The Veterinary Journal

Seasonal variation in canine anti-Leishmania infantum antibody titres

--Manuscript Draft--

Manuscript Number:	YTVJL-D-20-00623R1
Article Type:	Original article
Keywords:	Antibody titers; Canine leishmaniosis; IFAT; Inter-seasonal decline; Sand fly season
Corresponding Author:	Andrea Zatelli Consultant TA'XBIEX, MALTA
First Author:	Maria Alfonsa Cavalera
Order of Authors:	Maria Alfonsa Cavalera
	Roberta Iatta
	Rossella Panarese
	Jairo Alfonso Mendoza-Roldan
	Floriana Gernone
	Domenico Otranto
	Saverio Paltrinieri
	Andrea Zatelli
Abstract:	Quantitative anti-Leishmania antibody titres are critical in the management of dogs with leishmaniosis, from diagnosis to treatment and follow-up. and there is a paucity of data relating changes in antibody titres to sand fly vector seasonality. This study aimed to evaluate seasonal variations in anti-Leishmania infantum antibody titres in dogs from a hyperendemic area for canine leishmaniosis (CanL). Leishmania infantum-seropositive and clinically healthy dogs (n=65) were sampled in June 2019 (sand fly season) and again in February-March 2020 (non-transmission season) to monitor clinical status and serological titres. There was a reduction in anti-L. infantum antibody titres during the non-transmission season in most dogs (n=36; 55.4%), and 44% of those dogs (n=16/36) became seronegative (i.e. below the cut-off value of 1:80). Given the relevance of serology to epidemiological, preventive and clinical studies related to CanL, seasonal variations in antibody titres are important in areas where phlebotomine vectors have seasonal patterns of activity. Sand fly seasonal period must be considered in the interpretation of annual anti-L. infantum antibody screening test results in asymptomatic dogs, to inform clinical decisions about staging, treatment and prevention.

Seasonal variation in canine anti-*Leishmania infantum* antibody titres

M.A. Cavalera [a], R. Iatta [a], R. Panarese [a], J.A. Mendoza-Roldan [a], F. Gernone [a], D. Otranto [a, b], S. Paltrinieri [c], A. Zatelli [a, *]

[a] *Dipartimento di Medicina Veterinaria, Università degli Studi di Bari, Bari, Italy*
[b] *Faculty of Veterinary Sciences, Bu-Ali Sina University, Hamedan, Iran*
[c] *Dipartimento di Medicina Veterinaria, Università degli Studi di Milano, Milano, Italy*

* Corresponding author: Tel +39 80 467 9804
E-mail address: andrea.zatelli@uniba.it (A. Zatelli)

Abstract

Quantitative anti-*Leishmania* antibody titres are critical in the management of dogs with leishmaniosis, from diagnosis to treatment and follow-up. and there is a paucity of data relating changes in antibody titres to sand fly vector seasonality. This study aimed to evaluate seasonal variations in anti-*Leishmania infantum* antibody titres in dogs from a hyperendemic area for canine leishmaniosis (CanL). *Leishmania infantum*-seropositive and clinically healthy dogs (*n*=65) were sampled in June 2019 (sand fly season) and again in February-March 2020 (non-transmission season) to monitor clinical status and serological titres.

There was a reduction in anti-*L. infantum* antibody titres during the non-transmission season in most dogs (*n*=36; 55.4%), and 44% of those dogs (*n*=16/36) became seronegative (i.e. below the cut-off value of 1:80). Given the relevance of serology to epidemiological, preventive and clinical studies related to CanL, seasonal variations in antibody titres are important in areas where phlebotomine vectors have seasonal patterns of activity. Sand fly seasonal period must be considered in the interpretation of annual anti-*L. infantum* antibody screening test results in asymptomatic dogs, to inform clinical decisions about staging, treatment and prevention.

