28 | 512 | 1024 | 256 | Positive | Negative | Negative | - |
| HCV-31 | 64 | 64 | 128 | Positive | Negative | Negative | - |
| MO-1 | 512 | 128 | 64 | Positive | Positive | Negative | <i>A. platys</i> (99%) |
| MO-4 | 256 | 64 | 1024 | Negative | - | Positive | <i>H. canis</i> (100%) |
| MED-5 | 64 | <64 | <64 | Positive | Positive | Negative | <i>A. platys</i> (100%) |
| MED-7 | <64 | <64 | 1024 | Positive | Negative | Negative | - |

5. Discussion

Previous studies have speculated about how CanL could be affected by other vector-borne pathogens. De Tommasi et al. (2013) stated that an infection with two or more pathogens could complicate the clinical presentation of the diseases and the severity of haematological abnormalities in dogs and Mekuzas et al. (2009) examined naturally exposed dogs with *L. infantum* and *E. canis* co-infection and described that the increase of clinical signs could support the postulation of a synergistic pathological effect between both pathogens. Furthermore, Mekuzas et al. (2009) suggested that *E. canis* could be contributing to the establishment of CanL and Baneth et al. (2015) examined three dogs with *E. canis* and *H. canis* co-infection and suggested that infection with one pathogen could permit or enhance invasion of another. Conversely, Tabar et al. (2013) examined dogs with leishmaniosis and/or filariasis to detect filarial species, *Wolbachia* species and *Leishmania* and, although an increase of severity and clinical signs was observed when *Leishmania*-filarial co-infection, it was also suggested that *Wolbachia* infection could have a protective role against *Leishmania* infection.

Our results demonstrated the existence of co-infections with vector-borne pathogens in dogs with clinical leishmaniosis living in the Mediterranean basin. To the best knowledge of the authors, a statistical significant relationship was found, for the first time, between sick dogs and a higher number of detected co-infections and the detection of *R. conorii* or *A. phagocytophilum* antibodies when compared with healthy dogs. Accordingly, a recent study documented that co-infection with several tick-borne diseases caused clinical progressions of leishmaniosis in foxhounds living in the USA (Toepp et al. 2017). In disagreement with previous reports (Mekuzas et al. 2009; De Tommasi et al. 2013; Estevez et al. 2017; Lima et al. 2017; Rodriguez Sánchez et al. 2017), no association was found between seroreactivity to *E. canis* antigens and sick dogs with leishmaniosis.

One of the more interesting findings in the present study is the fact that more pronounced clinicopathological abnormalities such as decrease in albumin or RBC numbers or increase in globulins were noted in dogs with clinical leishmaniosis and co-infections. Similar findings were previously reported by other studies. These studies demonstrated more marked thrombocytopenia, an evident reduction of platelet aggregation response, a significant increase in activated partial thromboplastin time

(APTT) and a reduction of the albumin/globulin ratio in Italian dogs with clinical leishmaniosis co-infected with *E. canis* (Cortese et al. 2006; Cortese et al. 2009; Cortese et al. 2011). Here, in the present study, we reported for the first time that certain clinicopathological abnormalities are more marked in dogs with co-infections with *R. conorii*, or *Rickettsia* related species, *A. platys*, *E. canis* and *H. canis*. It is important to highlight that based on the present findings, moderate to marked hypoalbuminemia or hyperglobulinemia in dogs with clinical leishmaniosis should be very suspicious of co-infections with other vector-borne pathogens. It is well known that infection with tick-borne pathogens such as *R. conorii*, *A. platys*, *A. phagocytophilum* and *E. canis* may induce a decrease in serum levels of negative acute phase proteins while an increase of positive acute phase proteins occurs in canines with these infections (Kohn et al. 2008; Al Izzi et al. 2013; Dondi et al. 2014; Solano-Gallego et al. 2015; Bouzouraa et al. 2016). Interestingly, an association was found between antibodies against *A. phagocytophilum* and more advanced clinical stages of leishmaniosis (Leishvet stage III and IV) in agreement with the recent previous study (Toepp et al. 2017). Moreover, a significant association was also found between dogs positive for *E. canis* and *Anaplasma* spp. PCR and a lower haematocrit, RBC and platelets, which are typical clinicopathological findings in canine ehrlichiosis or anaplasmosis (Little 2010; Procajlo et al. 2011; Dondi et al. 2014; Pantchev et al. 2015). Further studies are needed to understand the relationship between co-infections and clinical leishmaniosis in dogs.

