Management, prevention and treatment of Canine Leishmaniasis in NE Spain: an online questionnaire-based survey in the province of Girona with special emphasis on new preventive methods (Canileish® vaccine and Domperidone).

Silvia Lladró[1,2], Albert Picado[2], Cristina Ballart[1,2], Montserrat Portús[1], Montserrat Gállego[1,2*]

[1]Section of Parasitology, Departament of Biology, Healthcare and the Environment, Faculty of Pharmacy, Universitat de Barcelona, Avda. Joan XXIII 27-31, 08028 Barcelona, Spain.

[2]ISGlobal, Barcelona Ctr. Int. Health Res. (CRESIB), Hospital Clínic - Universitat de Barcelona, Barcelona, Spain.

*Corresponding author at: Section of Parasitology, Faculty of Pharmacy, Universitat de Barcelona, Avda. Joan XXIII 27-31, 08028 Barcelona, Spain. Tel: +34934024502; fax: +34934024504.

e-mail address: mgallego@ub.edu (M. Gállego)

ABSTRACT:

Knowledge of how canine leishmaniasis (CanL) is being managed clinically and its epidemiology are very important, since dogs are the main reservoir of human leishmaniasis.

This study reports the results obtained through a questionnaire-based survey of veterinary practitioners in Girona province, a recognized, but non-documented endemic area in NE Spain. The primary objective was to obtain data on the clinical management of CanL, focusing particularly on new preventive methods and therapeutic tools. The results show an extensive routine management of CanL cases and a widespread use of the CaniLeish® (Virbac) vaccine and domperidone (Leisguard®, Esteve). Adverse reactions were detected by a vast majority of the vaccine users (82%), the most frequent being local reactions, apathy, fever and gastroentheritis. All the respondents had treated confirmed cases, and the therapeutic protocol most used was the combination of meglumine antimoniate (Glucantime ®, Merial) and allopurinol (Zyloric®, GlaxoSmithKline).

KEY WORDS: canine leishmaniasis, vaccine, prevention, web-questionnaire; NE Spain

40 INTRODUCTION

41

42 Canine leishmaniasis (CanL) is a global vector-borne disease caused by protozoan
43 parasites of the genus *Leishmania*, which in the Old World is transmitted by blood-
44 sucking sand flies belonging to the genus *Phlebotomus* (Alvar and others 2004;
45 Gállego, 2004). The Mediterranean basin is an endemic area of *L. Infantum*
46 transmission, dogs being the main reservoir, and humans acting as an incidental host
47 (Ashford, 1996; Dantas-Torres, 2007). Zoonotic human leishmaniasis (HL) is a public
48 health concern in Southern Europe (Alvar and others 2012).
49 In Spain, the Mediterranean coast registers the highest prevalence of CanL (de Ybáñez
50 and others 2009). CanL is endemic in Girona but epidemiological data are scarce
51 (Portús and others 2007). In fact, no official data on CanL are available for the
52 province, as for the rest of other endemic Spanish areas, despite notification being
53 compulsory at national and international levels (B.O.E., 2009; OIE, 2014).
54 Similarly, there is limited information on the current CanL management practices in
55 endemic areas. This is especially relevant nowadays, as the number of available tools to
56 prevent, diagnose and treat CanL has increased significantly in recent years.
57 Early diagnosis of CanL cases is critical for a good prognosis (Maia and Campino,
58 2008). However, clinical presentation is variable and the gold standard for diagnosis
59 (i.e. observing parasites in bone marrow or lymph node aspirates) has a low sensitivity
60 and requires trained personnel. Serological tests, mainly rapid diagnostic tests, are often
61 used in veterinary practices (Maia and Campino, 2008; Paltrinieri and others, 2010).
62 Meglumine antimoniate combined with allopurinol is still the first line of therapy for
63 CanL, but new treatments such as miltefosine (Milteforan®, Virbac), the only oral
64 antileishmanial drug, are now available (Miró and others 2008; Solano-Gallego and