Keywords: Antibody titers; Canine leishmaniosis; IFAT; Inter-seasonal decline; Sand fly season

Introduction

Canine leishmaniosis (CanL) caused by *Leishmania infantum* (Kinetoplastida, Trypanosomatidae) is a widespread zoonotic sand fly-borne disease. Clinical outcome depends on the fine balance between the parasite and the host immune response (Paltrinieri et al., 2010; Otranto et al., 2013; Hosein et al., 2017) and disease progression occurs when the cell-mediated immune response targeting the *Leishmania* organism is ineffective (Hosein et al., 2017). Because of the wide spectrum of clinical presentations, from subclinical infection to severe and potentially fatal clinical disease, and the variety of clinicopathological abnormalities, the diagnosis of CanL can be challenging (Solano-Gallego et al., 2017). Anti-*Leishmania* antibody screening is often the initial organism-specific diagnostic tool in dogs (Paltrinieri et al., 2010; ESCAAP, 2019). Timing of seroconversion after an infective phlebotomine bite can occur from 1 to 22 months, with a median time of 3 and 5 months in experimentally and naturally infected dogs, respectively (Moreno et al., 2002). Even though point-of-care tests based on immunochromatographic methods have been developed, indirect fluorescent antibody test (IFAT) and enzyme linked immunosorbent assays (ELISA) are required due to their higher diagnostic performance and antibody quantification. The IFAT test for the diagnosis of CanL has a sensitivity of 96% and specificity of 98%, which is similar to the ELISA (OIE, 2018).

Quantitative anti-*Leishmania* antibody titre testing is frequently used in veterinary practice and is a reliable for the confirmation *L. infantum* infection in dogs, for monitoring the clinical evolution of the disease, and for monitoring response to treatment (Oliva et al., 2010; Paltrinieri et al., 2010; Proverbio et al., 2014; Paltrinieri et al., 2016; Solano-Gallego et al., 2017). Because of inter- and intra-test variability, values that are ≥1-2 fold and ≥3-4 fold higher than the threshold value for the laboratory are interpreted as ‘low titres’ and ‘high

62 titres', respectively (Paltrinieri et al., 2010). High antibody titres in the presence of clinical
63 signs and/or clinicopathological abnormalities associated with CanL are diagnostic for active
64 CanL, whereas low antibody titres indicate subclinical infection, or exposure without
65 infection (Paltrinieri et al., 2010; Solano-Gallego et al., 2011; Travi et al., 2018). High anti-
66 *Leishmania* antibody IFAT titres are most often correlated with parasite dissemination and
67 more severe clinical signs (Paltrinieri et al., 2010; Proverbio et al., 2014). Antibody titres are
68 also used to guide treatment (Paltrinieri et al., 2010; Oliva et al., 2010), as dogs with high
69 antibody titres and one or more clinical signs consistent with CanL are staged as 'sick', thus
70 requiring anti-*Leishmania* therapy (Oliva et al., 2010).
71
72 Phlebotomine sand flies colonize a wide range of environments (e.g. primary forest,
73 crop plantations, animal shelters, and human houses), from coastal plains to hilly areas.
74 According to the macro geographical area, the development of sand flies may occur year-
75 round (e.g. in the New World) or during a defined period or 'season' (e.g. in the
76 Mediterranean region; Dantas-Torres et al., 2012). In the Mediterranean region, dogs can be
77 exposed to sand fly bites from early April to November (Alten et al., 2016), potentially
78 leading to new infection by *L. infantum* (Vlkova et al., 2011). Although anti-*Leishmania*
79 antibody titres are of paramount importance in the management of CanL cases from diagnosis
80 to treatment and follow-up, limited data are available comparing sand fly seasonality with
81 antibody titres.
82
83 This study aimed to evaluate the role of sand fly seasonality on quantitative anti- *L.*
84 *infantum* antibody titres in seropositive and clinically healthy dogs living in an area endemic
85 for CanL.
86

Materials and methods

Study area

Clinical and serological examinations were performed from late June 2019 to March 2020 in dogs from a shelter in southern Italy (40° 25′ 09.6″N, 18° 09′ 56.1″E, Lecce, Apulia region). This area is endemic for CanL (Mendoza-Roldan et al., 2020) and the sand fly season usually lasts from late May to late October, with two density peaks during July and August. Vector species of *L. infantum* (e.g. *Phlebotomus perniciosus, Phlebotomus perfiliewi*, and *Phlebotomus neglectus*; Tarallo et al., 2010) are also prevalent.