Previous studies have evaluated the serological and molecular evidence of exposure to vector-borne pathogens in dogs in Catalonia (Spain) (Roura et al. 2005; Solano-Gallego et al. 2006; Tabar et al. 2009; Miró et al. 2013). Taking these studies into account, our results demonstrated a high increase of seropositivity rates when studying dogs with clinical leishmaniosis. Another study done by Amusatogui et al. (2008) in North-western Spain found similar seroprevalences as the ones found in this study: 50% for *R. conorii*, 54.7% for *E. canis* and 45.3% for *A. phagocytophilum* antigens. The difference is that those seroprevalences were found in dogs living in community dog shelters, not in sick dogs with clinical leishmaniosis. In this same study, it was reported that the seroprevalence of *E. canis* and *A. phagocytophilum* was higher in dogs in a community shelter than in dogs evaluated at local veterinary clinics (Amusatogui et al. 2008).

Another important point is the results obtained by the PCRs performed in this study. Tabar et al. (2009) examined 153 dogs and found the following percentages of infection

by blood PCR. Four per cent for *Ehrlichia* spp. and *Anaplasma* spp., 3.3% for *H. canis* and 2% for *B. vogeli*. In the present study, eight dogs (10.7%) had a positive PCR result for *E. canis* and *Anaplasma* spp. and only two of them remained positive by conventional PCR confirming *A. platys* infection in both cases. *Hepatozoon canis* was only found in one sick dog with leishmaniosis while *Babesia* DNA was not detected in any of the dogs studied in agreement with previous studies performed in Sicily (Italy) although *B. vogeli* was encountered in febrile Sicilian dogs (Solano-Gallego et al. 2015). Combining the serological and molecular results of the present study with the present literature, it is noteworthy to remark that co-infections patterns will be different depending on the geographical region where the dogs with leishmaniosis live, their life style, their exposure to ticks and fleas, the species of ectoparasites present in the area, and also on the preventative measures applied against ticks and fleas. For example, in the present study, *A. platys* and *H. canis* were only confirmed by PCR in dogs from the Tarragona area. In the Mediterranean basin, where *R. sanguineus* ticks are common, it would be expected that the pathogens related to these ticks would be also prevalent (Beugnet and Marié 2009; Hornok et al. 2017), however, comparing the present study with other recent studies from Croatia (Mrljak et al. 2017), Greece (Latrofa et al. 2017), Cyprus (Attipa et al. 2017) and Israel (Azmi et al. 2017), it is evident that *E. canis*, *Hepatozoon* spp. and *Babesia* spp. are circulating abundantly in those countries while, in the Spanish regions studied in this present study, the results suggest that they are less common.

Moreover, it is noteworthy to take into account the difference of the diagnostic techniques used in this study. PCR is a technique that can detect pathogen DNA and, therefore, confirm infection but a negative result does not completely exclude infection while serology techniques, as ELISA or IFAT, can detect antibodies, which could be interpreted as current infection, past exposure to the pathogen tested or be used to detect seroconversion, but may also be the result of cross-reaction with antibodies formed against other organisms with similar antigens. PCR also allows speciation of DNA of the pathogen amplified. Due to the aforementioned characteristics, it is recommended to use both techniques for diagnosis (Tabar et al. 2009; Nicholson et al. 2010; Otranto et al. 2010). In the present study, the results found for the different PCR performed showed an important limitation in the detection of positive samples, possibly due to the low concentration of pathogen in blood. It is important to remark that with these type of

pathogens, serial evaluations of blood parasitemia by PCR are recommended to enhance the likelihood of PCR detection (Kidd et al. 2017). Additionally, in this case, no repeated testing of the same dogs was performed and serology was not used to detect seroconversion, although seroconversion could have been helpful in the detection of a higher number of acute infections (Solano-Gallego et al. 2015; Kidd et al. 2017). Furthermore, in the present study, no PCR was performed to detect *Rickettsia* spp. such as *R. conorii* due to the low rickettsiemia usually found in dogs (Solano-Gallego et al. 2008; Tabar et al. 2009; Solano-Gallego et al. 2015).