65 others 2009). The level of uptake of these new treatments in endemic areas has still not
66 been reported. Similarly, the use and acceptance of the first canine leishmaniasis
67 vaccine commercialised in Europe (LiESP/QA-21, CaniLeish®) has not been
68 independently evaluated. The new vaccine, which, according to the manufacturer,
69 reduces the risk of developing clinical CanL by four (Moreno and others 2012), should
70 complement the traditional preventive measures: spot-on insecticides and treated collars
71 (Deltamethrin, Permethrin, Imidacloprid ,…). These vector control methods aim to
72 reduce contact with the sand fly-host. Finally, domperidone is an immunomodulator
73 recently postulated as a preventive method against CanL (Sabaté and others 2014) and
74 data on its use are still scarce.
75 The primary objective of this study was to report on the clinical management of CanL,
76 focusing particularly on the uptake of new preventive methods and therapeutic tools, in
77 an endemic area (North-eastern Spain). The data gathered from veterinary practices
78 were also used to study the epidemiology of CanL in this endemic area. This
79 preliminary study would be useful for the design of a more extensive and protocolized
80 analysis of CanL control and management.
81
82 MATERIAL AND METHODS
83
84 Study area and population
85 The study took place in Girona province in North-eastern Spain. Girona has 7 counties,
86 761,267 inhabitants and 5,905km² (Idescat, 2013). Habitats and climates vary from
87 Mediterranean on the coast to high mountains in the Pyrenees bordering France in the
88 north. Girona is a major national and international tourist destination and a key traffic
89 node between North Africa, Southern Spain and the rest of Europe.

90 The study population were the veterinary practitioners in Girona province. There were
91 100 veterinary practices registered at the regional veterinary college (*Col·legi Oficial de*
92 *Veterinaris de Girona* (COVGi)) in May 2013.
93
94 Questionnaire design
95 A semi-structured questionnaire was used to collect the information from the
96 veterinarians. The questionnaire was adapted from one developed to study leishmaniasis
97 in other parts of Europe (de Ybáñez and others 2009; Morosetti and others 2009; Gálvez
98 and others 2011; Alcover and others 2013; Ballart and others 2013) as part of the
99 Emerging Diseases in a Changing Environment (EDEN) Project.
100 In the first of two parts, the questions focused on the causality and epidemiology of
101 CanL, and on the perceptions of the veterinarians regarding this disease. The second
102 part contained questions about clinical manifestations, diagnostic methods, treatment
103 protocols, prognosis and prophylactic measures for CanL. This part also had questions
104 previously not included in similar surveys, concerning the treatment protocol and the
105 use and safety of the new methods available to prevent CanL: LiESP/QA-21
106 CaniLeish® vaccine and domperidone. A copy of the questionnaire is provided as
107 Supplementary Material (S1).
108 The questionnaire was distributed to the veterinary practices from May to October 2013
109 via email using online survey software: www.encuestafacil.com. To encourage
110 participation, a conference at the COVGi headquarters was organized in May 2013 to
111 explain the purpose of the study. Non-responding veterinarians were contacted by
112 telephone and/or by email at least twice.
113 For the analysis of the results regarding epidemiology in the area and causality, Chi-
114 square tests were carried out using R software (R Development Core Team, 2013).

RESULTS

By October 2013, 54% (54 out of 100) of the veterinary practices had responded to the questionnaire. The responses were from clinics in each of the seven counties of Girona province (Fig 1). The results of the questionnaire are presented as absolute numbers, percentages and 95% confidence intervals to show the distribution of the responses. The p-value is presented in Table 1.

The results show that 67% of the treated dog population was preferentially mixed (urban and rural) and around 80% of the clinics examined more than 11 dogs per week (Table 1). Approximately half the respondents reported between 11 and 50 suspected CanL cases in the previous year. About 75% confirmed between 1 and 20 CanL cases, most of which had not been diagnosed before.

As for the origin of the infection, 87% of the respondents believed that the dogs had been infected within their own activity area and almost 50% thought that the presence of the disease had increased in the previous 10 years. Only 17% of the veterinarians had ever come across an HL case and only 19% had ever suspected/confirmed a leishmaniasis case in animal species other than dogs (mainly cats).

Regarding clinical management, the most frequent clinical signs seen in infected animals were squamosis (78%), adenopathy (52%), loss of weight (50%) and localized alopecia (52%), the first two being the most valuable for diagnosis (60% and 54%, respectively) together with epistaxis (70%) and onychogryphosis (65%). All the veterinarians with suspected cases confirmed clinical diagnosis by laboratory methods and 59% used more than one laboratory diagnostic method. All of them reported using serology to confirm CanL (100%), mainly ELISA (80%). Clinical signs were used as a

reference by 52% of the clinics, followed by PCR (33%), microscopy (24%), epidemiology (9%) and proteinogram (5%). To confirm the clinical diagnosis, 65% of the veterinarians used their own private laboratory, while 76% used an external private laboratory; 41% used both their own and private laboratories.

All the confirmed cases were treated by the veterinarians (Table 2). The most favoured therapeutic protocol was the combination of meglumine antimoniate and allopurinol (Zyloric®), both as the first and second line of treatment (91% and 41%, respectively). In the 'Others' category, veterinary practitioners mainly reported the use of domperidone, alone (4 clinics) or combined with allopurinol (2 clinics), as first and second line treatment, followed by vitamin supplements, renal diet, fluoroquinolones and euthanasia.