Dog population, sampling and follow-up

This study was approved by the ethical committee of the Department of Veterinary Medicine of the University of Bari, Italy (Approval number, Prot. Uniba 8/19; Approval date, *14 June 2019* dd Month yyyy). In late June 2019 (baseline; sand fly season), dogs were enrolled in the study if they fulfilled the following criteria: (1) seropositive to *L. infantum*; (2) no treatment for CanL in the previous 6 months; and (3) no current clinical signs compatible with CanL infection (Paltrinieri et al., 2010; Solano-Gallego et al., 2011). Anamnestic data and signalment information (i.e. age, sex, breed, reproductive status, weight, body condition score and microchip number), as well as details of any previous treatments, were recorded. In February-March 2020 (non-transmission season) all dogs underwent repeated clinical examination and serological testing.

At both timepoints, a 5 mL blood sample was collected from the cephalic vein and placed in a serum (clot activator) tube. Samples were transported to the laboratory within 4 h of collection, and serum was obtained by centrifugation (1500 *g* for 15 min). Serological analysis was performed within 24 h.

Dogs with negative serology in the non-transmission season underwent repeat clinical and serological examination 8 weeks after their last serological test. Additionally, fine needle aspirates (FNA) of lymph node and/or bone-marrow were tested by molecular means, as described below. Bone marrow aspirates were obtained from the costochondral junction, after the infiltration of 1-2 mL of local anaesthetic (2% lidocaine), using a 21 G needle; samples were placed in EDTA tubes. FNA samples from popliteal or submandibular lymph nodes were placed in sterile tubes containing 500 µL saline. Lymph node and bone marrow samples were stored at -20 °C until DNA extraction and molecular processing.

Serological testing

Serum samples were tested for anti-*L. infantum* IgG antibodies by IFAT as previously described (Otranto et al., 2009). Samples were considered positive if there was clear cytoplasmic and membrane fluorescence of *L. infantum* promastigotes from a cut-off dilution of 1:80. Positive sera were titrated by serial dilutions (i.e., 1:80, 1:160, 1:320, 1:640, 1:1280 and 1:2560) until negative results were obtained. Samples were considered negative if they failed to produce a positive result at 1:80. All serological tests were read in a double-masked manner by two different operators.

Molecular testing

Lymph node and bone-marrow aspirates were subjected to DNA extraction using the DNeasy Blood and Tissue Extraction Kit (Qiagen), according to manufacturer's instructions. Detection of 120 base pair fragments of *L. infantum* kinetoplast DNA minicircle was achieved by real time-PCR (qPCR), using primers, probes, and protocol as previously described (Francino et al., 2006).

137

138 *Statistical analysis*

139 Statistical analyses were performed using Analyse-it software v 5.01 (Analyse-it
140 Ltd.). Since a Kolmogorov-Smirnov test demonstrated data were not normally distributed,
141 seasonal differences in *L. infantum*-antibody titres were evaluated using Wilcoxon signed
142 rank tests. $P<0.05$ was considered statistically significant.

143

144 **Results**

145 Sixty-five *L. infantum*-seropositive, clinically healthy dogs (n=26 males; n=39
146 females) were enrolled. Age range was 1-14 years (median, 8 years) and weight range was
147 10-38 kg (median, 25 kg); most dogs were Mixed breed (n=64) and neutered (n=62). Anti-*L.*
148 *infantum* antibody titres for the 65 dogs, at both sampling times, are shown in Table 1.

149

150 Antibody titres decreased in 36 dogs (55.4%), became seronegative in 16 dogs
151 (44.4%), remained static in 12 dogs (18%), and increased in 17 dogs (26%). Median antibody
152 titre increased from enrolment (1:80) to the non-transmission season (1:160; P=0.016). Of the
153 dogs with a lower titre in the non-transmission season, median titre decreased from 1:320 at
154 enrolment to 1:80 in the non-transmission season; 16 dogs were considered negative.
155 Conversely, of the dogs with an increased titre in the non-transmission season, the median
156 titre increased from 1:80 at enrolment to 1:320 in the non-transmission season. In dogs with
157 titres of 1:80 at enrolment, median antibody titre was unchanged in the non-transmission
158 season (P=0.234). In dogs with titres > 1:80 at enrolment, median antibody titre decreased
159 from 1:1280 to 1:320 in the non-transmission season ($P<0.001$).