Another important point to consider would be the different cross-reactions that could have occurred in this study. It has been reported that many of the reactions made in serological tests for *R. conorii* might be due to other spotted fever group (SFG) *Rickettsia* species as *R. massiliae*, *R. slovaca* or *R. aeschlimannii* among others, which are more common in ticks in the Mediterranean basin countries (Merino et al. 2005; Fernández-Soto et al. 2006; Solano-Gallego et al. 2015). Furthermore, serocrossreactivity between *A. phagocytophilum* and *A. platys* is common, and at similar antibody titration, due to their high antigenic similarity (Aguirre et al. 2006; Miró et al. 2013; Estrada-Peña et al. 2017). In Europe, *A. phagocytophilum* is usually transmitted by *I. ricinus* ticks while *A. platys* is suspected as transmitted by *R. sanguineus* (Beugnet and Marié 2009; Little 2010; Chomel 2011). Taking into account that the main tick that inhabits the Barcelona area is *R. sanguineus* (Estrada-Peña et al. 2004), it can be suggested that the positive serologic reactivity was probably aimed at *A. platys* and not *A. phagocytophilum*. In similar way, *E. canis* can also have a serocross-reaction with *Anaplasma* species (Waner et al. 2001; Gaunt et al. 2010). In the present study, 22 dogs seroreacted to both, *E. canis* and *A. phagocytophilum*, and without a positive result by PCR and sequencing, it could be suggested that the dogs were exposed to only one of the two vector-borne pathogens detected and, actually, they could have been infected by *A. platys*, the only Anaplasmataceae species detected by PCR.

Another finding of the present study was the detection of a higher number of pathogens by IFAT and PCR in older dogs compared to young dogs. It is reasonable that an older dog would have had more time and opportunities to be exposed to the different pathogens studied, although young dogs could be more susceptible to infections due to the immaturity of the immune system (Greeley et al. 2001; Blount et al. 2005; Day

2007; Day 2010). In agreement, Amusatogui et al. (2008) stated that *R. conorii* infection was significantly associated with older age. But, a recent study (Santana Almeida et al. 2017) found that young animals are more susceptible to co-infection of *Leishmania* and *Babesia* spp. and Miró et al. (2013) found that dogs under one year of age showed higher seropositivity rates for *E. canis* and *Borrelia Burgdorferi* compared to dogs older than one year. Further studies need to be performed to understand the relationship between age and vector-borne diseases, taking into account other factors such as lifestyle and location.

When studying vector-borne pathogens, it is also expected to find a relationship between the time of the detection of the infection and the season when the vector is more active. In this study, only the results of IFAT for *A. phagocytophilum* antigen showed an association between seropositivity and season, in this case spring or winter. The vectors for *A. phagocytophilum* present in Spain is *I. ricinus* (Beugnet and Marié 2009; Little 2010; Chomel 2011), which have the highest activity between April and June, a decrease of activity thereafter and a slight increase in the autumn-winter months (Barandika et al. 2011). When evaluating our results, it could be suggested that the dogs with a positive IFAT result for *A. phagocytophilum* antigen were infested with these ticks and a subsequent infection occurred. However, we have suggested earlier in this study, antibodies against *A. phagocytophilum* are likely to be antibodies against *A. platys*, and, as mentioned above, the main vector for this pathogen is probably *R. sanguineus*. Different studies (Lorusso et al. 2010; Dantas-Torres et al. 2011) have evaluated the seasonal dynamics of this tick in the Mediterranean basin and, although it has been stated that the highest activity of *R. sanguineus* is in summer, it has also been observed that this tick can infest dogs during all seasons. Furthermore, *A. platys* is known to cause subclinical infections (Bradfield et al. 1996; Little 2010; Bouzouraa et al. 2016) and, in fact, that the detection of the infection might not be associated with the season. On the other hand, no association was found between the other vector-borne pathogens and seasonality. This could be due to the high probability of subclinical and chronic ehrlichiosis during canine infection with *E. canis* (Little 2010) with the consequent delay in detection of infection as well as with leishmaniosis (Baneth et al. 2008; Solano-Gallego et al. 2009; Pennisi 2015). *Rickettsia conorii* infection in dogs is also typically a subclinical or mild disease (Solano-Gallego et al. 2015).