The majority of the practitioners treating dogs diagnosed with CanL followed up the cases at least once a year: 24% every 6 months or less, and 56% every 6-12 months. Additional laboratory tests were always used to follow up cases in 50% of the clinics, while 43% applied them only occasionally, and 7% only when suspecting therapeutic failure. Almost half of the respondents (48%) reported between 1% to 10% treatment failure, while 37% of all the polled veterinarians reported up to 35% of treatment failure.

All the veterinary practitioners recommended at least one preventive measure, which in most cases was an insecticide-impregnated collar (98%)(Table 3). A high percentage also recommended spot-on insecticide (69%) and vaccine (67%). The 'other' methods, recommended by 22% of the veterinarians, were basically preventing exposure to sand flies by keeping the dog indoors at night. Almost all the respondents (96%) recommended products specifically indicated against phlebotomine sand flies, but 78% did not think that the owners protected their dogs properly. In relation to already

infected animals, 89% advised prophylactic measures to avoid sand fly bites.

Among the respondents that had used the vaccine (83%), 37% of them had applied 30 or more primo-vaccination courses in the previous 12 months (one practice reported 200) and 48% had applied between 4 and 25 complete vaccine courses. Approximately 1,300 dogs had received the vaccine in Girona province, according to the data provided in the questionnaires. Most of the clinicians (82% of the vaccine appliers) observed adverse reactions, the most common being local effects (33%) (inflammation, wounds) and apathy (27%) (Table 4). Severe adverse reactions (anaphylactic shock and/or death) were classified as a 'sporadic presentation' by 16.2% of those who reported adverse reactions.

DISCUSSION

Veterinary questionnaire surveys are fast becoming established as an alternative approach to assessing the current status of CanL and its clinical management, since they allow accurate data to be obtained over large geographic areas rapidly and cost-effectively (Ballart and others 2013). They have been used in studies at regional (Gálvez and others 2011; Alcover and others 2013; Ballart and others 2013), national (Oliveira and others 2010) and multinational levels (Bourdeau and others 2014; Mattin and others 2014). The classical questions used in the EDEN project were extended in the present work to cover the use of new immunomodulating preventive methods in clinical practice, including questions on the use of domperidone and the use and safety of vaccination (CaniLeish®).

Apparently, the majority of the responses were from areas with a high concentration of veterinary clinics corresponding to regions with the highest human population density

(central and coastal areas) (Figure 1) and with the greatest number of HL cases according to official data (Generalitat de Catalunya, 2014).

The veterinarians of the area see a large number of dogs (60% see more than 20 dogs per week) and a high percentage thought that the presence of CanL was increasing, as in the Balearic Islands (Alcover and others 2013) and the province of Lleida (Ballart and others 2013). However, as mentioned, there are no official or published data to corroborate this. This supposed increase of cases is despite routine veterinary recommendations to use preventive methods.

Several vaccine candidates are under development and three have been commercialized: two in Brazil (Leishmune®, Fort Dodge, and Leish-Tec®, Hertape) and one in Europe (CaniLeish®) (Gradoni, 2015). Given the novelty of these tools, the results of the present study are important because they provide preliminary data about their use, efficacy and safety. Two studies have previously collected information regarding the opinion of veterinarians about vaccine recommendation (Oliveira and others 2010; Bourdeau and others 2014) and one about vaccine use in France, the country where it was first available (mean 0.31% of dogs receiving the vaccine per department) (Mattin and others 2014).

Although scarcely a year had passed since the CaniLeish® vaccine was released in Spain, it was the second preventive method most recommended by the polled veterinarians (67%) after insecticide-based products. The percentage of veterinarians who applied the vaccine (83%) was higher than those who recommended it, suggesting that in some cases it was the dog owner who requested its application. Not enough time has passed to show its efficacy, but some CanL cases have been detected in vaccinated dogs. In the present study, 10 practices (18% of respondents) reported cases in vaccinated dogs, amounting to 18 dogs in total. This preliminary data should be treated