160

Fourteen of 16 dogs with negative titres in the non-transmission season were resampled 8 weeks later, and were negative on serology and qPCR of lymph node and/or bone marrow aspirates. The remaining 2/16 dogs were not re-sampled because they were lost to follow up.

During the study period, 64/65 dogs remained non-clinical for CanL (Paltrinieri et al., 2010; Solano-Gallego et al., 2011). One dog developed uveitis and dermatologic lesions (consistent with CanL) and had a 1-fold increase in antibody titre (from 1:1280 to 1:2560).

Discussion

This study demonstrated that variations in antibody titres against *L. infantum* between sand fly and non-sand fly transmission periods occurred in dogs from an endemic area for CanL characterized by vector seasonality. Median antibody titres increased significantly at the second sampling time compared with baseline, which was attributed to increases in titre of up to 4-fold in 12/34 dogs that had titres of 1:80 at enrolment. However, when dogs with titres of 1:80 at enrolment were considered separately, this finding was lost. Most dogs experienced a reduction in *Leishmania* IFAT titres during the non-transmission season, and almost half of these dogs became seronegative. Reduced anti-*L. infantum* antibody titres during the non-sand fly period may be related to the progressive reduction of exposure to vectors and/or to an effective cellular immune response. During the transmission period, dogs are exposed to sand fly bites and rapidly develop a strong anti-saliva antibody response which increases with the number of sand fly bites and declines inconsistently when exposure ceases (Vlkova et al., 2011; Kostalova et al., 2015). There is a positive relationship between anti-saliva and anti-parasite responses (Kostalova et al., 2015; Quinnell et al., 2018), and the immune response is likely up-regulated during the transmission period, as a consequence of

uninfected and *L. infantum*-infected sand fly bites and the immunogenic effect of the parasite. This hypothesis could explain decreased antibody titres from 1:2560 at time of enrolment to as low as 1:80 during the non-transmission season in some dogs in our study. Moreover, a protective cell-mediated immune response leading to a complete remission of infection could have developed in dogs with reduced antibody titres and, in particular, in dogs with IgG titres < 1:80 and negative by qPCR techniques in the non-transmission season. When *L. infantum* is under control by the immune response, antibody titres tend to either remain low, or eventually become negative in clinically healthy dogs (Paltrinieri et al., 2016). It has recently been reported that not only infective sand fly bites, i.e. those in which infectious mature metacyclic promastigotes are regurgitated and inoculated into the host, but also non-infective sand fly bites, i.e. those where non-infectious immature promastigotes are regurgitated and inoculated, can elicit *Leishmania*-specific antibodies in animals, although this report has not yet undergone peer review (Gradoni et al., 2019). Non-infective sand fly bites could explain the existence of 'exposed but uninfected dogs' that develop and maintain low (1:40-1:160) IFAT titres. This hypothesis could also explain stable titres over both sampling seasons in some dogs in our study, since most of those dogs maintained antibody titres of 1:80.

The reduction in antibody titres during the non-transmission season (up to 3-fold lower than the previous antibody titre determination) in over half of the enrolled dogs impacts the interpretation of CanL seroepidemiological results and case management. Variations in seroprevalence and antibody titres could be associated with time of year, with higher values more likely during the sand fly season. Additionally, sampling time should be carefully assessed in field studies evaluating the efficacy of preventive measures for CanL. In CanL endemic areas, data from clinically normal dogs that were initially seropositive for *L. infantum* but became seronegative after 6 or 12 months have been reported in time frames

that were within (Acedo-Sánchez et al., 1998) or between (Nejjar et al., 2000) transmission seasons. However, in our study, decreased antibody titres, including some that became seronegative, were recorded over the relatively short period between sand fly season and the following non-transmission season. It is noteworthy that most dogs that became seronegative had low positive antibody titres (1:80) at enrolment. Sand fly season-dependent seropositive *L. infantum* results could have several implications, for example in shelter dogs being rehomed, where movement from endemic to non-endemic areas could depend on the results of CanL serological tests. Therefore, asymptomatic dogs that are seropositive for *L. infantum* during sand fly season should be reassessed during the non-transmission season to confirm infection status.