A number of limitations are present in this study due to its retrospective nature. It would be interesting to increase the spectrum of clinical stages in the dogs with clinical leishmaniosis as the majority of them were classified as being in stage II of the LeishVet clinical staging, and also to increase the numbers of dogs both sick and apparently healthy dogs included in the study. Another limitation, that was found when analysing the data, was the lack of information about the lifestyle of the dogs. It would be highly important to know if they were living indoors or outdoors and therefore how much exposure did they have to sand flies and ticks. Moreover, the use of serial testing to detect seroconversion and associate the results with changes in the clinical signs and clinicopathological findings would be also interesting to evaluate. In addition, it could be suggested to increase the number of pathogens tested and include other canine pathogens such as *Bartonella* spp. which may also be encountered in Spain (Roura et al. 2005; Solano-Gallego et al. 2006; Amusatogui et al. 2008; Tabar et al. 2009; Miró et al. 2013). Future studies should be designed as case control longitudinal studies to obtain additional information on vector-borne diseases and their co-infections in Spain.

6. Conclusion

This study demonstrates that dogs with clinical leishmaniosis from the Barcelona and Tarragona area have a higher rate of co-infections with other vector-borne pathogens when compared with healthy controls. Furthermore, positivity to other vector-borne pathogens was associated with more pronounced clinicopathological abnormalities as well as disease severity with canine clinical leishmaniosis.

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9. Annexes

Annex 1. LeishVet Clinical Staging (Solano-Gallego et al. 2009; Solano-gallego et al. 2011)

| Clinical stages | Serology | Clinical signs | Laboratory findings | Therapy | Prognosis |
|---|--|--|---|---|-----------------|
| Stage I Mild disease | Negative to low positive antibody levels | Dogs with mild clinical signs such as peripheral lymphadenomegaly, or papular dermatitis | Usually no clinicopathological abnormalities observed. Normal renal profile: creatinine < 1.4 mg/dl; non-proteinuric: UPC < 0.5 | Scientific neglect/allopurinol or meglumine antimoniate or miltefosine/allopurinol + meglumine antimoniate or allopurinol + miltefosine** | Good |
| Stage II Moderate disease | Low to high positive antibody levels | Dogs, which apart from the signs listed in stage I, may present: diffuse or symmetrical cutaneous lesions such as exfoliative dermatitis/onychogryposis, ulcerations (planum nasale, footpads, bony prominences, mucocutaneous junctions), anorexia, weight loss, fever, and epistaxis | Clinicopathological abnormalities such as mild non-regenerative anaemia, hyperglobulinemia, hypoalbuminemia, serum hyperviscosity syndrome. Substages a) Normal renal profile: creatinine < 1.4 mg/dl; non-proteinuric: UPC < 0.5 b) Creatinine < 1.4 mg/dl; UPC = 0.5-1 | Allopurinol + meglumine antimoniate or allopurinol + miltefosine | Good to guarded |
| Stage III Severe disease | Medium to high positive antibody levels | Dogs, which apart from the signs listed in stages I and II, may present signs originating from immune-complex lesions: vasculitis, arthritis, uveitis and glomerulonephritis | Clinicopathological abnormalities listed in stage II. Chronic kidney disease (CKD) IRIS stage I with UPC > 1 or stage II (creatinine 1.4-2 mg/dl) | Allopurinol + meglumine antimoniate or allopurinol + miltefosine Follow IRIS guideline for CKD. | Guarded to poor |
| Stage IV Very severe disease | Medium to high positive antibody levels | Dogs with clinical signs listed in stage III. Pulmonary thromboembolism, or nephrotic syndrome and end stage renal disease | Clinicopathological abnormalities listed in stage II CKD IRIS stage III (creatinine 2-5 mg/dl) and stage IV (creatinine > 5 mg/dl). Nephrotic syndrome marked proteinuria UPC > 5 | Allopurinol (alone) Follow IRIS guidelines for CKD | Poor |