215 cautiously because the total number of vaccinated animals is unknown and no
216 information was available on whether the vaccine protocol was correctly followed.
217 Oliva et al. (2014) have recently reported a clinical efficacy of 68.4% over two
218 transmission seasons with correct application of protocol. The reported clinical efficacy
219 of Leishmune® and Leish-Tec® were around 80 and 60%, respectively (Gradoni, 2015;
220 Oliva et al., 2014; Wylie et al., 2014).
221 According to the manufacturer, a serological test should be done before the first
222 vaccination dose to ascertain whether the dogs are healthy and seronegative. Thanks to
223 this policy, 33% of the veterinary clinics that applied the vaccine detected asymptomatic
224 cases. In fact, cryptic CanL has been seen in other studies, revealing that the proportion
225 of apparently healthy seropositive dogs can be considerable in highly endemic areas
226 (Iniesta and others 2002; Baneth and others 2008).
227 Additionally, although the vaccine has been described as very safe in relation to the
228 dosage and dog age and breed (Lemesre and others 2007; Oliva and others 2014), this
229 has always been a polemical issue. The most notable adverse event seems to be local
230 swelling and pain (Oliva and others 2014). In the present study, 82% of the vaccine
231 appliers reported adverse reactions. In many cases, they were local reactions (mild to
232 severe) and apathy. Severe reactions, such as vasovagal syncope, anaphylactic shock
233 and even death, were also reported, albeit in a very low incidence. It should be noted
234 that the attribution of these adverse effects to the vaccine is based on the criteria of the
235 veterinarian (with or without checking it), and there is a risk of bias towards over-
236 diagnosis of vaccine secondary effects due to a high awareness. Although these data
237 ought to be treated carefully, given that they are the result of an opinion poll, the results
238 can serve as a basis for more extensive and protocolised studies on this subject.
239 Another novelty of the present study was the questions about the use of domperidone.

240 In the present study, half of the polled veterinarians reported the use of this drug, at
241 least as a preventive tool (Table 3). This anti-dopaminergic drug has proved effective in
242 reducing clinical signs and antibody titters in most treated dogs, with mild or severe
243 clinical signs (74.3% and 38%, respectively) (Gómez-Ochoa and others 2009) and
244 avoiding the development of clinical disease with a prevention rate around 80% (Sabaté
245 and others 2014). However, as its veterinary use is still quite novel, available data are
246 scarce. Indeed, it was only registered in Spain for preventive use at the end of 2011,
247 which would explain its limited application, and its greater use as a preventive (50%)
248 than a therapeutic drug (11%). In fact, it was the third most used preventive method
249 after insecticide-based products (collars and spot-on) and the vaccine. There were no
250 specific questions about the safety of domperidone, but it is reported to have only a few
251 mild or no side effects (Sabaté and others 2014).
252 A high percentage of the respondents recommended the use of insecticide-impregnated
253 collars (98%) and topical spot-on insecticide capsules (69%) to prevent sand fly bites,
254 as reported in other survey-based studies (de Ybáñez and others 2009; Oliveira and
255 others 2010; Gálvez and others 2011; Kotnik and others 2012; Ballart and others 2013).
256 A significant outcome of the current study is that 89% of the veterinarians advised
257 owners of already infected dogs to apply methods to avoid sand fly bites, which would
258 help prevent the dispersion of the disease. Nevertheless, according to the majority of
259 respondents, the presence of the disease has increased, as in other areas (Ballart and
260 others 2013) and 78% thought that dog owners do not properly protect their dogs
261 against the vector.
262 Another measure to control leishmaniasis transmission is the detection and management
263 of infected animals (Maroli and others 2010) using different methods, such as clinical
264 signs, laboratory tests, etc. (Paltrinieri et al., 2010). As dogs are the main reservoir of *L*

infantum the measures to control canine leishmaniasis would affect the prevention of zoonotic leishmaniasis (Maroli and others 2010). Other routes of transmission and the existence of other animals acting as reservoirs should be considered (Quinell and Courtenay, 2010).

Regarding the clinical signs, those that were most valuable and most frequently observed were the same as indicated in the common diagnosis guidelines (squamosis, epistaxis, onychogryphosis, adenopathy, loss of weight and localized alopecia) (Alvar and others 2004; Paltrinieri and others 2010; Solano-Gallego and others 2011). The use of serological methods was widespread and all the clinics used at least one test for diagnosis confirmation, as in other questionnaire-based studies in Spain (de Ybáñez and 2009; Gálvez and others 2011; Alcover and 2013; Ballart and others 2013) and other European countries (Oliveira and others 2010; Kotnik and others 2012; Bourdeau and others 2014). Although IFAT is the 'gold standard' in serological diagnosis (Maia and Campino, 2008; OIE, 2014), ELISA was the one most used (80%). In fact, depending on the commercial kit and the clinical status of the dog, some ELISA tests are more reliable, so the 'gold standard' status of IFAT should be revised, as mentioned by Rodríguez-Cortés and others (2013).