Dogs with high titres in the sand fly season (*n*=36) had reduced antibody titres during the non-transmission season (12/12 dogs with titres of 1:2560 and 4/6 dogs with titres of 1:1280). According to published guidelines, dogs with high anti-*Leishmania* antibody titres (i.e. 2-4 fold higher than the threshold positive value) with at least one CanL-related clinical sign and/or associated laboratories abnormalities, are usually classified as 'sick' and require treatment with appropriate anti-*Leishmania* therapy (Paltrinieri et al., 2010; Oliva et al., 2010). However, since CanL-related clinical signs and laboratory abnormalities are non-specific, particularly in regions where other arthropod-borne pathogens are common, dogs incorrectly classified as 'sick' based on high antibody titres could receive unnecessary treatment, potentially contributing to the emergence of drug resistance (Yasur-Landau et al., 2016). Therefore, dogs with high antibody titres during sand fly season should undergo direct diagnostic testing to confirm the presence of the parasite before treatment for CanL is initiated.

About a quarter of the dogs in our study had higher antibody titres in the non-transmission season than at enrolment. This could have been related to the progression of infection, leading to the development of clinical disease (Solano-Gallego et al., 2011). However, in our study, clinical signs suggestive of active CanL (Paltrinieri et al., 2010; Solano-Gallego et al., 2011) were only observed in one dog, although assessment for active CanL was incomplete due to lack of further relevant laboratory testing. This result could be related to individual variability in the immunological response to *L. infantum* in dogs (Paltrinieri et al., 2016) and to the precipitin reaction of immune complexes (Day, 1999), which has a widely recognized pathogenic role in CanL. Dogs with high antibody titres but limited circulating *L. infantum* antigen might not have enough soluble immune complexes in circulation to trigger the inflammatory response and, therefore, clinical disease or organ damage (Day, 1999).

This study had a number of potential limitations. Firstly, enrolled dogs were considered clinically healthy based on physical examination alone, and not in conjunction with the evaluation of other laboratory test results such as haematology, serum biochemistry, and urinalysis. Additionally, seropositive dogs were not classified as either exposed or infected based on the detection of *L. infantum* by qPCR in lymph node and/or bone-marrow aspirates at enrolment or follow-up. Finally, although a titre of 1:80 is frequently considered to be the threshold for positivity in CanL endemic areas, and was used in this study, there is no consensus regarding the anti-*L. infantum* titre threshold.

Conclusions

In areas where phlebotomine vectors have clear seasonal patterns of activity, the direct impact of the sand fly season on canine *L. infantum* antibody titres must be considered

when interpreting results from asymptomatic dogs, so that appropriate clinical staging and treatment decisions can be made. Sand-fly seasonality should also be considered in the design of studies investigating the treatment and prevention of CanL.

Conflict of interest statement

The authors have no financial or personal relationships that could inappropriately influence or bias the content of the paper.

Acknowledgments

The Authors would like to thank the shelter employees for their valid and commendable support during field activities.

References

Acedo-Sánchez, C., Morillas-Márquez, F., Sanchíz-Marín, M.C., Martín-Sánchez, J. 1998. Changes in antibody titres against *Leishmania infantum* in naturally infected dogs in southern Spain. Veterinary Parasitology, 75, 1–8.

Alten, B., Maia, C., Afonso, M.O., Campino, L., Jiménez, M., González, E., Molina, R., Bañuls, A.L., Prudhomme, J., Vergnes, B., et al. 2016. Seasonal dynamics of phlebotomine sand fly species proven vectors of Mediterranean leishmaniasis caused by *Leishmania infantum*. PLoS Neglected Tropical Diseases, 10, e0004458.

Dantas-Torres, F., Solano-Gallego, L., Baneth, G., Ribeiro, V.M., de Paiva-Cavalcanti, M., Otranto, D. 2012. Canine leishmaniosis in the Old and New Worlds: Unveiled similarities and differences. Trends in Parasitology, 28, 531–538.

Day M. 1999. Immunopathological mechanisms. In: Clinical Immunology of the Dog and Cat, First Edn. Iowa State University Press/Manson Publishing, Ames, IA, USA, pp. 47-58.

ESCCAP Guidelines 05 Third Edition, March 2019. Control of vector-borne diseases in dogs and cats.