Although the treatment of dogs is not considered a useful control method, due to the high rate of therapeutic failures, infectivity is positively associated to the severity of symptoms and the parasite load that decrease after treatment (Quinnell and Courtenay, 2009). There are studies that refer to recommended treatment protocols (Alvar and others 2004; Oliva and others 2010; Solano-Gallego and others 2009), but few address their use in daily practice as in the present study. The most frequently used was found to be the combination of meglumine antimoniate and allopurinol as both the first line

289 (91%) and second line treatment (41%), as previously described in Portugal or SW
290 Europe (France, Italy and Spain) (Bourdeau and others 2014; Oliveira and others 2010).
291 The second option most chosen by the veterinarians was the combination of miltefosine
292 and allopurinol, again present in the main guidelines. The daily use of miltefosine in
293 CanL treatment has been very little investigated (Kotnik and others 2012; Mattin and
294 others 2014) and its scarce use could be due to higher prices and a shorter period on the
295 market.
296 Unfortunately, the lack of previous data on CanL in the region does not allow the trend
297 of the disease to be compared or validated. Thanks to the feedback of the veterinarians
298 and the existing data of HL (Portús et al., 2007), it is widely accepted that CanL is
299 endemic in Girona province, and 87% of the respondents thought that the animals had
300 been infected within their own area. However, further prospective studies are necessary
301 to validate the true extent of the infection and more data about the efficacy and safety of
302 the vaccine need to be collected, due to its very recent implementation.
303 Despite their recent incorporation into the market, the new CanL prophylactic tools are
304 quite well implemented. Among them, the CaniLeish® vaccine is the second most used,
305 right after barrier methods. Domperidone is used less than the vaccine, and it is being
306 also used as a treatment rather than only for prevention, for which it is registered.
307 The results obtained by this veterinary questionnaire strongly suggest the existence of
308 an autochthonous endemic focus of leishmaniasis in the province of Girona.
309

310 ACKNOWLEDGEMENTS
311 The authors would like to thank the veterinary practitioners that participated in the
312 survey for their kind collaboration and valuable information. This study would have not
313 been possible without the collaboration of the Col·legi Oficial de Veterinaris de Girona,

and especially of its president B.Serdà, the secretary M.Zurutuzar, the administrative officer M.Peñalosa, and its member J. Cairó. Thanks are also due to Ms. Sonia Ares who kindly performed the statistical analysis of data

Work supported by Ministerio de Ciencia e Innovación, Spain (CG12010-22368-CO2-01), Departement d' Universitats, Recerca i Societat de la Informació de la Generalitat de Catalunya (Spain) (2009SGR385) and Red de Investigación Cooperativa en Enfermedades Tropicales (RICET).

BIBLIOGRAPHY

Alcover, M.M., Ballart, C., Serra, T., Castells, X., Scalone, A., Castillejo, S., Riera, C., Tebar, S., Gramiccia, M., Portús, M., Gállego, M., 2013. Temporal trends in canine leishmaniosis in the Balearic Islands (Spain): A veterinary questionnaire. Prospective canine leishmaniosis survey and entomological studies conducted on the Island of Minorca, 20 years after first data were obtained. Acta Tropica. 128, 642–651. doi:10.1016/j.actatropica.2013.09.008

Alvar, J., Cañavate, C., Molina, R., Moreno, J., Nieto, J., 2004. Canine leishmaniasis. Advances in Parasitology. 57, 1–88. doi:10.1016/S0065-308X(04)57001-X

Alvar, J., Vélez, I.D., Bern, C., Herrero, M., Desjeux, P., Cano, J., Jannin, J., den Boer, M., 2012. Leishmaniasis worldwide and global estimates of its incidence. PLoS One 7, e35671. doi:10.1371/journal.pone.0035671

Ashford, R.W., 1996. Leishmaniasis reservoirs and their significance in control. Clinics in Dermatology 14, 523–532. doi:10.1016/0738-081X(96)00041-7

B.O.E, 2009. BOLETÍN OFICIAL DEL ESTADO. BOE 31990.

Ballart, C., Alcover, M.M., Picado, A., Nieto, J., Castillejo, S., Portús, M., Gállego, M., 2013. First survey on canine leishmaniasis in a non classical area of the disease in Spain (Lleida, Catalonia) based on a veterinary questionnaire and a cross-sectional study. Preventive Veterinary Medicine 109, 116–127. doi:10.1016/j.prevetmed.2012.09.003

Baneth, G., Koutinas, A.F., Solano-Gallego, L., Bourdeau, P., Ferrer, L., 2008. Canine leishmaniosis - new concepts and insights on an expanding zoonosis: part one. Trends in Parasitology 24, 324–330. doi:10.1016/j.pt.2008.04.001

345 Bourdeau, P., Saridomichelakis, M.N., Oliveira, A., Oliva, G., Kotnik, T., Gálvez, R.,
346 Foglia Manzillo, V., Koutinas, A.F., Pereira da Fonseca, I., Miró, G., 2014.
347 Management of canine leishmaniosis in endemic SW European regions: a
348 questionnaire-based multinational survey. Parasites and Vectors 7, 110.
349 doi:10.1186/1756-3305-7-110