Francino, O., Altet, L., Sánchez-Robert, E., Rodriguez, A., Solano-Gallego, L., Alberola, J., Ferrer, L., Sánchez, A., Roura, X. 2006. Advantages of real-time PCR assay for diagnosis and monitoring of canine leishmaniosis. Veterinary Parasitology, 137, 214–221.

Gradoni, L., Bongiorno, L., Fiorentino, E., Foglia Manzillo, V., Gizzarelli, M., Oliva, G. 2019. A hamster model of defective sand-fly transmission may explain the occurrence of canine *Leishmania* seroreactors without evidence of infection in endemic areas of zoonotic visceral leishmaniasis. Proceeding of the 10th International Symposium on Phlebotomine Sandflies, San Cristóbal - Galápagos (Ecuador) 16-19 July 2019.

Hosein, S., Blake, D.P., Solano-Gallego, L. 2017. Insights on adaptive and innate immunity in canine leishmaniosis. Parasitology, 144, 95–115.

Kostalova, T., Lestinova, T., Sumova, P., Vlkova, M., Rohousova, I., Berriatua, E., Oliva, G., Fiorentino, E., Scalone, A., Gramiccia, M., et al. 2015. Canine antibodies against salivary recombinant proteins of *Phlebotomus perniciosus*: A longitudinal study in an endemic focus of canine leishmaniasis. PLoS Neglected Tropical Diseases, 9, e0003855.

Mendoza-Roldan, J., Benelli, G., Panarese, R., Iatta, R., Furlanello, T., Beugnet, F., Zatelli, A., Otranto, D. 2020. *Leishmania infantum* and *Dirofilaria immitis* infections in Italy, 2009-2019: Changing distribution patterns. Parasites and Vectors, 13, 193.

Moreno, J., Alvar, J. 2002. Canine leishmaniasis: Epidemiological risk and the experimental model. Trends in Parasitology, 18, 399–405.

Nejjar, R., Lemrani, M., Boucedda, L., Amarouch, H., Benslimane, A. 2000. Variation in antibody titres against *Leishmania infantum* in naturally infected dogs in northern Morocco. Revue de Médecine Vétérinaire, 151, 841-846.

Oliva, G., Roura, X., Crotti, A., Maroli, M., Castagnaro, M., Gradoni, L., Lubas, G., Paltrinieri, S., Zatelli, A., Zini, E. 2010. Guidelines for treatment of leishmaniasis in dogs. Journal of the American Veterinary Medical Association, 236, 1192–1198.

Otranto, D., Dantas-Torres, F. 2013. The prevention of canine leishmaniasis and its impact on public health. Trends in Parasitology, 29, 339–345.

Otranto, D., Paradies, P., de Caprariis, D., Stanneck, D., Testini, G., Grimm, F., Deplazes, P., Capelli, G. 2009. Toward diagnosing Leishmania infantum infection in asymptomatic dogs in an area where leishmaniasis is endemic. Clinical and Vaccine Immunology, 16, 337–343.

Paltrinieri, S., Gradoni, L., Roura, X., Zatelli, A., Zini, E. 2016. Laboratory tests for diagnosing and monitoring canine leishmaniasis. Veterinary Clinical Pathology, 45, 552–578.

Paltrinieri, S., Solano-Gallego, L., Fondati, A., Lubas, G., Gradoni, L., Castagnaro, M., Crotti, A., Maroli, M., Oliva, G., Roura, X., et al. 2010. Guidelines for diagnosis and clinical classification of leishmaniasis in dogs. Journal of the American Veterinary Medical Association, 236, 1184–1191.

Proverbio, D., Spada, E., Bagnagatti de Giorgi, G., Perego, R., Valena, E. 2014. Relationship between *Leishmania* IFAT titer and clinicopathological manifestations (clinical sore) in dogs. BioMed Research International, 2014, 412808.

Quinnell, R.J., Soremekun, S., Bates, P.A., Rogers, M.E., Garcez, L.M., Courtenay, O. 2018. Antibody response to sand fly saliva is a marker of transmission intensity but not disease progression in dogs naturally infected with *Leishmania infantum*. Parasites and Vectors, 11, 7.