350 Catalunya, I. d' E. de, 2012. IDESCAT [WWW Document].

351 Dantas-Torres, F., 2007. The role of dogs as reservoirs of Leishmania parasites, with
352 emphasis on Leishmania (Leishmania) infantum and Leishmania (Viannia) braziliensis.
353 Veterinary Parasitology 149, 139–146. doi:10.1016/j.vetpar.2007.07.007

354 De Ybáñez, R.R., del Río, L., Martínez-Carrasco, C., Segovia, M., Cox, J., Davies, C.,
355 Berriatua, E., 2009. Questionnaire survey on Canine Leishmaniosis in southeastern
356 Spain. Veterinary Parasitology 164, 124–133. doi:10.1016/j.vetpar.2009.06.013

357 Gállego, M., 2004. Zoonosis emergentes por patógenos parásitos : las leishmaniosis.
358 OIE 23, 661–676.

359 Gálvez, R., Miró, G., Descalzo, M. a, Molina, R., 2011a. Questionnaire-based survey on
360 the clinical management of canine leishmaniosis in the Madrid region (central Spain).
361 Preventive Veterinary Medicine 102, 59–65. doi:10.1016/j.prevetmed.2011.07.002

362 Generalitat de Catalunya, 2014. Bulletí Epidemiològic de Catalunya. Inst. d' Estadística
363 Catalunya 0, 163–177.

364 Gómez-Ochoa, P., Castillo, J. a, Gascón, M., Zarate, J.J., Alvarez, F., Couto, C.G.,
365 2009. Use of domperidone in the treatment of canine visceral leishmaniasis: a clinical
366 trial. The Veterinary Journal 179, 259–263. doi:10.1016/j.tvjl.2007.09.014

Gradoni, L., 2015. Canine Leishmania vaccines: Still a long way to go. Veterinary Parasitology 208, 94–100. doi:10.1016/j.vetpar.2015.01.003

Gramiccia, M., Gradoni, L., 2005. The current status of zoonotic leishmaniases and approaches to disease control. Internarional Journal for Parasitology 35, 1169–1180. doi:10.1016/j.ijpara.2005.07.001

Iniesta, L., Fernández-Barredo, S., Bulle, B., Gómez, M.T., Piarroux, R., Gállego, M., Alunda, J.M., Portús, M., 2002. Diagnostic Techniques To Detect Cryptic Leishmaniasis in Dogs. Clinical and Diagnostic Laboratory Immunology 9, 1137–1141. doi:10.1128/CDLI.9.5.1137

Kotnik, T., Ahačič, K., Rostaher, A., Bourdeau, P., 2012. Canine Leishmaniosis (Leishmania infantum) in Slovenia: A questionnaire-based survey. Slovenian Veterinary Research 49, 103–112.

Lemesre, J.-L., Holzmuller, P., Gonçalves, R.B., Bourdoiseau, G., Hugnet, C., Cavaleyra, M., Papierok, G., 2007. Long-lasting protection against canine visceral leishmaniasis using the LiESAp-MDP vaccine in endemic areas of France: double-blind randomised efficacy field trial. Vaccine 25, 4223–4234. doi:10.1016/j.vaccine.2007.02.083

Maia, C., Campino, L., 2008. Methods for diagnosis of canine leishmaniasis and immune response to infection. Veterinary Parasitology 158, 274–287. doi:10.1016/j.vetpar.2008.07.028

Maroli, M., Gradoni, L., Oliva, G., Castagnaro, M., Crotti, A., Lubas, G., Paltrinieri, S., Roura, X., Zini, E., Zatelli, A., 2010. Guidelines for prevention of leishmaniasis in

389 dogs. Journal of the American Veterinary Medical Association 236, 1200–1206.
390 doi:10.2460/javma.236.11.1200

391 Mattin, M.J., Solano-Gallego, L., Dhollander, S., Afonso, A., Brodbelt, D.C., 2014. The
392 frequency and distribution of canine leishmaniosis diagnosed by veterinary practitioners
393 in Europe. The Veterinary Journal 200, 410–419. doi:10.1016/j.tvjl.2014.03.033

394 Miró, G., Cardoso, L., Pennisi, M.G., Oliva, G., Baneth, G., 2008. Canine
395 leishmaniosis--new concepts and insights on an expanding zoonosis: part two. Trends in
396 Parasitology 24, 371–377. doi:10.1016/j.pt.2008.05.003

397 Moreno, J., Vouldoukis, I., Martin, V., McGahie, D., Cuisinier, A.-M., Gueguen, S.,
398 2012. Use of a LiESP/QA-21 vaccine (CaniLeish) stimulates an appropriate Th1-
399 dominated cell-mediated immune response in dogs. PLoS Neglected Tropical Diseases
400 6, e1683. doi:10.1371/journal.pntd.0001683

401 Morosetti, G., Bongiorno, G., Beran, B., Scalone, A., Moser, J., Gramiccia, M.,
402 Gradoni, L., Maroli, M., 2009. Risk assessment for canine leishmaniasis spreading in
403 the north of Italy. Geospatial Health 4, 115–127.

404 OIE, 2014. Leishmaniosis OIE Terrestrial Manual 2014.

405 Oliva, G., Nieto, J., Foglia Manzillo, V., Cappiello, S., Fiorentino, E., Di Muccio, T.,
406 Scalone, A., Moreno, J., Chicharro, C., Carrillo, E., Butaud, T., Guegand, L., Martin,
407 V., Cuisinier, A.-M., McGahie, D., Gueguen, S., Cañavate, C., Gradoni, L., 2014. A
408 randomised, double-blind, controlled efficacy trial of the LiESP/QA-21 vaccine in naïve
409 dogs exposed to two leishmania infantum transmission seasons. PLoS Neglected
410 Tropical Diseases 8, e3213. doi:10.1371/journal.pntd.0003213

411 Oliva, G., Roura, X., Crotti, A., Maroli, M., Castagnaro, M., Gradoni, L., Lubas, G.,
412 Paltrinieri, S., Zatelli, A., Zini, E., 2010. Guidelines for treatment of leishmaniasis in
413 dogs. Journal of the American Veterinary Medical Association 236, 1192–1198.
414 doi:10.2460/javma.236.11.1192

415 Oliveira, A.M., Diaz, S., Santos, C., Bourdeau, P., Pereira, I., 2010. Geographical
416 distribution, clinical presentation, treatment and prevention of canine leishmaniosis in
417 Portugal: a 2007 field survey. Revista Portuguesa Ciências Veterinárias 109, 21–29.

418 Paltrinieri, S., Solano-Gallego, L., Fondati, A., Lubas, G., Gradoni, L., Castanaro, M.,
419 Crotti, A., Maroli, M., Oliva, G., Roura, X., Zatelli, A., 2010. Guidelines for diagnosis
420 and clinical classification of leishmaniasis in dogs. Journal of the American Veterinary
421 Medical Association 236, 1184–1191. :doi: 10.2460/javma.236.11.1200

422 Portús, M., Gállego, M., Riera, C., Fisa, R., Aisa, M.J., Botet, J., Carrió, J., Castillejo,
423 S., Iniesta, L., López, P., Montoya, L., Muñoz, C., Serra, T., Gallego, J., 2007. A review
424 of human and canine leishmaniosis in Catalonia, and associated vector distribution.
425 Revista Ibérica de Parasitologia 67, 59–67.

426 R Development Core Team, 2013. R Software. R: A Language and Environment for
427 Statistical Computing. ISBN 3-900051-07-0. http://www.R-project.org/

428 Rodríguez-Cortés, A., Ojeda, A., Todolí, F., Alberola, J., 2013. Performance of
429 commercially available serological diagnostic tests to detect Leishmania infantum
430 infection on experimentally infected dogs. Veterinary Parasitology 191, 363–366.
431 doi:10.1016/j.vetpar.2012.09.009

Sabaté, D., Llinás, J., Homedes, J., Sust, M., Ferrer, L., 2014. A single-centre, open-label, controlled, randomized clinical trial to assess the preventive efficacy of a domperidone-based treatment programme against clinical canine leishmaniasis in a high prevalence area. Preventive Veterinary Medicine 115, 56–63. doi:10.1016/j.prevetmed.2014.03.010

Solano-Gallego, L., Koutinas, A., Miró, G., Cardoso, L., Pennisi, M.G., Ferrer, L., Bourdeau, P., Oliva, G., Baneth, G., 2009. Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis. Veterinary Parasitology 165, 1–18. doi:10.1016/j.vetpar.2009.05.022

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, 1–16. doi:10.1186/1756-3305-4-86

Wylie, C.E., Carbonell-Antoñanzas, M., Aiassa, E., Dhollander, S., Zagmutt, F.J., Brodbelt, D.C., Solano-Gallego, L., 2014. A systematic review of the efficacy of prophylactic control measures for naturally occurring canine leishmaniosis. Part II: topically applied insecticide treatments and prophylactic medications. Preventive Veterinary Medicine 117, 19–27. doi:10.1016/j.prevetmed.2014.06.016

Figure 1 - Area of study (Girona, NE Spain) and the veterinary clinics surveyed.