Solano-Gallego, L., Cardoso, L., Pennisi, M.G., Petersen, C., Bourdeau, P., Oliva, G., Miró, G., Ferrer, L., Baneth, G. 2017. Diagnostic challenges in the era of canine *Leishmania infantum* vaccines. Trends in Parasitology, 33, 706–717.

Solano-Gallego, L., Miró, G., Koutinas, A., Cardoso, L., Pennisi, M.G., Ferrer, L., Bourdeau, P., Oliva, G., Baneth, G., The LeishVet Group. 2011. LeishVet guidelines for the practical management of canine leishmaniosis. Parasites and Vectors, 4, 86.

Tarallo, V.D., Dantas-Torres, F., Lia, R.P., Otranto, D. 2010. Phlebotomine sand fly population dynamics in a leishmaniasis endemic peri-urban area in southern Italy. Acta Tropica, 116, 227–234.

Travi, B.L., Cordeiro-da-Silva, A., Dantas-Torres, F., Miró, G. 2018. Canine visceral leishmaniasis: Diagnosis and management of the reservoir living among us. PLoS neglected tropical diseases, 12, e0006082.

Vlkova, M., Rohousova, I., Drahota, J., Stanneck, D., Kruedewagen, E. M., Mencke, N., Otranto, D., Volf, P. 2011. Canine antibody response to *Phlebotomus perniciosus* bites negatively correlates with the risk of *Leishmania infantum* transmission. PLoS Neglected Tropical Diseases, 5, e1344.

Yasur-Landau, D., Jaffe, C.L., David, L., Baneth, G. 2016. Allopurinol resistance in *Leishmania infantum* from dogs with disease relapse. PLoS Neglected Tropical Diseases, 10, e0004341.

World Organisation for Animal Health (OIE). 2018. Leishmaniosis. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, pp 491–502.

Table 1.

Variation in antibody titres against *Leishmania infantum* detected by indirect fluorescent antibody test (IFAT) according to sampling time and serial dilution (1:80 to 1:2560). Enrolment of seropositive dogs was in the sand fly season (late June 2019), and follow-up was in the non-transmission season (February-March 2020).

		Antibody titre during non-transmission season						
Antibody titre during sand fly season		Negative at 1:80	1:80	1:160	1:320	1:640	1:1280	1:2560
1:80	*n*=34 (52.3%)	*n*=13/34 [a] (38.2%)	*n*=9/34 [b] (26.5%)	*n*=4/34 [c] (11.8%)	*n*=3/34 [c] (8.8%)	*n*=2/34 [c] (5.9%)	*n*=3/34 [c] (8.8%)	-
1:160	*n*=5 (7.7%)	*n*=1/5 [a] (20%)	*n*=1/5 [a] (20%)	*n*=1/5 [b] (20%)	*n*=2/5 [c] (40%)	-	-	-
1:320	*n*=5 (7.7%)	*n*=1/5 [a] (20%)	*n*=3/5 [a] (60%)	-	*n*=1/5 [b] (20%)	-	-	-
1:640	*n*=3 (4.6%)	-	-	*n*=1/3 [a] (33.3%)	-	*n*=1/3 [b] (33.3%)	-	*n*=1/3 [c] (33.3%)
1:1280	*n*=6 (9.2%)	*n*=1/6 [a] (16.7%)	*n*=1/6 [a] (16.7%)	*n*=1/6 [a] (16.7%)	-	*n*=1/6 [a] (16.7%)	-	*n*=2/6 [c] (33.3%)
1:2560	*n*=12 (18.5%)	-	*n*=2/12 [a] (16.7%)	*n*=1/12 [a] (8.3%)	*n*=1/12 [a] (8.3%)	*n*=6/12 [a] (50%)	*n*=2/12 [a] (16.7%)	-
Total	*n*=65	*n*=16/65 (24.6%)	*n*=16/65 (24.6%)	*n*=8/65 (12.3%)	*n*=7/65 (10.8%)	*n*=10/65 (15.4%)	*n*=5/65 (7.7%)	*n*=3/65 (4.6%)

[a] Reduced antibody titres at follow-up during non-transmission season

[b] Stable antibody titres at follow-up during non-transmission season

[c] Increased antibody titres at follow-up during non-transmission season