TABLES

	N	% (95% CI)	p-value		N	% (95% CI)	p-value
Type of dog population				**New cases of CanL**			
Mixed	36	66.7 (54.2-79.2)	p < 0.001	0	1	1.9 (0-5.5)	p < 0.001
Urban	12	22.2 (11.2-33.2)		1-5	23	42.6 (29.4-55.8)	
Rural	6	11.1(2.7-19.5)		6-10	16	29.6 (17.4-41.8)	
Dogs examined/week				11-20	11	20.4 (9.7-31.1)	
1-3	1	1.9(0-5.5)	p < 0.001	21-50	2	3.7 (0-5)	p < 0.001
4-10	9	16.7 (6.8-26.7)		>50	1	1.9 (0-5.5)	
11-20	12	22.2(11.2-33.2)		**Dogs infected in the area**			
>20	32	59.3(46.2-72.4)		Yes	47	87 (78-95.9)	p < 0.001
Dogs suspected				No	3	6 (0-12.3)	
1-5	9	16.7 (6.8-26.7)	0.2286	No answer	4	7 (0.2-13.8)	

6-10	9	16.7 (6.8-26.7)		**Evolution (past 10 years)**			
11-20	17	31.5 (19.1-43.9)		Increase	26	48 (34.7-61.3)	p < 0.001
21-50	12	22.2 (11.2-33.2)		No evolution	20	37 (24.1-49.9)	
>50	7	13 (4-22)		Decrease	4	7 (7 (0.2-13.8)	
CanL cases confirmed				No answer	4	7 (7 (0.2-13.8)	
1-5	13	24.1 (12.7-35.5)	0.1301	**HL**			
6-10	13	24.1 (12.7-35.5)		Yes	9	17 (7-27)	p < 0.001
11-20	15	27.8 (15.9-39.8)		No	45	83 (73-93)	
21-50	9	16.7 (6.8-26.7)		**Other reservoirs**			
>50	4	7.4 (0.4-14.4)		Yes	10	19 (8.5-29.5)	p < 0.001
				No	44	81 (70.5-91.5)	

452 Table 1. Type of dog population and CanL trends

453

454

Drugs	First line		Second line	
	N	% (95 %CI)	N	% (95% CI)
Allopurinol	11	20.4 (9.7-31.1)	9	17 (7-27)
Meglumine antimoniate	4	7 (0.2-13.8)	4	7 (0.2-13.8)
Meglumine antimoniate+ Allopurinol	49	90.8 (87.1-94.5)	22	40.7 (27.6-53.8)
Miltefosine	3	5.6 (0.5-11.7)	4	7 (7 (0.2-13.8)
Miltefosine + Allopurinol	8	14.8 (5.3-24.3)	18	33.3 (20.7-45.9)
Amphotericin B	0	0	1	1.9 (0-5.5)
Liposomal Amphotericin B	0	0	1	1.9 (0-5.5)
None	0	0	5	9.3 (1.6-17.1)
Other	9	17 (7-27)	13	24.1 (12.7-35.5)

455 Table 2. Treatment protocols used by the veterinary practitioners

456

457

458

Preventive measures recommended	N	% (95% CI)
Collar	53	98.1 (94.5-100)
Spot on	37	68.5 (56.1-80.9)
Spray	3	5.6 (0.5-11.7)
Shampoo	0	0.0
CaniLeish® vaccine	36	66.7 (54.16-79.2)
Domperidone formulations	27	50 (36.6-63.4)
None	0	0.0
Others	12	22.2 (11.2-33.2)

459

460 Table 3. Preventive measure protocols recommended by the veterinary practitioner

461

462

463

Adverse reaction	Very frequent		Relatively frequent		Sporadically	
	N	**% (95% CI)**	**N**	**% (95% CI)**	**N**	**% (95% CI)**
Local reaction (inflammation, wounds)	15	33.3 (22.9-43.7)	4	8.9 (0.6-17.2)	12	26.7 (13.8-39.6)
Gastrointestinal symptoms	3	6.7 (0-14)	3	6.7 (0-14)	8	17.8 (6.6-29)
Fever	7	15.6 (5-26.2)	6	13.3(3.4-23.2)	1	2.2 (0-6.5)
Apathy	12	26.7 (13.8-39.6)	8	17.8 (6.6-29)	8	17.8 (6.6-29)
Vasovagal syncope	1	2.2(0-6.5)	2	4.4 (0-10.4)	7	15.6 (5-26.2)
Anaphylactic shock	0	0	1	2.2 (0-6.5)	7	15.6 (5-26.2)
Death	0	0	0	0	2	4.4 (0-10.4)

464 Table 4. Frequency of adverse reactions to CaniLeish® vaccine observed by the veterinary practitioners

465